The cause, effects, dynamics, and distribution of *Cochlodinium polykrikoides* blooms and cells in the Peconic Estuary, Suffolk County, NY

Progress report to Suffolk County, Department of Health Services

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Table of Contents:

Executive Summary:

Cochlodinium polykrikoides is notorious for causing toxic algal blooms in coastal waters around the world. In 2004, a bay-wide *C. polykrikoides* occurred in the Peconic Estuary and blooms have recurred there and Shinnecock Bay every year since. Observed impacts during blooms have included the complete mortality of captive finfish in both estuaries and mortality of caged and wild shellfish in the Peconic Estuary. Since the precise causes of *C. polykrikoides* blooms in NY and the nature of bloom toxicity are both poorly understood, this study was initiated to address these issues. Studies indicated that *C. polykrikoides* blooms are highly toxic, displaying the ability to rapidly (minutes to hours) kill other phytoplankton and zooplankton allowing blooms be sustained and proliferate without predation or competition. Regarding nutrients, during blooms, the addition of different N compounds significantly increased the growth of *C. polykrikoides* more frequently than other phytoplankton groups suggesting blooms were promoted by N-loading. *C. polykrikoides* was also a nutritionally flexible species, capable of assimilating a variety of N compounds, with the compound yielding maximal growth or uptake depending on prevailing nutrient conditions. Studies also confirmed the highly toxic nature of *C. polykrikoides* blooms and isolates to multiple species and life stages of fish and shellfish. While this project made great progress on understanding these newly occurring events in NY waters, there remains a series of critically important questions regarding *C. polykrikoides* blooms which still need to be addressed. Specifgically, the factors which lead to bloom intiation remain unknown since deterrence of predators and competitors only occurred once blooms were established. While this project could not find *C. polykrikoides* cysts in the field, we were able to create cysts in culture, suggesting cycts formation this may be a key aspect of this species' ecology and one reason for annual bloom recurrence. The impacts of current and future climate change may be driving the sudden occurrence and recurrence of blooms, but has not been investigated. The impact of blooms on fish and shellfish populations in the wild must be better understood to sustain local fisheries. Finally, potential mechanisms for successfully mitigating blooms are not known, but should be explored.

Chapter one, Introduction and background:

Harmful algal blooms (HABs) pose a significant threat to fisheries, public health, and economies around the world. HABs have increased in frequency, duration, and distribution in recent decades and the Peconic Estuary is representative of this global phenomenon. The Suffolk County estuaries were first introduced to HABs in 1985 when brown tides caused by the phytoplankton *Aureococcus anophagefferens* occurred through this system destroying eel grass beds and the bay scallop fishery. While everyone welcomed the disappearance of brown tide in the Peconics after 1995, a new HAB has emerged to become an annual visitor to our estuary. *Cochlodinium polykrikoides* is a phytoplankton known as a dinoflagellate and when it grows to large densities, it can discolor waters a reddish – brown color. Since 2004, *Cochlodinium polykrikoides* blooms have occurred every year across the Peconics, starting in August and persisting through September and sometimes into October. Blooms are visually distinct, as they consist of very dark patches of more than 10,000 cells per milliliter. Globally, *Cochlodinium* blooms were previously rare, but they have emerged across the globe during the past decade from Chesapeake Bay to California to Indonesia, Malaysia, and the Arabian Gulf. Historically, blooms have been most common in Japan and South Korea where blooms have caused more than $100 million in annual losses to fisheries due to fish kills.

Because *Cochlodinium* blooms are a new and recent phenomenon, the causes and complete ecosystem impacts of these events are only starting to be understood. During the past four years, this study has investigated the impacts and causes of *Cochlodinium polykrikoides* blooms in the Suffolk County estuaries. We have isolated cells to form laboratory cultures which have been investigated in conjunction with the collection of bloom water from estuaries. We have published some of their results in a series of manuscripts in international, peer-review journals in 2008 and 2009 and have several more papers in the works. We have

found that bloom water and cultures isolated from the Peconic Estuary are capable of killing phytoplankton, zooplankton, fish, and juvenile and larval shellfish including bay scallops, hard clams, and oysters in a matter of hours to days. During widespread blooms of 2008 and 2009, fishermen have reported mass mortality of fish held in pound nets in the Peconics and neighboring Shinnecock Bay. Moreover, the Southampton Town Trustees reported a mass mortality of scallops in Little Peconic and Noyack Bay during the 2009 bloom.

Chapter two: Characterization, dynamics, and ecological impacts of harmful *Cochlodinium polykrikoides* blooms on eastern Long Island, NY, USA

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Abstract: We report on the emergence of *Cochlodinium polykrikoides* blooms in the Peconic Estuary and Shinnecock Estuary, NY, USA, during 2004 - 2006. Blooms occurred during late summer when temperatures and salinities ranged from 20-25°C and 22-30 ppt, respectively. Bloom patches achieved cell densities exceeding 10^5 ml^{-1} and chlorophyll *a* levels exceeding 100 μg L^{-1}, while background bloom densities were typically 10^3 - 10^4 cells ml^{-1}. Light, scanning electron and ultrathin-section transmission electron microscopy suggested that cells isolated from blooms displayed characteristics of *C. polykrikoides* and provide the first clear documentation of the fine structure for this species. Sequencing of a hypervariable region of the large subunit rDNA confirmed this finding, displaying 100% similarity to other North American *C. polykrikoides* strains, but a lower similarity to strains from Southeast Asia (88-90%). Bioassay experiments demonstrated that 24 h exposure to bloom waters (> 5 x 10^4 cells ml^{-1}) killed 100% of multiple fish species (1-week old *Cyprinodon variegates*, adult *Fundulus majalis,* adult *Menidia menidia*) and 80% of adult *Fundulus heteroclitus*. Microscopic evaluation of the gills of moribund fish revealed epithelial proliferation with focal areas of fusion of gill lamellae, suggesting impairment of gill function (e.g. respiration, nitrogen excretion, ion balance). Lower fish mortality was observed at intermediate *C. polykrikoides* densities (10^3 - 10^4 cells ml^{-1}), while all fish survived for 48 hr at cell densities below 1 x 10^3 cells ml^{-1}. The inability of frozen and thawed-, or filtered (0.2 μm)-bloom water to cause fish mortality suggested that the thick polysaccharide layer associated with cell membranes and/or a toxin principle within this layer may be responsible for fish mortality. Juvenile bay scallops (*Argopecten irradians*) and American oysters (*Crassostrea virginica*) experienced elevated mortality compared to control treatments during a nine-day exposure to bloom water (~5 x 10^4 cells ml^{-1}). Surviving scallops exposed to bloom water also experienced significantly reduced growth rates. Moribund shellfish displayed hyperplasia, hemorrhaging, squamation, and apoptosis in gill

and digestive tissues with gill inflammation specifically associated with areas containing *C. polykrikoides* cells. In summary, our results indicate *C. polykrikoides* blooms have become annual events on eastern Long Island and that bloom waters are capable of causing rapid mortality in multiple species of finfish and shellfish.

1. Introduction

Harmful algal blooms (HABs) represent a significant threat to fisheries, public health, and economies around the world and have increased in frequency, duration, and distribution in recent decades. HABs are most commonly caused by dinoflagellates which, under bloom conditions, can discolor effected waters red and thus have also been deemed red tides, particularly in Southeast Asia (Okaichi 2003). Many harmful dinoflagellates synthesize potent biotoxins which can poison humans when shellfish which have concentrated such toxic cells are consumed. However, these toxins often do not harm marine life (Landsberg 2002). Other dinoflagellates can cause direct harm to or even kill marine animals, such as fish, although the precise modes of impairment to the animals are diverse and sometimes not known. One dinoflagellate which is well known for causing fish kills in Southeast Asian waters is *Cochlodinium polykrikoides*.

Cochlodinium has been implicated in kills of wild and impounded fish around the globe (Onoue et al., 1985; Yuki, and Yoshimatsu, 1989; Guzmán et al., 1990; Qi et al., 1993; Gárrate-Lizárraga et al., 2004; Whyte et al., 2001) and has been the cause of fisheries losses exceeding $100 million in Korea (Kim, 1998; Kim et al., 1999). Studies have also indicated that metamorphosis of oyster (*Crassostrea gigas*) larvae was slowed during *Cochlodinium* blooms (Matsuyama *et al*. 2001) and that mortality of larvae of the American oyster, *Crassostrea virginica*, was elevated by exposure to *Cochlodinium* (Ho and Zubkoff, 1979). The most common *Cochlodinium* species, *C. polykrikoides*, grows optimally at temperatures between 21 and 26 °C and at salinities between 30 and 36 (Kim et al 2004; Yamatogi et al., 2006). *Cochlodinium* is a mixotrophic alga (Larsen and Sournia, 1991; Jeong et al., 2004) and thus likely employs flexible nutrient acquisition strategies during blooms. Moreover, since this alga is noxious to some planktonic grazers (Ho and Zubkoff, 1979; Shin et al.,

185 2003), it may escape top-down control by zooplankton which most phytoplankton experience
186 (e.g. Gobler et al., 2002). Prior to this special issue, peer-reviewed reports of *Cochlodinium*
187 blooms in the US have been rare and blooms in NY waters have never been noted in peer-
188 reviewed literature.
189
190 The occurrence of HABs in Long Island estuaries have been well documented for
191 more than 50 years. During the 1950s, Ryther (1954) described the occurrence of green tide
192 blooms caused by the chlorophytes *Nannochloris* and *Stichococcus* in the south shore
193 estuaries, Great South Bay and Moriches Bay. These blooms negatively impacted the oyster
194 fishery in these systems (Ryther 1989). More recently, brown tides caused by the
195 pelagophyte *Aureococcus anophagefferens* occurred in both south shore bays (Great South,
196 Moriches, and Shinnecock Bays) and on eastern Long Island (Peconic Estuary; Gobler et al.,
197 2005). Chronic recurrence of these blooms in the Peconic Estuary for 10 years and on the
198 south shore for more than 15 years led to the destruction of eel grass beds (*Zostera marina*),
199 scallop fisheries (*Argopecten irradians*), and hard clam fisheries (*Mercenaria mercenaria*;
200 Gobler et al., 2005). The absence of brown tides on Long Island for nearly five years has
201 buttressed hope that local fisheries may recover.
202
203 Here, we report on the emergence and dynamics of red tides caused by *Cochlodinium*
204 sp. in some of the same estuaries which formerly hosted brown tides, the Peconic Estuary and
205 Shinnecock Bay. We describe the initial occurrence of blooms in 2002 and 2004, and the
206 spatial and temporal dynamics of blooms in 2005 and 2006. We present light, scanning and
207 thin-section transmission electron micrographs of algal isolates, as well as sequences of the
208 large subunit ribosomal DNA. We describe the results of experiments with multiple species
209 of finfish and shellfish to elucidate the potential for blooms to impact marine life in these

systems and present histopathological analysis of moribund individuals. Finally, we analyze current and historical water quality data to assess possible bloom causes and to compare and contrast these blooms to those of the brown tide, *A. anophagefferens*.

2. Methods

2.1. Field sampling and sample processing

Fixed stations within the Peconic Estuary and Shinnecock Bay and their respective tributaries (Fig 1) were sampled via small research vessels sporadically in 2004 and 2005 and weekly to biweekly during the summer of 2006. Moreover, in 2006, bloom patches were sampled in addition to fixed stations. Primary stations in 2006 included the two most western basins of the Peconic Estuary, Flanders Bay (40.923°N, 72.587°W) and Great Peconic Bay (40.936°N, 72.512°W), as well as Meetinghouse Creek (40.938°N, 72.619°W), a tributary which empties into Flanders Bay, and Old Fort Pond (40.868°N, 72.446°W), a tidal tributary in eastern Shinnecock Bay. On station, temperature and salinity were determined using a Hydrolab Quanta probe. Surface water samples at each station were collected from the bow of boats in replicated 20 L carboys which were transported to the Stony Brook-Southampton Marine Science Center for immediate sample processing and analysis.

2.2. Quantification of chlorophyll a *and cell densities in field samples*

Triplicate chlorophyll *a* samples were collected on GF/F glass fiber filters and stored frozen. Chlorophyll *a* was size-fractionated using a 5 µm Nitex© mesh (Gobler et al., 2002). Whole seawater samples were preserved in Lugol's iodine for microscopic examination of phytoplankton. Chlorophyll *a* (chl *a*) was analyzed in triplicate by standard fluorometric methods (Parsons et al., 1984). Preserved plankton samples were settled in counting chambers and enumerated on an inverted light microscope (Hasle 1978). At least 100 cells

were enumerated per sample, yielding a relative standard deviation of less than 20% for *Cochlodinium* enumeration at bloom and non-bloom densities. The relative abundance of *Cochlodinium* among autotrophs in selected samples was estimated from lengths and widths of the most common phytoplankton present and using volumetric equations corresponding to the geometric shape each organism most resembled (Smayda, 1978; Stoeker et al., 1994).

2.3. Culture establishment, DNA analysis, and morphological observations with LM, SEM and TEM

Culture isolates were obtained by pipetting single cells to polystyrene 12-well cell culture plates containing culture medium under an inverted microscope. Cells were cultured in sterile f/20 medium supplemented with 10^{-8} M selenium at 22° C in an incubator with a 14:10h light:dark cycle, illuminated by a bank of fluorescent lights that provided a light intensity of ~100 µmol quanta m^{-2} sec^{-1} to cultures. Cultures were transferred once they reached stationary phase which was approximately three weeks. Light microscope (LM) photographs were obtained using an inverted microscope and a Spot Insight (model 3.2) camera.

For genetic analysis, cells were pelleted by centrifugation of 3ml of culture at 5K RPM for 2 minutes. The cell pellet was immediately resuspended in 600µL CTAB solution (100 mM Tris-HCl [pH 8], 1.4 M NaCl, 20 mM EDTA, 2% [w/v] cetyltrimethylammonium bromide [CTAB], 0.4% [v/v] β-mercaptoethanol, 1% [w/v] polyvinylpyrollidone; Dempster et al., 1999) and cells were disrupted by vortexing and pipetting. The resuspended cells were frozen at -80°C for at least 1 hour. Nucleic acids were extracted as in Coyne et al. (2001). The hypervariable D1-D3 region of the rDNA was amplified by PCR using forward primer 'D1RF' (5'-ACCCGCTGAATTTAAGCATA- 3') and reverse primer 'D3Car' (5'-

ACGAACGATTTGCACGTCAG -3'; Mikulski et al., 2005). The D4-D6 region was amplified with forward primer 6F (5'-TAGTAGCTGGTTCCCTCCGA- 3') and reverse primer 11R (5'-TTGCCGACTTCCCTTACCTA - 3'; Iwatake et al., this issue). PCR was performed with the following components: 80-100 ng nucleic acids, 0.5 uM of each primer, 1x reaction buffer, 3 mM MgCl2, 200 µM dNTPs mix (New England Biolabs), 0.5 µM of each primer, 2.5 U polymerase enzyme mix (GeneAmp® High Fidelity PCR Applied Biosystems). We performed 50 µl reactions with the following cycling parameters: 94°C for five minutes, followed by 31 cycles of 94°C for 45 sec, 45°C for 60 sec and 72°C for 45 sec, followed by 72°C final extensions for 7 min (Mikulsi et al, 2005). Sequencing was done directly on the unmodified PCR product using 50 ng of PCR product and 3.2 pmol primer on a ABI3730 Genetic Analyzer using BigDye Terminator ® Cycle sequencing kit (Applied Biosystems) at the Stony Brook University DNA Sequencing Facility.

To preserve field samples for electron microscopy, equal volumes of buffered glutaraldehyde fixative and algal suspension from the sampling site were mixed to produce a final solution of 2% glutaraldehyde in 0.05 M cacodylate buffered seawater (pH = 8.0) at 5^{o}C. The fixed cells were gently sedimented to form a pellet, the supernatant was decanted, and 5 ml of 4% osmium tetroxide solution in the same cacodylate buffer was added to the pellet as a post stain and fixative. After 1 hour at 5 °C, the osmium-fixed cells were sedimented, washed by addition of cacodylate buffer and again sedimented to form a pellet and enrobed in 0.8 % agar matrix. Small segments of the agar-embedded pellet (~ 3 mm in size) were washed in deionized water, dehydrated in a graded acetone/aqueous series, embedded in TAAB epoxy resin (Energy Beam Sciences, Granby, Conn.) and polymerized in BEEM capsules at 70^{o}C for 18 hours. Ultrathin sections, collected on uncoated copper grids, were obtained with a Porter-Blum MT-2 ultramicrotome fitted with a diamond knife, post-

stained with Reynold's lead citrate, and observed with a Philips 201 transmission electron microscope (TEM). A portion of the osmium-fixed cells was set aside for scanning electron microscopic (SEM) observation. The fixed cells were sedimented, washed with deionized water, and brought to a volume of 2 ml in 10% ethanol solution. Cells were collected on 0.45 µm pore-size Millipore nitrocellulose filters, dehydrated in a graded ethanol/aqueous series, and critical point dried using a Balzer critical point dryer. The dried filters were attached to SEM stubs, plated with gold using a Denton Desktop 2 sputter coater, and observed with a LEO 1455VP scanning electron microscope. Higher resolution images of cell surface detail were obtained with a Hitachi 4700 SEM.

2.4. Bioassay experiments

Experiments were conducted to elucidate the potential impact of *Cochlodinium* sp. bloom water on fish and shellfish. One set of experiments was performed with juvenile (1 – 3 week old) sheepshead minnows (*Cyprinodon variegates*) spawned from a line of *C. variegates* which has been laboratory reared for more than a decade (E.M. Cosper, pers. comm.). For these experiments, individual minnows were transferred using a modified 200 µl pipette to 3 ml of treatment water held in 24-well sterile, polystyrene plates (n = 24 per treatment). In the first experiment, *C. variegates* were transferred to plates containing one of four treatment waters: 1. Bloom water from Flanders Bay (Fig 1) containing 5×10^4 cells ml^{-1}, 2. Filtered bloom water (0.2 µm), 3. Water from Great Peconic Bay (Fig 1), which had < 1×10^2 cells ml^{-1}, or 4. Filtered (0.2 µm) Great Peconic Bay water. Subsequent experiments were conducted using 100% bloom water from Flanders Bay or Old Fort Pond (Fig 1), as well as bloom water diluted with 0.2 µm-filtered bloom water to final concentrations of bloom water of 50, 25, 10%, and 0% to elucidate a dose response. To further understand modes of mortality, experiments were conducted using boiled or frozen and thawed bloom

water as treatments, as well as water which was passed through 2.0 µm polycarbonate filters, a 10 µm nylon mesh or a 20 µm nylon mesh. In all experiments, control treatments of non-bloom Great Peconic Bay water and/or filtered bloom water (0.2 µm) were established which yielded 100% survival for the full duration of all experiments ($\leq$ 72 h). Fish in experiments were checked several times daily and dead individuals were immediately removed and placed in 10% neutral buffed formalin for later histopathological evaluation.

Larger fish for experiments (*Fundulus majalis, Fundulus heteroclitus, Menidia menidia*) were obtained via seine nets from regions of Shinnecock Bay with undetectable levels of *Cochlodinium* sp. Fish were maintained in aerated seawater with low levels of *Cochlodinium* sp at the Stony Brook-Southampton Marine Science Center for 24 h prior to experiments. Experiments were established by placing ten individuals in triplicate five-liter plastic containers holding three liters of bloom water from Flanders Bay or Eastern Shinnecock Bay ($> 10^4$ cells ml^{-1}) or water from Great Peconic Bay (Fig 1) which had $< 1 \times 10^2$ cells ml^{-1}. All containers were placed in flow through seawater to maintain ambient temperature and were bubbled with air, maintaining oxygen levels above 5 mg L^{-1}. Containers were covered with white, nylon screening with 1 mm mesh openings. Dilutions of bloom water with filtered (0.2 µm) bloom water (50, 25, 10%) were created for experiments with *F. majalis and M. menidia* to elucidate a dose response in the fish. Fish survival was examined every 1 – 8 hours and dead fish were immediately removed and placed in 10% neutral buffed formalin for later histopathological evaluation. Experiments with *F. majalis, F. heteroclitus,* and *M. menidia* lasted 24 – 48 h. Spectrophotometrically analyzed ammonium levels (Parsons et al., 1984) remained < 20 µM during fish experiments.

334 *Argopecten irradians* and *Crassostrea virginica* bioassay experiments were conducted
335 using juvenile bay scallops (~11 mm) and American oysters (~21mm) obtained from the
336 Cornell Cooperative Extension shellfish hatchery facility in Southold, NY. Shellfish were
337 maintained in flowing seawater at the Stony Brook-Southampton Marine Science Center for
338 24 h prior to experiments. Experiments were established by measuring the lengths of ten
339 marked individuals and placing them in triplicate, five-liter plastic buckets (10 individuals
340 per bucket) containing three liters of: 1. Bloom water from Flanders Bay (Fig 1) containing
341 ~5 x 10^4 cells ml^{-1}, 2. Filtered (0.2 μm) treatment #1 water (Flanders Bay), 3. Water from
342 Great Peconic Bay (Fig 1), which had < 1 x 10^2 cells ml^{-1}, or 4. Filtered (0.2 μm) treatment
343 #3 water (Great Peconic Bay). All buckets were kept in a temperature-controlled room
344 maintained at 24°C with light from a bank of fluorescent bulbs which provided 100 μE m^{-2} s^{-1}
345 of light on a 14h:10h light:dark cycle. All buckets were bubbled with air, maintaining
346 oxygen levels above 5 mg L^{-1}, and covered with white, nylon screening with 1mm mesh
347 openings. Experimental water was fully exchanged with newly obtained water from the field
348 with comparable cell numbers every other day during this nine day experiment. Mean cell
349 densities (± SD) for the experiment were 3.7 ± 1.3 x 10^4 cells ml^{-1} (range = 2.7 - 6.1 x 10^4
350 cells ml^{-1}). Samples for the enumeration of phytoplankton cell densities and chlorophyll *a*
351 levels were obtained from the old and new water and analyzed every other day as described
352 above. Survival was examined daily and dead individuals were immediately measured,
353 shucked and placed in 10% neutral buffed formalin for later histopathological evaluation.
354 After nine days, lengths of surviving individuals were measured.
355
356 Experiments that involved larger fish and scallops were analyzed via one-way
357 ANOVAs followed by Tukey multiple comparisons tests of treatments (Sokal and Rolf,
358 1994). Non-normally distributed data sets were log transformed. Experiments using

359 sheepshead minnows were analyzed using Chi-square tests. In all cases, significance levels
360 were set at $p < 0.05$.
361
362 *2.5. Histopathology*
363 All specimens were fixed in 10 % neutral buffered formalin for a minimum of one
364 week before being processed for histology using standard techniques. For scallops and
365 oysters, a cross-section of about 6 mm in thickness (typically containing mantle, gills,
366 digestive gland, stomach, intestine, heart and kidney) was transferred to a pre-labeled histo-
367 cassette, and dehydrated in graded ethanol and xylene series. Tissues were then embedded in
368 paraffin and sectioned (5 μm thick). Resulting sections were stained with hematoxylin and
369 eosin, before being examined with a Nikon Eclipse TE-200 microscopy equipped with a Spot
370 Insight QE digital camera. Fish were decalcified with sodium EDTA (Luna, 1968). In the
371 case of the smallest fish (1-3 wk old *C. variegates*), the specimens were sectioned whole in
372 the median plane in three step sections: 1/8-way toward the mid-median plane, ½-way toward
373 the mid-median plane and at the mid-median plane. For all other fish, gills were dissected
374 from the fish for processing. Tissues were then embedded in paraffin, sectioned and stained
375 with hematoxylin and eosin stains for histological evaluation (Luna, 1968).
376
377 **3. Results**
378 *3.1. Bloom dynamics*
379 The first noted appearance of *Cochlodinium* on eastern Long Island was in 2002,
380 when it was identified as the organism responsible for a red water event in West Neck Bay,
381 on Shelter Island (Fig 1). The first geographically extensive bloom occurred within the
382 Peconic Estuary in September and October of 2004 when cell densities ranging from 1 – 2 x
383 10^3 ml^{-1} were recorded in the two western basins of this system, Flanders Bay and Great

384 Peconic Bay (Fig 1). A bloom occurred within the same regions in 2005, with station cell
385 densities exceeding 10^3 ml^{-1} in the western Peconic Estuary (Fig 1) and patch densities
386 exceeding 2 x 10^4 cells ml^{-1}. In contrast, low cell densities (< 10^3 ml^{-1}) were found in the
387 major basins of the eastern Peconic Estuary, although some tributaries and sub-embayments
388 such as West Neck Bay, had higher densities (Fig 1).
389
390 In 2006, a more robust sampling approach allowed the details of *Cochlodinium* bloom
391 temporal dynamics to be refined. *Cochlodinium* was detected for the first time in the water
392 column in July at all major sampling locations as temperatures were approaching 25°C (Fig
393 2; Table 1). *Cochlodinium* maintained moderate cell densities (10^2 – 10^3 cell ml^{-1}) through
394 mid-August as temperatures reached an annual maximum above 25 °C at all sites (Fig 2;
395 Table 1). During the period of 20 August through 21 September, cell densities within four
396 major sampling locations (Flanders Bay, Meetinghouse Creek, Great Peconic Bay, eastern
397 Shinnecock Bay) were consistently > 10^3 cell ml^{-1} and were commonly > 10^4 cell ml^{-1} (Fig
398 2). Concurrently, chlorophyll *a* levels often exceeded 100 µg L^{-1} (range 54 – 370) with
399 nearly all chlorophyll being > 5 µm, as temperatures were declining to 20 °C (Table 1).
400 During the same period, dense bloom patches were chronically present throughout the four
401 major stations, with patch cell densities ranging between 10^4 and 10^5 cells ml^{-1} (Fig 2). By
402 late September, cell densities once again declined to < 10^2 cell ml^{-1} at all locations as
403 temperatures dropped below 20 °C (Fig 2; Table 1). Salinities at three of four sites generally
404 ranged from 25 to 30 and were highest during the late August bloom peak (Fig 2; Table 1).
405 Meetinghouse Creek had lower salinities throughout the study (22 – 25; Table 1).
406
407 *3.2. Molecular and microscopic characterization of cells*

There were considerable variations in cell sizes of the unpreserved *Cochlodinium* sp. isolates, with an average of 34 ± 4.7 μm long (range 21 – 35 μm) and 27 ± 4.1 μm wide (range 24 – 48 μm; n = 100). A LM view of isolated, living cells (Fig. 3) exhibits the major morphological features of paired and catenated cells in a chain. The leading cell in a chain frequently contains a more tapered, semi-circular epicone. The sulcus extending from the apex toward the cingulum bisects the apex into two lobes of unequal size. The hypocone is bigger than the epicone in both width and length, slightly or heavily bilobbed at the antapex, most easily observed in single cell or the last cell in a chain. The left spiral cingulum is deeply grooved, with about two turns of torsion. The sulcus is narrow, with a torsion of about one turn. Intermediate cells within a chain are slightly compressed longitudinally and thus more rounded to ovate. A reddish stigma is visible in the left side of the apical region of the epicone (Fig. 3, arrowhead). The nucleus is small and located at the center of the epicone (Fig. 3, white arrow).

A SEM micrograph of a field-collected and possibly stressed four-cell chain displays the extensive exocellular organic fibrillar matrix, presumably trichocysts (Fig. 4A, arrow). More slender tubular extensions from the surface of some cells appear to be discharged trichocysts. A higher magnification of SEM (Fig. 4B) shows the network organization of the secretory fibrils enclosing the cell surface. In addition to the exocellular organic matrix, the cell is surrounded by a more closely enclosing organic envelope (Fig. 4C) that partially obscures the underlying cingulum (thick arrow) and is more clearly evidenced at places where it is partially fractured (thin arrow) revealing the underlying cellular surface. An enlarged view of a terminal cell in a chain (Fig. 4E) exhibits the transverse flagellum (thick arrow) encircling the cell slightly anterior to the depression of the cingulum and situated on the surface of the epicone.

An overview of the nuclear region and peripheral cytoplasm (Fig 5A) as observed by transmission electron microscopy shows the mesocaryotic nucleus (N), approximately 12 μm in diameter, with condensed chromosomes (~ 1 μm diameter), and some of the large peripheral cytoplasmic vacuoles (V) and plastids (P) that are typically distributed near the periphery of the cytoplasm. The inset (Fig 5A) illustrates the organization of the two kinds of ejectosomes, elongated trichocyst-like organelles that appear to be quadrangular in cross-section (large arrow) and elongated in longitudinal section (L), and smaller mucocyst ejectosomes (small arrows) that have a dense central core. An enlarged view of the peripheral cytoplasm (Fig 5B) displays a mitochondrion (arrow) with tubular cristae and, adjacent to it, the peripheral alveolar membranes surrounding the cell with a thin somewhat electron dense organic deposit within the lumen of the alveolar membranes. A higher magnification image of the cell periphery (Fig 5C) shows the alveolar membranes (thick arrow) in more detail, including the organic deposit (~ 60 nm thick) within the alveolar space. An organic outer envelope or pellicle, external to the alveolar membranes, is limited externally by an osmiophilic thin layer (thin arrow). This outer layer of the organic envelope lies approximately 1 μm or greater from the plasma membrane and underlying alveolar membranes at the surface of the cell. A thin layer of cytoplasm encloses the cell beneath the organic envelope and immediately external to the alveolar membranes. A segment of a mitochondrion (M) and an ejectosome, that appears to be a mucocyst (E) docked at the plasma membrane, are shown near the peripheral cell membranes. An enlarged view (Fig 5D) of a typical mitochondrion (M) shows the elongated profile (up to 3 μm long) and the tubular cristae (~ 40 nm in diameter) in greater detail. An oblique tangential section, passing through the nearby alveolar membrane complex at the periphery of the cell, shows the texture of the internal dense organic deposit (arrow) and the less dense enclosing membranes on either side.

458 Since this is an oblique tangential section, the image displays the membranes and internal
459 organic deposit in a partial sheet-like perspective. The chloroplasts (Fig 5E&F) are
460 surrounded by three membranes and contain a simple pyrenoid (Py), without internal
461 thylakoids. There are occasional osmiophilic granules (60 nm diameter) within the stroma of
462 the plastid as seen more clearly at the base of the chloroplast section in Fig 5F. A portion of
463 an elongated mitochondrion (M) is also visible within a cytoplasmic lobe near the plastid.
464 Each chloroplast lamella (~ 40 nm thick and 60 nm apart), shown in high magnification (Fig
465 5G), contains up to three thylakoids (arrow) and is suspended in a somewhat finely granular
466 stroma.
467
468 Our sequencing of LSU rDNA from two separate isolates from Flanders Bay, NY,
469 USA (CpFB-06-1, CpFB-06-2), showed identical sequences among the isolates for both the
470 D1-D3 and D4-D6 regions (Accession Nos. EF110556 and EF110557, respectively). The
471 D1-D3 region sequence of NY isolates showed similarity to three GenBank sequences from
472 Korean isolates of *Cochlodinium polykrikoides* (Accession Nos. AY347309, AY725423,
473 AF067861) with identities of 90%, 90% and 88%, respectively. The D4-D6 region of our
474 strains showed 89% identity with the Korean strain (AY347309). The sequencing efforts of
475 Matsuoka, Iwatake, Mikulski and Doucette (this issue) permit our isolates to be compared to
476 additional clones in North American and southeast Asia. The D1-D3 and D4-D6 region
477 sequences of our isolates displayed 100% identity with two North American *C. polykrikoides*
478 strains: CPCB10 isolated from Cotuit Bay, MA, USA, and CPPV-1, isolated from Bahia de
479 La Paz, Mexico. Regarding the Korean and Hong Kong strains of Mikulski and Doucette
480 (this issue; Accession Nos. pending), we had 90.4% alignment with their Korean and Hong
481 Kong strains for the D1-D3 region and 97.5% alignment with the D4-D6 region. Therefore, it

482 is clear our isolates are not con-specific with the isolates from Korea and Hong Kong
483 identified as *C. polykrikoides*.
484
485 *3.3. Bioassay experiments - shellfish*
486 During the shellfish experiments, the bloom water treatment maintained mean
487 *Cochlodinium* densities of $3.7 \pm 1.3 \times 10^4$ cells ml^{-1} (range = 2.7 - 6.1 x 10^4 cells ml^{-1}),
488 accounting for 91 ± 5% of the algal biomass. *Cochlodinium* was not detected in filtered
489 water treatments throughout this experiment and was always < 10^2 cell ml^{-1} in the Great
490 Peconic Bay water treatment. Bay scallops (*Argopecten irradians*) and oysters (*Crassostrea*
491 *virginica*) exposed to waters containing bloom concentrations of *Cochlodinium* experienced
492 significantly increased mortality and significantly decreased growth rates (scallops only)
493 relative to filtered bloom water, non-bloom water and filtered non-bloom water ($p < 0.001$ for
494 all; Tukey test; Fig 6). For scallops, filtered bloom water, non-bloom water and filtered non-
495 bloom water treatments displayed growth rates of ~0.2 mm d^{-1} and 100% survival of all
496 individuals in all replicates during the 9-day experiment (Fig 6A&B). In contrast, scallops
497 exposed to bloom water grew half as fast, began to die within 48 h of exposure and
498 experienced 67 ± 13 % mortality by the end of the 9-day experiment (Fig 6A&B). Oysters
499 exposed to *Cochlodinium* bloom water displayed lower mortality than scallops (16 ± 3.3 %;
500 Fig 6A), but significantly greater mortality than control treatments (Fig 6A; $p < 0.05$; Tukey
501 test).
502
503 Histopathological evaluation of scallop tissue revealed gill hyperplasia, as well as
504 hemorrhaging in gills and digestive tracts (Fig 7). Moreover, *Cochlodinium* cells, found
505 within scallop gills, were associated with tissue inflammation (Fig 7). By contrast, there
506 were no signs of starvation in morbid individuals. The digestive glands of oysters displayed

severe hemorrhaging and squamation, while apoptosis was observed in gill tissues (Fig 7). Apoptosis was not observed in scallop tissues. Examination of shellfish exposed to non-bloom water did not reveal any of the histopathological conditions described for the individuals exposed to bloom water.

3.4. Bioassay experiments - fish

Experiments were conducted with sheepshead minnows (*Cyprinodon variegates*) using bloom water with *Cochlodinium* densities ranging from 0.59 - 1.3 x 10^5 cells ml^{-1}. In all experiments, controls (non-bloom water, filtered non-bloom water, and filtered bloom water) always displayed 100% survival for the duration of experiments (> 96 hr), demonstrating that physical contact with cells was required for fish mortality. In contrast, individuals exposed to undiluted bloom water (0.59 - 1.3 x 10^5 cells ml^{-1}) began to expire within 10 minutes, and no individual (n = 144) survived longer than 9 h (Fig 8). In the most extreme experiment (*Cochlodinium* cell density = 1.3 ± 0.2 x 10^5 cells ml^{-1}), fish in all 24 wells perished within 30 minutes. While there was no obvious relationship between the initial cell density and the survival time of minnows, there was a hyperbolic relationship between the initial cell density and the fraction of minnows surviving after 24 h (Fig 8). Specifically, all fish survived 24 h at cell densities of $\leq$ 1.1 ± 0.1 x 10^3 and all experiments with > 5 x 10^4 cells ml^{-1} displayed 100% mortality (Fig 8). At intermediate densities, which represented dilutions of bloom water with 0.2 µm-filtered water, the percentages of fish populations which died (4 - 83%) were proportional to *Cochlodinium* cell densities (0.3 - 3.2 x 10^4 ml^{-1}; Fig 8). The filtration of water through a 0.2 and 2.0 µm filters resulted in 100% survival of fish for the duration of experiments, whereas mortality of fish in 10 and 20µm filtrations were proportional to the densities of *Cochlodinium* which passed through these filters (Fig 8). Minnows exposed to boiled or frozen cells at concentrations of 1.3 ± 0.4 x 10^5

532 cells ml^{-1} displayed 24h-survival rates of 83 and 96%, respectively, which were significantly
533 higher than that observed in the unamended bloom water (0% survival).
534
535 In the assays exposing *Fundulus heteroclitus* to the bloom water with $9.1 \pm 0.3 \times 10^4$
536 cells ml^{-1} of *Cochlodinium* (representing 94 ± 5% of the total algal biomass), the fish
537 displayed increased mortality with time: two-thirds of individuals died within the first 4 h of
538 exposure and 83 ± 10% expired within 24 h, in contrast to 100% survival in the control using
539 filtered bloom water (Fig 9A). In the experiment with *Fundulus majalis*, the *Cochlodinium*
540 cell densities were $9.2 \pm 0.4 \times 10^4$, $2.8 \pm 0.4 \times 10^4$, $7.2 \pm 1.1 \times 10^3$, and 0 (control) cells ml^{-1}.
541 In the first three treatments, *Cochlodinium* accounted for > 90% of the total algal biomass.
542 Fish exposed to the highest *Cochlodinium* density experienced 100% mortality after 15 h (Fig
543 9B), whereas the fish exposed to bloom water with lower cell densities ($2.8 \pm 0.4 \times 10^4$ and
544 $7.2 \pm 1.1 \times 10^3$ cells ml^{-1}) displayed significantly lower mortality (50 ± 9.6% and 22 ± 5.5%;
545 Fig 9B; $p < 0.05$; Tukey test). Fish in the filtered seawater control exhibited a 100% survival
546 (Fig 9B).
547
548 During the experiment with *Menidia menidia*, the bloom water treatments were 8.5 ±
549 1.5×10^4, $4.7 \pm 1.1 \times 10^4$, and $1.3 \pm 0.2 \times 10^4$ and 0 *Cochlodinium* cells ml^{-1}. In the first three
550 treatments, *Cochlodinium* accounted for > 90% of the algal biomass. *M. menidia* exposed to
551 *Cochlodinium* densities of $8.5 \pm 1.5 \times 10^4$ and $4.7 \pm 1.1 \times 10^4$ cells ml^{-1} experienced 100%
552 mortality after 1.5 and 5.5 h, respectively (Fig 9C), whereas individuals exposed to 1.3 ± 0.2
553 $\times 10^4$ cells ml^{-1} displayed 23 ± 5.7% mortality during this 24 h experiment (Fig 9C). By
554 contrast, fish in the filtered seawater control treatment exhibited a survival rate (100%) which
555 was significantly greater than all other treatments (Fig 9C; $p < 0.05$; Tukey test).
556

557 The impact of *Cochlodinium* exposure on fish was clearly shown via
558 histopathological examination of gill tissue. Microscopic evaluation of the gills of moribund
559 fish demonstrated the presence of mild to moderate multifocal to diffuse epithelial
560 proliferation (Fig 10). Focal areas of fusion of adjacent lamellae were common (Fig 10).
561 Histopathological examination of the gills of fish exposed to control, non-bloom water
562 displayed no signs of epithelial hyperplasia or fusion of adjacent lamellae.
563
564 **4. Discussion**
565 *4.1. Species identification*
566 There are multiple features of the *Cochlodinium* species blooming on eastern LI
567 which are consistent with the description of *C. polykrikoides* (Margelef 1961; Taylor et al.,
568 1995): the cells often formed short chains consisting of two, four, and rarely eight cells; the
569 individual cells possessed a rounded epicone; a cingulum making about two turns around the
570 cell; slightly or heavily bilobed at the antapex (Fig. 3); a sulcus with a torsion of about one
571 turn (Fig. 3); a red stigma located on the dorsal side of the episome (Fig. 3); numerous band-
572 shaped chloroplasts evenly distributed in the cell (Figs 3 and 5F); and the nucleus is located
573 in the episome (Fig. 4A). As seen in *C. heterolobatum* (Silva,1967), a synonym of *C.*
574 *polykrikoides* (Taylor et al., 1995; Steidinger and Tangen, 1997), our cells also displayed a
575 pellicle or organic envelope and two kinds of ejectosomes (an elongate trichocyst and a
576 mucocyst; Fig 4). Another feature of our species consistent with *C. polykrikoides* is its acute
577 toxicity to shellfish and finfish (see *Bloom impacts* section below). Although the presence
578 and shape of an apical groove is unknown in our isolates at the moment, the above-listed
579 overwhelming similarities encourage us to identify our species as *C. polykrikoides*.
580

581 Our designation of the New York bloom-forming *Cochlodinium* to the species *C.*
582 *polykrikoides* is also strongly supported by our LSU rDNA sequences. The New York
583 isolates showed 100% similarity with the *C. polykrikoides* isolate from Massachusetts Bay
584 and 99.9% with the La Paz, Mexico clone in the D1-D3 region. By contrast, there were
585 substantial differences in sequence alignment to strains from Korea and Hong Kong, with
586 similarities ranging from 89 – 90% in this region of the LSU. Such differences, coupled with
587 some outstanding morphological differences (e.g. cell compression) may require the
588 designation of a new species for either the North American *Cochlodinium* isolates or the
589 Korea and Hong Kong isolates in the future (Matsuoka, Iwatake, Mikulski and Doucette, this
590 issue). Considering *C. polykrikoides* (and *C. heterolobatum* as well) was originally described
591 from the North America, it seems it is more reasonable to create a new species for the Asian
592 isolates having been identified as *C. polykrikoides*.
593
594 *4.2. Bloom dynamics*
595 This study represents the first report of red tide dinoflagellate blooms caused by *C.*
596 *polykrikoides* within the Peconic and Shinnecock Estuaries of eastern Long Island. While
597 blooms caused by *C. polykrikoides* have been common in some parts of Asia, Korea and
598 Japan in particular (Yuki and Yoshimatsu. 1989; Kim, 1998; Kim et al., 1999; Park et al.,
599 2001; Yamatogi et al., 2006), blooms have been rarely reported in the US. Prior to this
600 special issue, the only noted occurrence of *C. polykrikoides* blooms in the US have been in
601 the York River, VA, and Barnegat Bay, NJ (Silva, 1967; Ho and Zuboff 1979).
602 Temperatures during the bloom initiation and peak period on eastern Long Island were within
603 the range at which *C. polykrikoides* grows optimally (21 and 26 °C), although the decline in
604 temperature during late August and September from 25°C to below 20°C may have
605 contributed to the bloom's demise (Table 1; Kim et al., 2004; Yamatogi et al., 2006). The

606 salinities found during this study (22 – 30; Table 1) were generally below the optimal range
607 for this species in Asian waters (30 and 36; Table 1; Kim et al., 2004; Yamatogi et al., 2006),
608 perhaps evidencing an ecological difference between Asian and North American strains of *C.*
609 *polykrikoides*.
610
611 Interestingly, the regions which are currently plagued with *C. polykrikoides* blooms in
612 Long Island (Fig 1) and Rhode Island (Smayda and Tomas, this issue) formerly hosted brown
613 tides caused by *Aureococcus anophagefferens* during the late 1980s and 1990s (Gobler et al.,
614 2005). In a manner similar to brown tides, *C. polykrikoides* blooms have been most intense
615 in the far western regions of the Peconic Estuary (Flanders, Great Peconic; Fig 1) which have
616 the highest ambient nitrogen concentrations and the longest residence times within this
617 system (Hardy, 1976; Nuzzi and Waters, 2004). Chlorophyll levels during the peak of *C.*
618 *polykrikoides* blooms often exceeded 100 $\mu g\ L^{-1}$ which is five-times greater than biomass
619 levels recorded during *Aureococcus* blooms (Gobler et al., 2005). It is likely that the higher
620 biomass of these blooms requires a larger nutrient supply for blooms to be maintained.
621 Unlike *Aureococcus* blooms, which achieved maximal cell densities within the major basins
622 of the Peconic Estuary and displayed lower concentrations within creeks and tributaries, *C.*
623 *polykrikoides* densities drop precipitously east of Great Peconic Bay, but are often maximal
624 within shallow tributaries, which have high nitrogen loads (Fig 1; Nuzzi and Waters., 2004).
625 The stronger association of *C. polykrikoides* with regions having high levels of nitrogen
626 suggests that it may be more directly linked with inorganic nutrient eutrophication than
627 *Aureococcus,* which exploited estuarine regions with copious supplies of dissolved organic
628 nitrogen but lower levels of dissolved inorganic nitrogen (LaRoche, et al., 1997; Gobler and
629 Sañudo-Wilhelmy, 2001; Gobler et al., 2005). The timing of *C. polykrikoides* blooms, which
630 emerge during late summer (August – September) also differs from that of *Aureococcus*

631 blooms, which typically developed during early summer (May, June; Gobler et al., 2005).
632 Since *C. polykrikoides* has well-documented phagotrophic capabilities (Larsen and Sournia,
633 1991; Jeong et al., 2004) and since blooms have been most prevalent in the regions of the
634 Peconic estuary where nitrogen and chlorophyll *a* are highest (Nuzzi and Waters, 2004),
635 initiation of the *C. polykrikoides* blooms may be promoted by peaks in smaller prey
636 phytoplankton that appear during August. August previously represented the annual peak in
637 chlorophyll *a* in the Peconic Estuary (Bruno et al., 1983).
638
639 *C. polykrikoides* blooms on eastern Long Island have been heterogeneous in space
640 and time. Our sampling of both fixed stations and specific bloom 'patches' within regions of
641 the Peconic Estuary revealed that ambient concentrations can differ by two orders of
642 magnitude between patches and ambient water (Fig 2). Consistent with prior research (Park
643 et al., 2001), our preliminary observations also indicate a strong vertical component to these
644 blooms, with 'patches' appearing in surface water during the late morning and persisting until
645 the evening. We have also noted cells within bloom patch water can sometimes aggregate,
646 sink, and die after a short period of containment (~ 2 hr). As such, recording precise cell
647 densities during *C. polykrikoides* blooms is challenging. Regardless, our rapid processing of
648 samples in 2006 (< 2 h) demonstrated that bloom patches, which can cover up to 1 km^2 and
649 occupied most of the western Peconic Estuary and eastern Shinnecock Bay during late
650 August of 2006, achieved cell densities > 10^5 cells ml^{-1}, a density consistent with prior
651 reports of blooms by this species in North America (Whyte et al., 2001), but lower than levels
652 reported by others in southeast Asia (Yuki and Yoshimatsu, 1989). Due to the heterogeneous
653 nature of *C. polykrikoides* events, it seems likely that fish and shellfish would be exposed to
654 both high (> 10^5 cells ml^{-1}) and lower concentrations of cells (~ 10^4 cells ml^{-1}) during blooms.
655

656 *4.3. Bloom Impacts:*

657 To date, multiple investigators have reported on fish mortalities associated with *C.*
658 *polykrikoides* blooms around the world (Yuki, and Yoshimatsu, 1989; Whyte et al., 2001;
659 Gárrate-Lizárraga. et al., 2000). Our experimental work demonstrates the very rapid death (<
660 24 hr) of four species of fish (*Cyprinodon variegates, Fundulus heteroclitus, Menidia*
661 *menidia, Fundulus majalis*) when exposed to dense *C. polykrikoides* blooms (Fig 8,9). No
662 individual from any species survived 24 h of exposure to 10^5 cells ml^{-1}, whereas intermediate
663 levels of mortality occurred when experimental densities ranged from 10^3- 10^4 cells ml^{-1} (Fig
664 8,9). These results are consistent with prior research conducted with salmon smolts (*Salmo*
665 *salar*) and juvenile slipmouths (*Leiognathus nuchalis*) which displayed 20 – 90% mortality
666 when exposed to *C. polykrikoides* cell densities ranging from 10^3- 10^4 cells ml^{-1} (Onoue et al.,
667 1985; Yuki and Yoshimatsu, 1989; Whyte et al., 2001).
668
669 To date, there has been substantial controversy surrounding the mechanism of fish
670 mortality associated with *C. polykrikoides.* While Kim et al. (1999, 2000) indicated fish
671 mortality was associated with reactive oxygen species made by the alga, Kim et al. (2002)
672 suggested polysaccharides were more likely to be the cause of fish mortality. To complicate
673 matters further, three toxic fractions (neurotoxic, hemolytic, and hemagglutinative) and two
674 paralytic shellfish poisons (a zinc complex of carbomoyl-N-sulfo-11α-hydroxyneosaxitoxin
675 sulfate (Ic-1) and its 11β epimer (epi-Ic-1) have been isolated from *Cochlodinium* sp. (as
676 *Cochlodinium* type '78 Yatushiro; Onoue and Nozawa, 1989a&b). Consistent with prior
677 findings, our experimental results demonstrate that physical contact with cells is required for
678 fish mortality (Onoue et al., 1985; Yuki and Yoshimatsu, 1989), as all fish exposed to 0.2 or
679 2 μm filtered seawater survived during all experiments. Exposure of fish to cells which had
680 been killed via freezing yielded almost no mortality (4%) in fish after 72 h while parallel

681 whole water treatments killed all fish (n=24) within 30 minutes. Our SEM and TEM
682 micrographs revealed the presence of a thick polysaccharide coat (> 1 μm) surrounding *C.*
683 *polykrikoides* cells (e.g., Fig 5), indicating this biochemical matrix may be the source of fish
684 mortality as had been reported for this species (Kim et al 2002) and other HABs (Gainey and
685 Shumway, 1990). The three-dimensional structure of polysaccharides collapses upon
686 freezing due to the loss of water and this structure is not recovered when thawed (Doerr et al.,
687 2000; Schwarzenbach et al., 2003). Hence, the loss of ichthyotoxicity of cells upon freezing
688 and thawing could be due to the degradation of polysaccharides, suggesting this matrix, or a
689 principle within this matrix, is the ichthyotoxic agent associated with *C. polykrikoides*.
690
691 Fish exposed to bloom levels of *C. polykrikoides* for < 48 h displayed fusion of
692 adjacent gill lamellae (Fig 10). The lesions observed were characteristic of an external insult
693 to the gills which is commonly seen in external parasitic infestations, particularly in protozoal
694 infestations (Roberts 2001). These hyperplastic lesions suggest severe impairment of gill
695 function (e.g. respiration, nitrogen excretion, ion balance) which could cause fish death
696 (Roberts, 2001)
697
698 *C. polykrikoides* blooms on eastern Long Island may have negative impacts on
699 ambient fish populations and, in turn, the entire ecosystem. While ambient densities of *C.*
700 *polykrikoides* densities were ~10^3 cells ml^{-1} at our primary sampling sites during June and
701 July of 2006, densities were consistently > 10^3 cells ml^{-1} and frequently > 10^4 cells ml^{-1}
702 during the end of August and beginning of September of 2006. Although only sporadic fish
703 kills were observed within tributaries during this time period, complete mortality of fish held
704 in flow through seawater chambers at the Stony Brook – Southampton Marine Science Center
705 was observed on multiple occasions at this time. This finding suggests that the combination

706 of high cell densities and captivation of fish may cause maximal mortality (Whyte et al.,
707 2001). Moreover, since most of the fish species examined during this study are important
708 prey items for commercially important finfish in the region (Juanes et al., 1993; Juanes and
709 Conover. 1995), *C. polykrikoides* blooms may impact the entire food web.
710
711 Previous studies have reported on the negative effects of *C. polykrikoides* on shellfish.
712 The metamorphosis of oyster (*Crassostrea gigas*) larvae is slowed during blooms
713 (Matsuyama *et al*. 2001), and mortality of larvae of the American oyster, *Crassostrea*
714 *virginica*, is elevated by exposure to *C. polykrikoides* (Ho and Zubkoff, 1979). To our
715 knowledge, this study represents the first report of mortality in juvenile-stage *Argopecten*
716 *irradians* and *Crassostrea virginica* and reduced growth rates in *Argopecten irradians* caused
717 by exposure to bloom densities of *C. polykrikoides* (Fig 6).
718
719 Histopathological evaluation of shellfish exposed to *C. polykrikoides* for
720 approximately one week revealed the severe hyperplasia and gill inflammation associated
721 with exposure to cells would cause a reduction of gas exchange within scallops (Fig 7).
722 Moreover, hemorrhaging and squamation of gill and digestive epithelia (Fig 7) would leave
723 scallops vulnerable to secondary bacterial infections which were occasionally observed in
724 analyzed specimen (data not shown). Although these symptoms clearly indicate a deleterious
725 effect of *C. polykrikoides* on bivalves, they are not specific enough to allow a precise
726 mechanistic determination of the cause. We did note that scallops exposed to bloom waters
727 were often closed, while control individuals were open and presumably feeding. Such
728 differences were not discernable among oysters. Regardless, the presence of *C. polykrikoides*
729 cells does elicits an inflammatory response in shellfish tissue (Fig 7), which might be related
730 to the polysaccharide coating of microalgal cells. It is noteworthy that Sogawa et al. (1998a

731 & b) have demonstrated that extracellular polysaccharides produced by another
732 dinoflagellate, *Gymnodinium* sp., are capable of inducing apoptosis in human lymphoid cells.
733 On the other hand, prior reports documented the production by *Cochlodinium* of hemolytic
734 toxins (Yatushiro et al., 1989a&b). Such toxins are capable of causing lesions and alterations
735 in epithelial barriers of vertebrates and invertebrates similar to those observed here in gills
736 and digestive epithelia of exposed shellfish (Landsberg, 2002).
737
738 Our results demonstrate that *C. polykrikoides* blooms may endanger native shellfish
739 populations on eastern Long Island. During the 2005 *C. polykrikoides* bloom, a massive soft
740 shell clam (*Mya arenaria*) mortality event occurred in Flanders Bay, when dead individuals
741 washed up in racks on the shore line and moribund individuals were found with dinoflagellate
742 cells in their hemorrhaged digestive tracts (B. Allam, unpubl.). Bay scallops on eastern Long
743 Island were formerly the top scallop fishery on the east coast of the US (Hoagland et al.,
744 2002). During the intense brown tide caused by *Aureococcus* during the 1980s, the
745 population experienced recruitment failure and a subsequent tremendous population
746 reduction (Gobler et al., 2005). A major brown tide has not occurred in the Peconic Estuary
747 in nearly a decade, but the scallop population has not recovered, despite ongoing efforts to
748 reseed and restore it. Our results demonstrate that the failure of this population to recover
749 could be due, in part, to the recent outbreaks of *C. polykrikoides* blooms in this system.
750
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Table 1. Temperature (T) in °C, salinity (S), *Cochlodinium polykrikoides* cell densities from fixed station sampling (Cells), and *Cochlodinium polykrikoides* cell densities from bloom patches during August and September (Patch) in Flanders Bay, Meetinghouse Creek, Great Peconic Bay, and eastern Shinnecock Bay. Cell densities are reported as means and standard deviation on parentheses. Dashes indicate samples from a given location and date were not taken.

	Flanders Bay				Meetinghouse Creek				Great Peconic Bay				eastern Shinnecock Bay			
Date	**T**	**S**	**Cells**	**Patch**	**T**	**S**	**Cells**	**Patch**	**T**	**S**	**Cells**	**Patch**	**T**	**S**	**Cells**	**Patch**
21-Jun	22.5	25.6	0.0 (0.0)	-	23.4	22.2	0.0 (0.0)	-	-	-	-	-	21.2	28.1	0.0 (0.0)	-
26-Jun	23	26	0.0 (0.0)	-	-	-	-	-	-	-	-	-	-	-	-	-
6-Jul	23.9	23.7	1 (0.4)	-	25.2	23.5	0.8 (0.1)	-	-	-	-	-	24	24.6	0.4 (0.0)	-
13-Jul	24.6	25.9	0.9 (0.1)	-	24.2	22.4	1.2 (0.0)	-	-	-	-	-	23.5	23	1.7 (0.0)	-
18-Jul	29.1	24.4	0.7 (0.1)	-	-	-	-	-	-	-	-	-	-	-	-	-
27-Jul	26.2	26.2	1.1 (0.2)	-	28.3	22.1	1.6 (0.2)	-	-	-	-	-	26.2	23.6	3.2 (0.5)	-
3-Aug	29.5	25.1	1.7 (0)	-	29.1	23.5	1.6 (0.0)	-	-	-	-	-	30.8	24.9	2.6 (0.4)	-
9-Aug	27.4	26.5	1.4 (0.2)	-	27.7	25.5	1.3 (0.4)	-	-	-	0.0 (0.0)	-	25.1	28.3	2.0 (0.2)	-
15-Aug	25	25.8	1.4 (0.2)	-	24.6	24.3	1.4 (0.2)	-	-	-	-	-	26.3	27.3	2.9 (0.0)	-
22-Aug	25.3	26.5	4.3 (0.3)	4.8 (0.6)	25.4	24.9	2.2 (0.2)	-	24.9	28	1.1 (0.4)	-	25.2	28.8	4.6 (0.0)	4.9 (0.4)
23-Aug	25.0	26.3	3.6 (0.0)	5.2 (0.3)	24.3	23.6	3 (0.2)	-			-	5.0 (0.4)	-	-	-	-
25-Aug	24.0	26.1	3.6 (0.1)	4.9 (0.4)	-	-	-	-	23.5	28.5	4 (0.4)	4.5 (0.4)	24.5	29	3.8 (0.0)	-
28-Aug	22.2	25.4	3.4 (0.00)	4.9 (0.4)	-	-	-	-	-	-	-	5.0 (0.3)	-	-	-	-
30-Aug	20.6	24	4.5 (0.8)	5.2 (0.1)	21.1	22	1.5 (0.1)	4.3 (0.2)	20.1	27.3	3.7 (0.8)	4.7 (0.8)	20.8	29.2	4.5 (1.2)	-
5-Sep	20.3	25.8	3.2 (0.1)	4.5 (0.3)	20.2	24.4	2.8 (0.5)	3.3 (0.2)	19.9	27.3	-	-			5.1 (0.7)	5.1 (0.1)
7-Sep	20.4	25.1	2.9 (0.2)	4.2 (0.0)	20.8	23.5	2.6 (0.02)	4.1 (1.0)	19.9	27.5	3.5 (0.5)	4.3 (0.1)	21.5	30	3.0 (0.2)	-
14-Sep	20.1	25.3	2.7 (0.0)	4.1 (0.2)	20.7	24	2.4 (0.1)	-	19.6	27.1	3.8 (0.0)	-	18.6	26.8	2.0 (0.0)	5.1 (0.1)
19-Sep	-	-	-	-	-	-	-	-	-	-	-	-			5.0 (0.0)	5.0 (0.6)
21-Sep	19.5	25.5	1.3 (0.0)	-	20.2	24.7	1.6 (0.0)	-	19.6	27.5	0.5 (0.4)	-	20.1	27.5	5.1 (0.1)	5.1 (0.1)
28-Sep	19.1	26.2	0.0 (0.0)	-	19	22.6	0.0 (0.0)	-	19.2	27.4	0.0 (0.0)	-	19.7	27.9	1.3 (0.3)	-
1-Oct	19	26.1	0.0 (0.0)	-	19.2	23.1	0.0 (0.0)	-	19.2	27.3	0.0 (0.0)	-	19.4	24.7	0.0 (0.0)	-

950 **Figure Legends**

951 Fig 1. Spatial distribution of *Cochlodinium* cells in the Peconic Estuary, early September
952 2005. Gradients are based log of cell densities measured at 25 samples across the estuary.
953
954 Fig 2. Log of *Cochlodinium* cell densities recorded during the summer of 2006 in Flanders
955 Bay (FB - circles), Meetinghouse Creek (MHC-squares), Great Peconic Bay (GPB-triangles),
956 and eastern Shinnecock Bay (SB-diamonds). Open symbols represent fixed stations, whereas
957 closed symbols represent dense bloom patches present at each location. Error bars are
958 standard error of triplicate field samples.
959
960 Fig. 3. Light microscopic views of paired and catenated cells of *Cochlodinium* sp. showing
961 the major morphological features, including the somewhat rounded form of the cells,
962 especially those more internal in a chain, and the surface details, e.g. sulcus (S) and the
963 deeply grooved cingulum (arrows). Scale bar = 10 μm.
964
965 Fig. 4. SEM views of surface details of *Cochlodinium* sp. (A) An overview of four
966 adlineated cells with an extensive exocellular fibrillar matrix (arrow) is shown uniting the
967 cells longitudinally and extending outward as filaments anchored to the surface of the
968 Millipore substratum. Scale bar = 20 μm. (B) A higher resolution image of the exocellular
969 matrix showing the fibrillar network in greater detail and the organization of the nodes where
970 the fibrils are joined. Scale bar = 10 μm. (C) An enlarged image of a terminal cell in a chain,
971 and its adjacent neighbor, showing the thin organic envelope or pellicle that encloses each
972 cell and underlies the exocellular fibrillar matrix. The cingulum (thick arrow), obscured by
973 the organic envelope and appearing as a surface depression, has an emergent transverse
974 flagellum (TF) that is partially dislocated from the groove and is stretched out across the

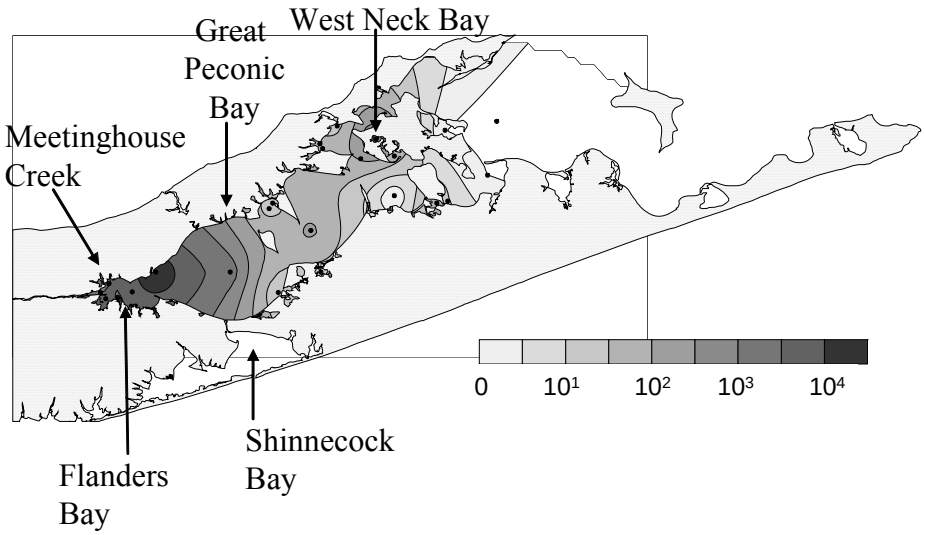
975 epicone onto the surrounding Millipore surface. A fracture in the organic envelope (thin
976 arrow) reveals the approximate thickness of the envelope and exposes the underlying surface
977 of the cell. Scale bar = 10 μm. (D) A chain of three cells exhibits the transverse flagella
978 (black arrows) in a more typical organization encircling the cell. Note that the middle cell of
979 the three shows a small pore in the organic envelope where the transverse flagellum emerges.
980 For purposes of comparison, an armored, theca-bearing smaller dinoflagellate (white arrow),
981 that was included in the water sample, is shown with a clearly exposed cingulum. Scale bar =
982 10 μm. (E) An enlarged view of a terminal cell of *Cochlodinium* in a chain, shows the
983 depression of the cingulum in clearer detail, and somewhat anterior to it, the location of the
984 transverse flagellum (black arrow) encircling the epicone. An apparent trailing flagellum
985 (white arrow) extends along the surface of the Millipore filter. Scale bar = 10 μm.
986
987 Fig. 5. Transmission electron microscopic images of ultrathin sections of *Cochlodinium* sp.
988 (A) An overview of the nuclear region and peripheral cytoplasm, showing the nucleus (N)
989 located eccentrically near one side of the cell, large vacuoles (V), and chloroplasts (P) near
990 the cell periphery. Scale bar = 5 μm. Inset displays sections of the two kinds of ejectosomes,
991 trichocyst-like organelles that are quadrangular in cross section (large arrow) and elongated
992 in longitudinal section (L), and smaller mucocysts (small arrows) that have a more circular
993 profile in cross section containing an electron dense central core. Compare to the ejectosome
994 (E) in Fig. 5C. Scale bar = 0.5 μm. (B) A higher magnification view of the cell periphery
995 shows a tubulocristate mitochondrion (arrow) within a cytoplasmic lobe near the alveolar
996 membranes at the surface of the cell. Scale bar = 0.2 μm. (C) A high magnification image of
997 the cell surface illustrates the dense organic deposit (thin arrow) within the alveolar lumen
998 and the limiting osmiophilic thin layer (arrowhead) of an organic envelope or pellicle lying
999 external to the cell surface. A thin layer of dense cytoplasm, enclosing the cell and delimited

1000 within closely spaced plasma membranes, lies immediately adjacent to the external surface of
1001 the alveolar complex. A segment of a mitochondrion (M) and an oblique section through the
1002 sac of an ejectosome (E), probably a mucocyst docked at the plasma membrane, are shown
1003 within the cytoplasm. Scale bar = 0.4 μm. (D) A high magnification view of a mitochondrion
1004 (M) illustrates the typical elongated profile and the details of the tubular cristae. An oblique
1005 tangential section through the peripheral alveolar membranes of the cell exhibits the
1006 somewhat electron dense organic deposit (arrow) surrounded on both sides by the less dense
1007 alveolar membranes that appear somewhat sheet-like due to the glancing plane of the
1008 ultrathin section. Scale bar = 0.1 μm. (E and F) Profiles of chloroplasts are shown in an
1009 oblique cross section (E), exhibiting the lenticular pyrenoid (Py), and in longitudinal section
1010 (F) more clearly showing the organization of the lamellae. A cytoplasmic lobe containing a
1011 mitochondrion (M) is shown nearby to a plastid. Scale bars = 1.0 μm. (G) A high-resolution
1012 image shows the plastid fine structure containing up to 3 thylakoids per lamella (arrow).
1013 Scale bar = 0.1 μm.
1014
1015 Fig 6. Shellfish bioassay experiments. A.) Percent mortality of juvenile bay scallops
1016 (*Argopecten irradians* – closed symbols) and American oysters (*Crassostrea virginica* – open
1017 symbols) exposed to bloom water (squares), filtered (0.2 μm) bloom water (triangles), control
1018 water from Great Peconic Bay (squares), filtered (0.2 μm) water from Great Peconic Bay
1019 (circles). B.) Shell growth rates of juvenile bay scallops (*Argopecten irradians*) during nine
1020 exposure to the four treatments. Error bars are SD of triplicate containers for both graphs.
1021
1022 Figure 7. Photomicrographs showing histopathological alterations in bay scallops
1023 (*Argopecten irradians*; a to d) and American oysters (*Crassostrea virginica*; e and f). A, b
1024 and c: the presence of dinoflagellate-like cells in gills was associated with a severe

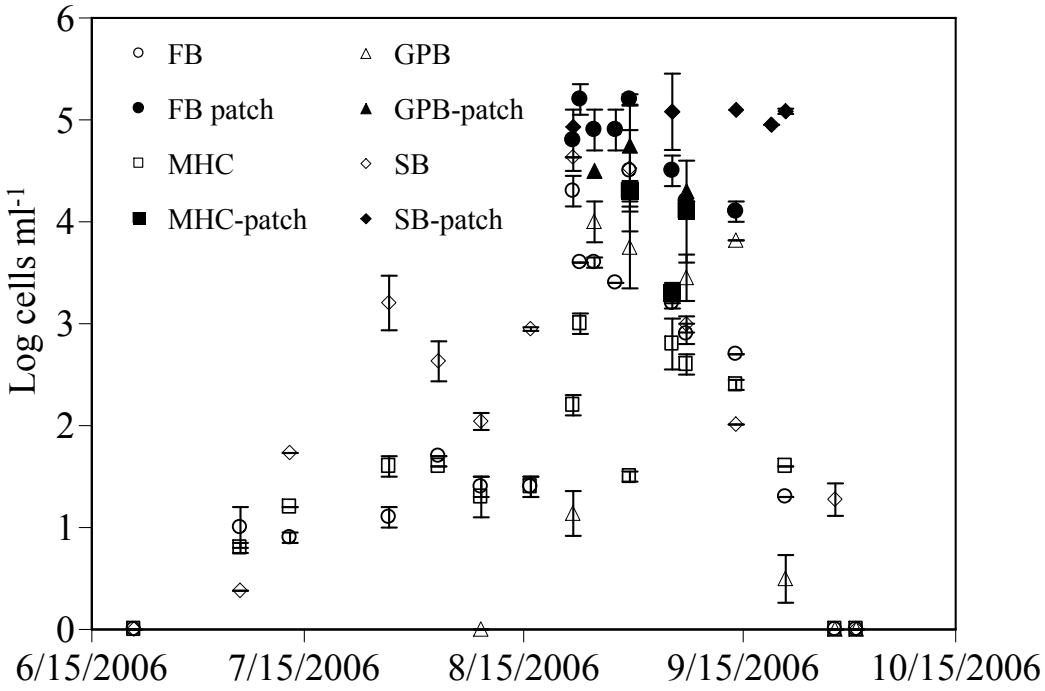
1025 inflammatory response and epithelial hyperplasia. Hemorrhage was also identified as free
1026 hemocytes in gill water tubules (c) and in the gut (d). In oysters, the inflammatory response in
1027 digestive gland (e) and gills (f) was associated with apoptotic figures (seen here as condensed
1028 chromatin in nucleus or round cells). Scale bar = 20 μm.
1029
1030 Fig 8. *Cyprinodon variegates* bioassay experiments. Percent mortality of *C. variegates* in 24-
1031 well plates after 24 h exposure to *Cochlodinium* bloom water containing cell densities
1032 ranging from 0 - 1.3 x 10^5 cells ml^{-1}.
1033
1034 Fig 9. Percent mortality of A.) *Fundulus heteroclitus* B.) *Fundulus majalis,*and C.) *Menidia*
1035 *menidia* during 24 h exposure to varying densities of *Cochlodinium* bloom water. Error bars
1036 are SD of triplicate containers.
1037
1038 Fig 10. Histopathological analysis of *Fundulus heteroclitus* gill tissue following 24 h
1039 exposure to *Cochlodinium* bloom water. Arrows depict regions of fused gill lamellae.
1040
1041
1042

1043

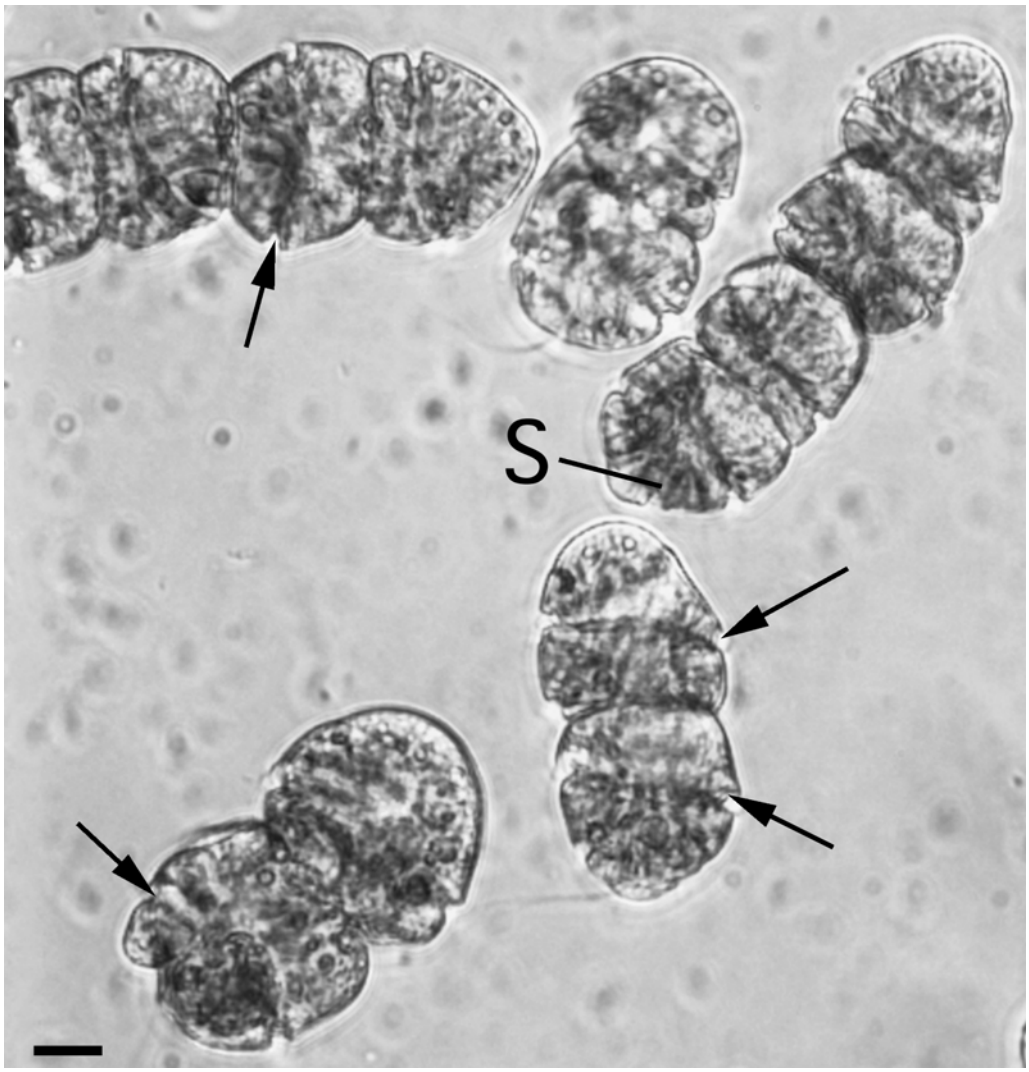
Fig 1


West Neck Bay
Great
Peconic
Bay
Meetinghouse
Creek
0
10^1
10^2
10^3
10^4
Shinnecock
Bay
Flanders
Bay

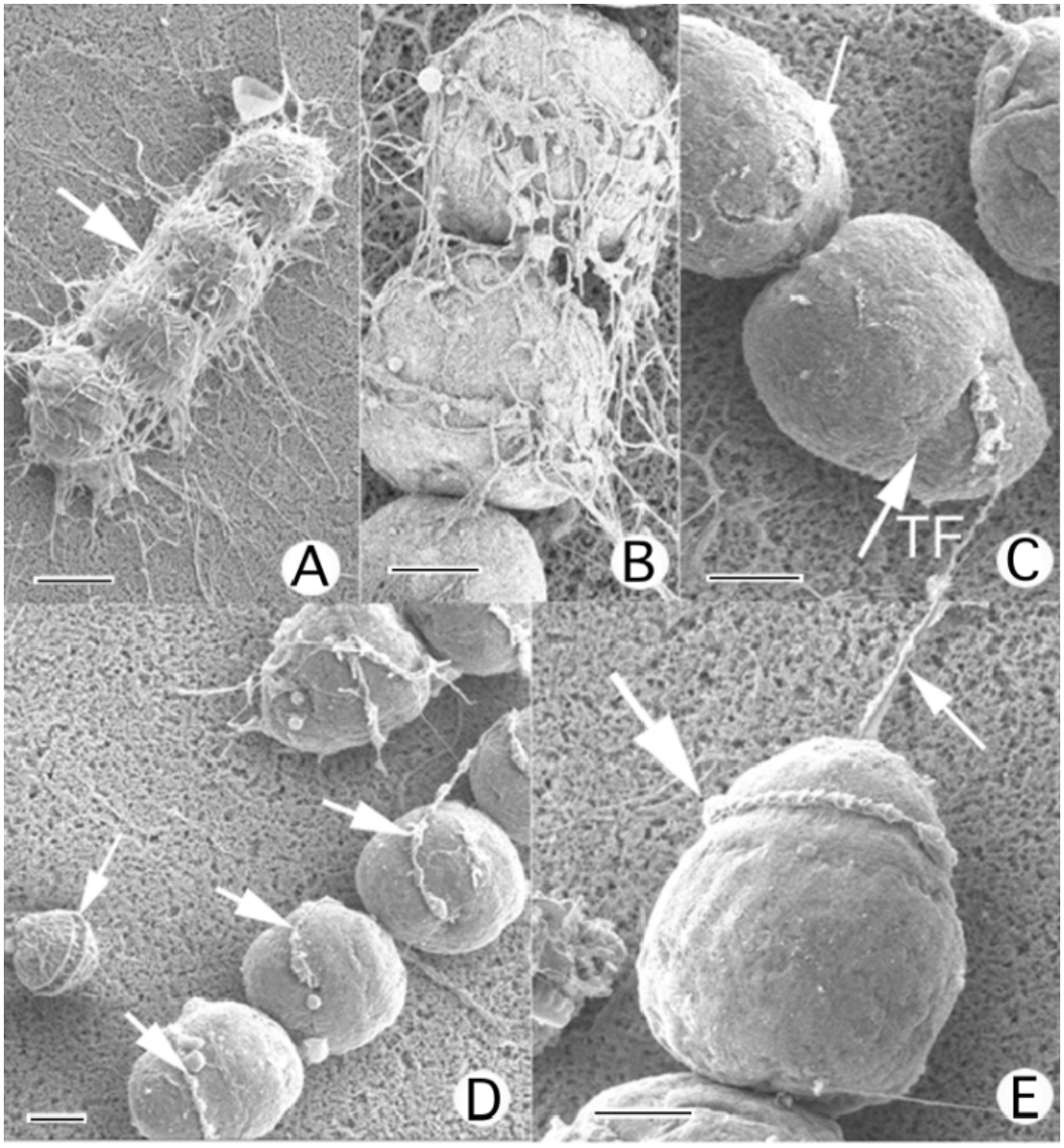
1044 **Fig 2**


FB
FB patch
MHC
MHC-patch
GPB
GPB-patch
SB
SB-patch
Log cells ml-1
0
1
2
3
4
5
6
6/15/2006
7/15/2006
8/15/2006
9/15/2006
10/15/2006

1045 **Fig 3**

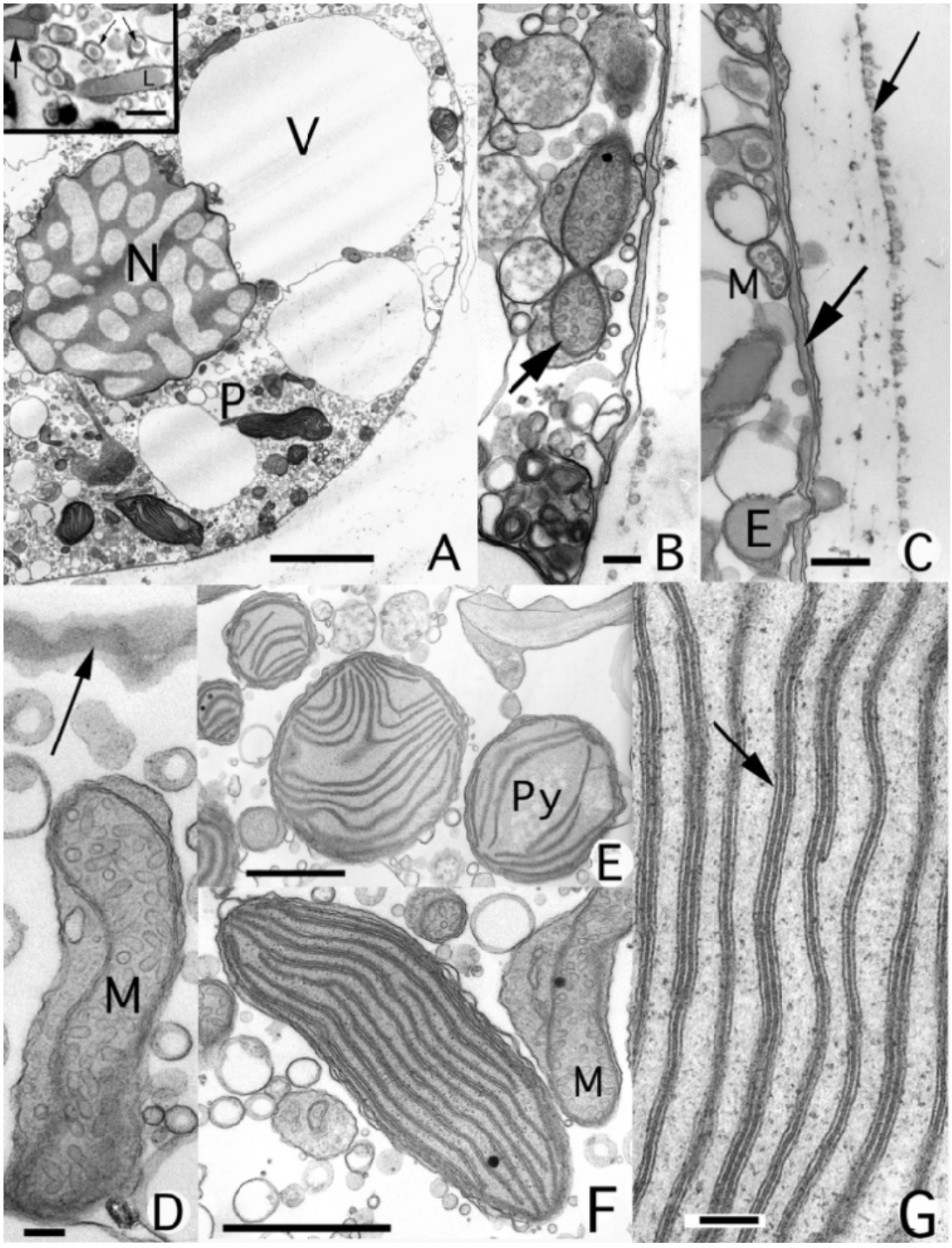


1046 Fig 4



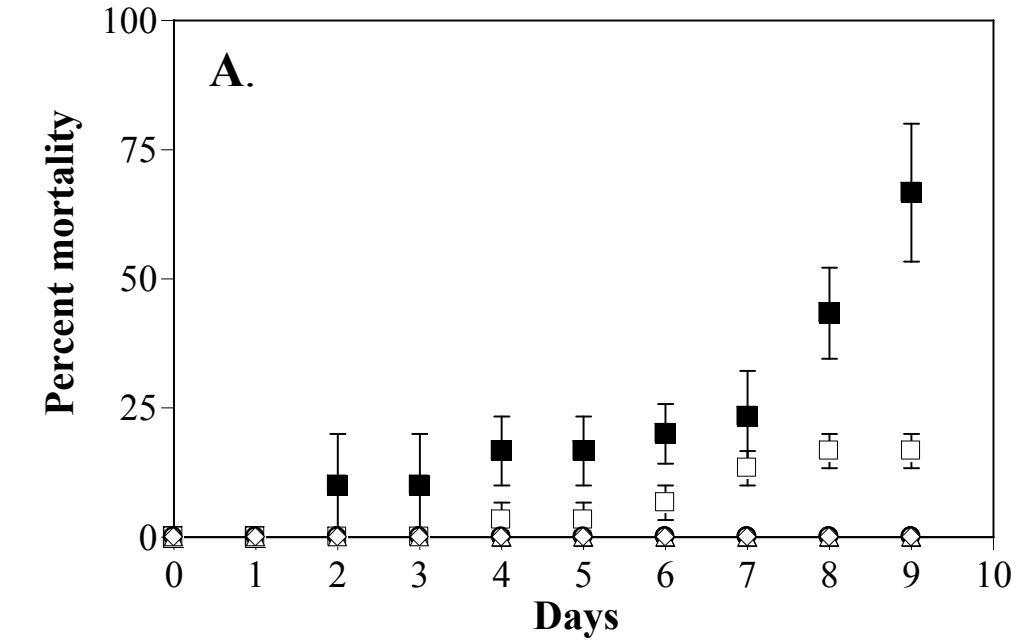
1047

1048 Fig 5



1049

1050 **Fig 6**


A.
Percent mortality
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10
Days

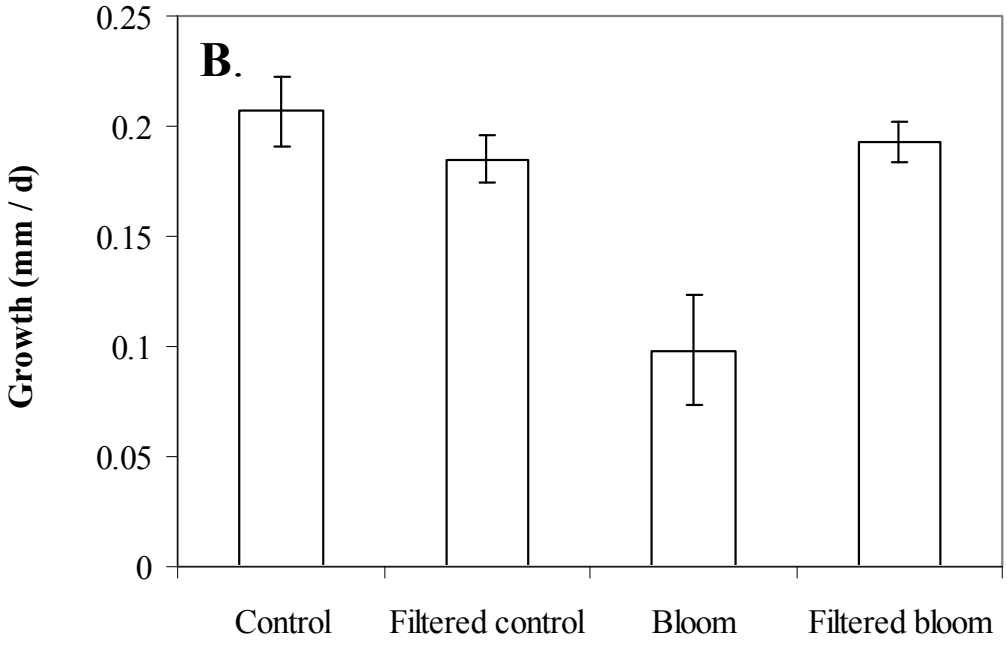
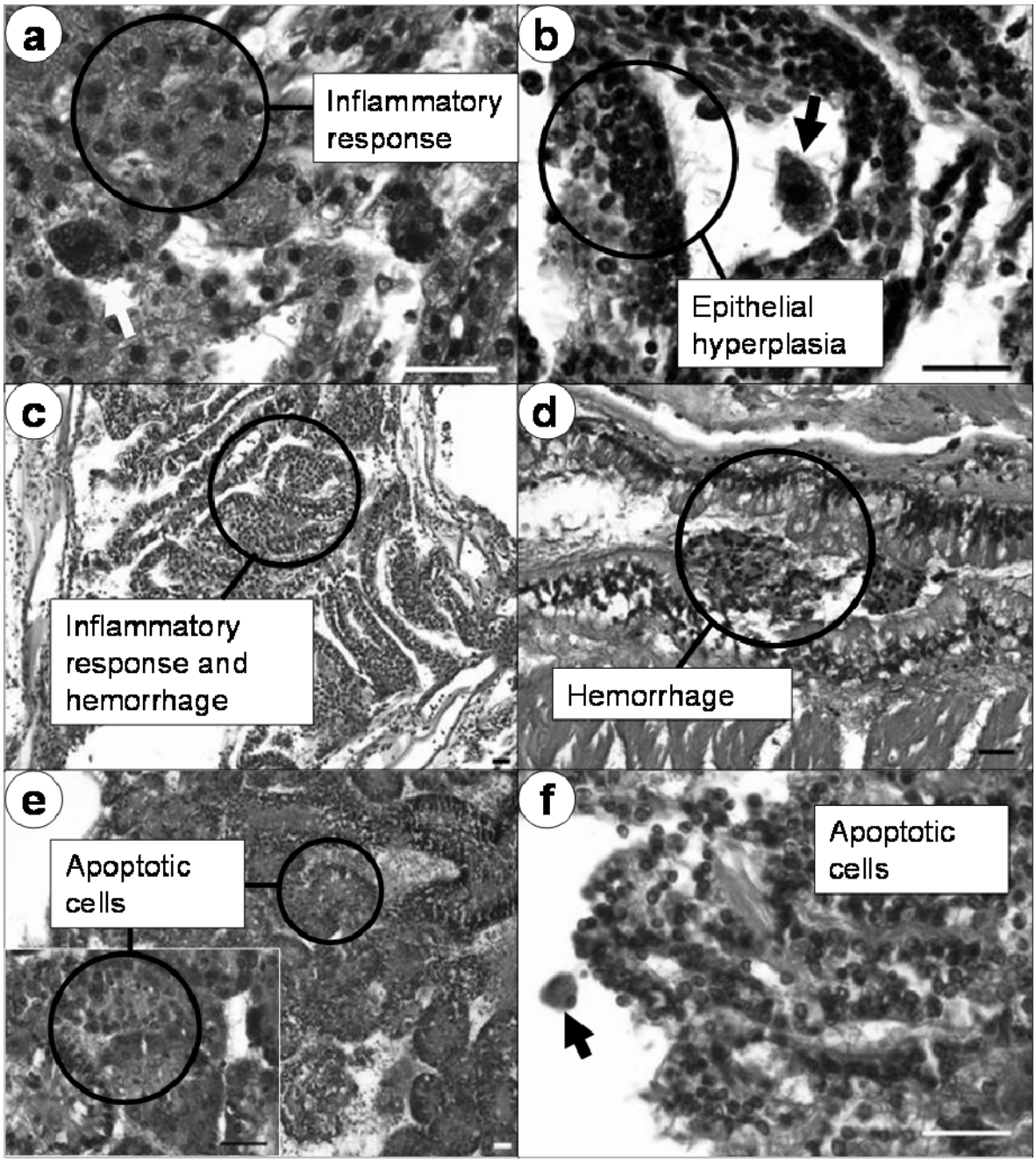
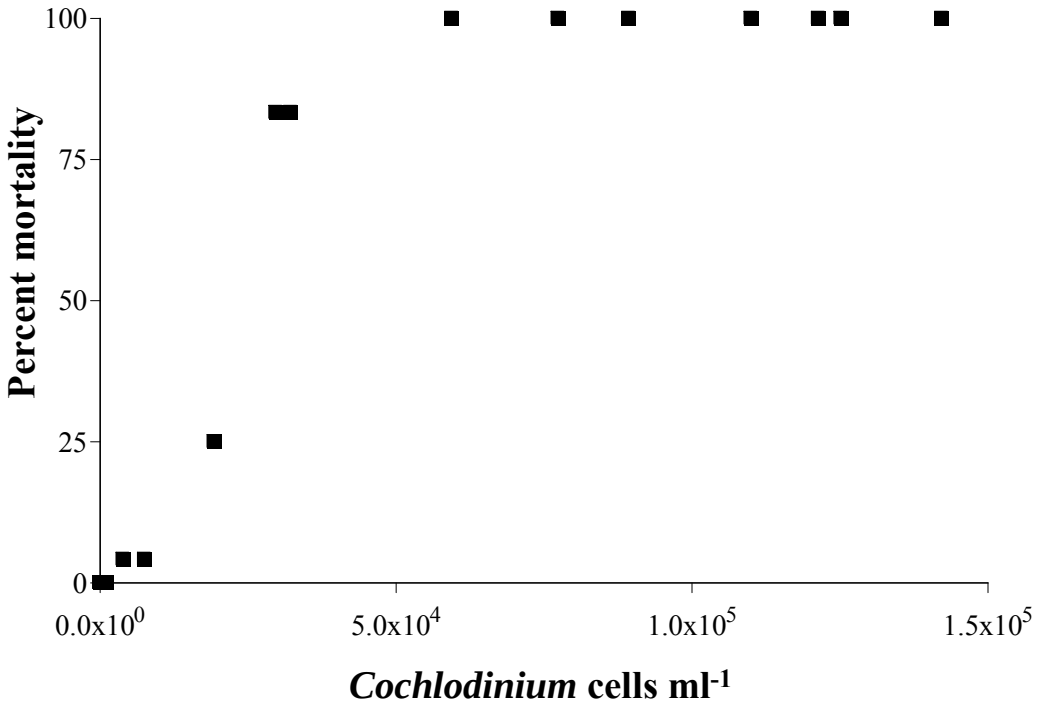

B.
Growth (mm / d)
0.25
0.2
0.15
0.1
0.05
0
Control
Filtered control
Bloom
Filtered bloom

Fig 7

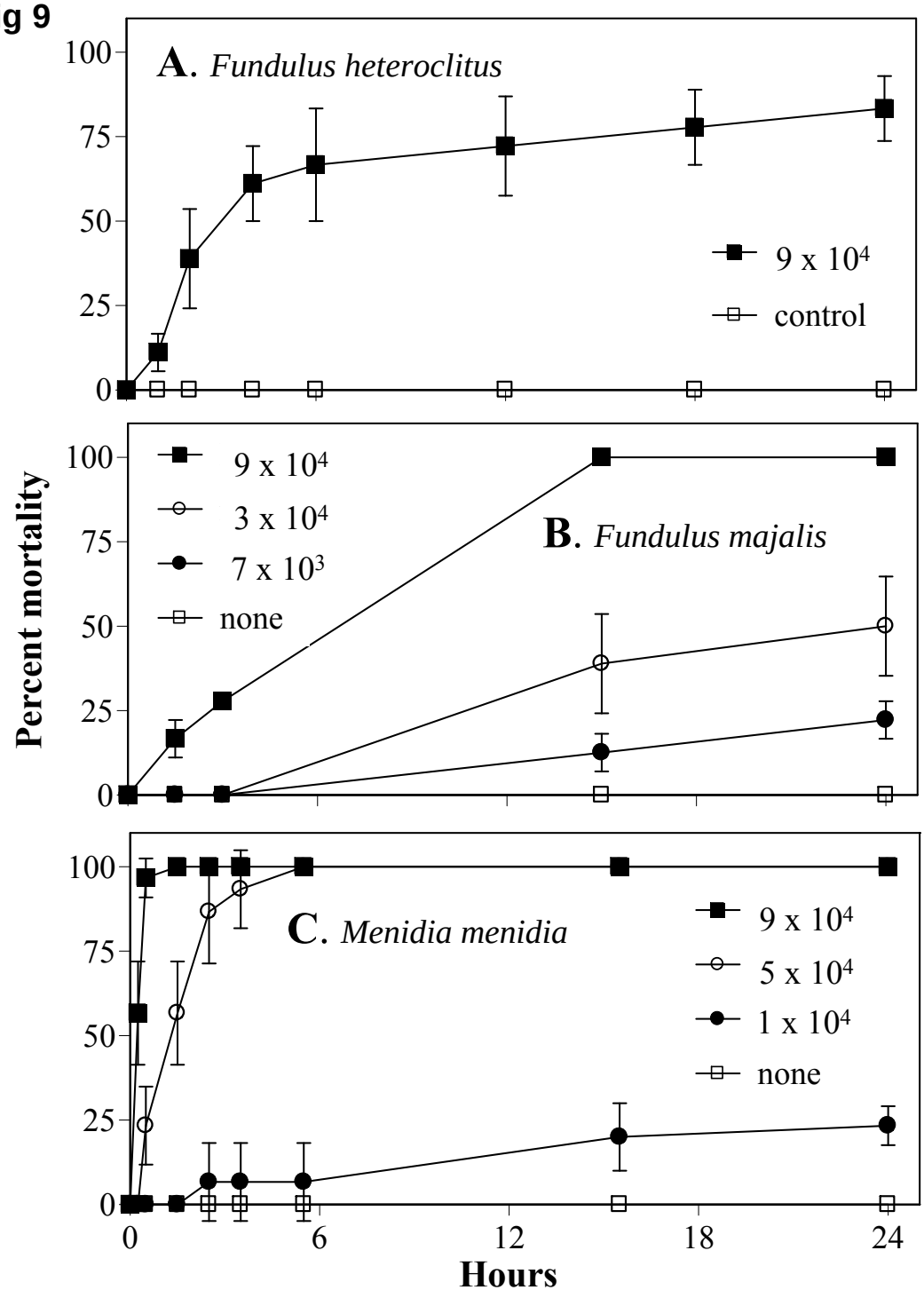

a
Inflammatory response
b
Epithelial hyperplasia
c
Inflammatory response and hemorrhage
d
Hemorrhage
e
Apoptotic cells
f
Apoptotic cells

1056 **Fig 8**


100
75
50
25
0
Percent mortality
0.0x10^0
5.0x10^4
1.0x10^5
1.5x10^5
Cochlodinium cells ml-1

1057

Fig 9

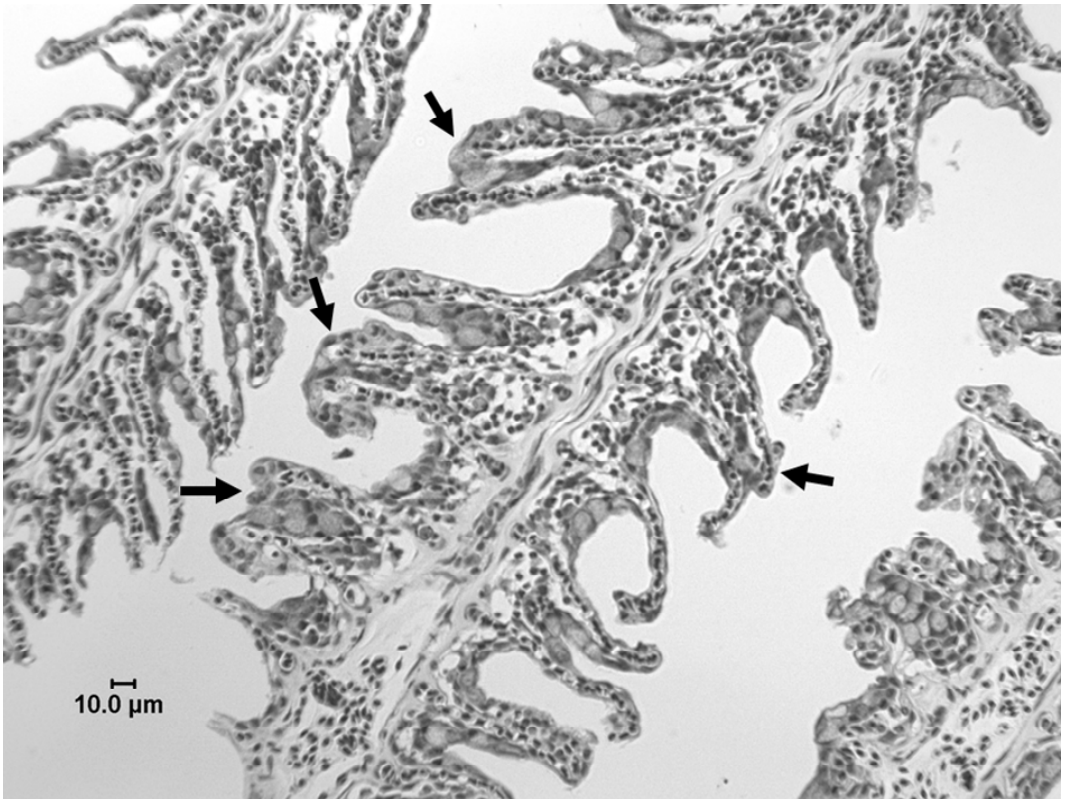

A. Fundulus heteroclitus
9 x 10⁴
control
B. Fundulus majalis
9 x 10⁴
3 x 10⁴
7 x 10³
none
C. Menidia menidia
9 x 10⁴
5 x 10⁴
1 x 10⁴
none
Percent mortality
Hours
0
25
50
75
100
0
6
12
18
24

1058

1059

1060 **Fig 10**

1061



Chapter three: Characterization of the toxicity of *Cochlodinium polykrikoides* isolates from Northeast US estuaries to finfish and shellfish

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Key words: *Cochlodinium polykrikoides,* harmful algal blooms, fish kill, ichthyotoxicity, shellfish, free radicals, reactive oxygen species (ROS)

Abstract: Harmful algal blooms caused by *Cochlodinium polykrikoides* are annual occurrences in coastal systems around the world. In New York (NY), USA, estuaries, bloom densities range from 10^3 - 10^5 mL^{-1} with higher densities ($\geq 10^4$ cells mL^{-1}) being acutely toxic to multiple fish and shellfish species. Here, we report on the toxicity of *Cochlodinium polykrikoides* strains recently isolated from New York and Massachusetts (USA) estuaries to juvenile fish (*Cyprinodon variegates*) and bay scallops (*Argopecten irradians*), as well as on potential mechanisms of toxicity. Cultures of *C. polykrikoides* exhibited dramatically more potent ichthyotoxicity than raw bloom water with 100% fish mortality occurring within ~1 h at densities as low as 3.3 x 10^2 cells mL^{-1}. More potent toxicity in culture was also observed in bioassays using juvenile bay scallops, which experienced 100% mortality during 3 days exposure to cultures at cell densities an order of magnitude lower than raw bloom water (~3 x 10^3 cells mL^{-1}). The toxic activity per *C. polykrikoides* cell was dependent on the growth stages of cultures with early exponential growth cultures being more potent than cultures in late-exponential or stationary phases. The ichthyotoxicity of cultures was also dependent on both cell density and fish size, as a hyperbolic relationship between the death time of fish and the ratio of algal cell density to length of fish was found (~10^3 cells mL^{-1} cm^{-1} yielded 100% fish mortality in 24 h). Simultaneous exposure of fish to *C. polykrikoides* and a second algal species (*Rhodomonas salina* or *Prorocentrum minimum*) increased survival time of fish, suggesting additional cellular biomass mitigated the ichthyotoxicity. Frozen and thawed-, sonicated-, or heat-killed-, *C. polykrikoides* cultures did not cause fish mortality. In contrast, cell-free culture medium connected to an active culture through a 5μm nylon membrane caused complete mortality in fish, although the time required to kill fish was significantly

longer than direct exposure to the whole culture. These results indicate that ichthyotoxicity of *C. polykrikoides* isolates is dependent on viability of cultures and that direct physical contact between fish and cells is not required to cause mortality. The ability of the enzymes peroxidase and catalase to significantly reduce the toxicity of live cultures and the inability of hydrogen peroxide to mimic the ichthyotoxicity of *C. polykrikoides* isolates suggests that the toxicity could be caused by non-hydrogen peroxide, highly reactive, labile toxins.

1. Introduction

Harmful algal blooms (HABs) have become a significant threat to fisheries, public health, and economies around the world and have increased in frequency, duration, and distribution in recent decades. Most of documented HABs are caused by dinoflagellates and, under bloom conditions they can discolor effected waters, poison humans and marine animals, and disrupt aquatic ecosystems (Sunda et al 2006). Many harmful dinoflagellates synthesize potent biotoxins that have been well characterized, while other HAB dinoflagellates can cause direct harm to or even kill marine animals (e.g. fish) with the precise modes of impairment to the animals unknown. One dinoflagellate that has been well known for causing fish kills in East and Southeast Asian waters and the Pacific coast of North America is *Cochlodinium polykrikoides* (Whyte et al. 2001; Matsuoka and Iwataki, 2004; Gárrate-Lizárraga et al., 2004), which, for instance, has been the cause of fisheries losses exceeding $100 million in Korea (Kim, 1998; Kim et al., 1999).

Cochlodinium species have been implicated in mass kills of wild and impounded fish around the globe (Onoue et al., 1985; Yuki and Yoshimatsu, 1989; Guzmán et al., 1990; Qi et al., 1993; Kim 1998; Kim et al. 1999; Kim et al. 2002; Gárrate-Lizárraga et al., 2004; Whyte et al., 2001; Curtiss et al. 2008; Anton et al. 2008; Azanza et al. 2008; Tomas and Smayda et al. 2008). Studies have also indicated that mortality of larvae of the American oyster (*Crassostrea virginica*) was elevated by exposure to *Cochlodinium* (Ho and Zubkoff, 1979), metamorphosis of oyster (*Crassostrea gigas*) larvae was slowed during *Cochlodinium* blooms (Matsuyama *et al*. 2001), and that coral reefs were seriously affected by blooms caused by a *Cochlodinium* species identified as *C. catenatum* Okamura (Guzmán et al., 1990), which is most likely conspecific with *C. polykrikoides* according to Iwataki et al. (2008). Recently, blooms caused by a *Cochlodinium* species (likely *C. fulvescens*; Iwataki et al. 2008) along

1147 the coastline of California in 1996 and 2004 were found to be associated with mass
1148 mortalities of mussels and oysters (Curtiss et al. 2008).
1149 Most reports of HABs caused by *Cochlodinium* have been identified as *C.*
1150 *polykrikoides*, with a broad geographic coverage from the Central and North America to the
1151 East and Southeast Asia along both the Pacific and Atlantic coasts. More detailed studies
1152 based on the phylogenetic analysis of the large subunit rDNA (LSU rDNA) sequences and
1153 morphology for specimens from different geographical origins, however, have recently
1154 demonstrated that some of those populations identified as *C. polykrikoides* (west Canada,
1155 Whyte et al. 2001; California, Kuedla et al 2008, Curtiss et al. 2008; Gulf of California,
1156 Mexico, Gárate-Lizárraa et al. 2004) are likely another species, *C. fulvescens* (Iwataki et al.
1157 2007, Matsuoka et al. 2008; Iwataki et al. 2008). A more complicated issue in the taxonomy
1158 of *C. polykrikoides* is that those isolates or specimens currently identified as different
1159 'ribotypes' of *C. polykrikoides* (Matsuoka et al. 2008) may belong to different species,
1160 because their differences in the LSU rDNA sequences are as high as 10%, which is generally
1161 more than enough to separate different species (Gobler et al. 2008). These complications in
1162 the taxonomy of *Cochlodinium* may be important in understanding the controversies
1163 surrounding the mechanisms or chemical nature of the toxicity of *C. polykrikoides*.
1164 Although the fish and shellfish killing activity of *Cochlodinium* species have been
1165 well documented, the associated toxic mechanism or chemical nature of toxicity has been a
1166 controversial issue (Onoue et al. 1985; Onoue and Nozawa, 1989a, b; Kim et al. 1999; Whyte
1167 et al. 2001; Kim et al. 2002). Landsberg (2002) categorized *Cochlodinium* species as taxa
1168 with multiple toxins. Two early studies reported three toxic fractions (i.e. neurotoxic,
1169 hemolytic, and hemagglutinative; Onoue and Nozawa 1989a) and zinc-bound paralytic
1170 shellfish poisoning (PSP) toxins (Onoue and Nozawa 1989b) from the red tide waters of
1171 *Cochlodinium* type' 78 Yatsushiro, which was considered to be conspecific with *C.*

1172 *polykrikoides* (Matsuoka et al. 2008). However, the PSP toxins and two of the non-PSP toxic
1173 fractions have not been identified further or described since their initial report (Onoue and
1174 Nozawa 1989a,b), while the third fraction, hemolytic agents, has been documented to be
1175 associated with fatty acids (Lee 1996). Because these toxic fractions were originally extracted
1176 from a mixed biomass sample concentrated from a large volume of field bloom water (1000
1177 L; Onoue and Nozawa 1989b), it is possible that these toxins or toxic fractions came from
1178 other sources. Others have demonstrated that reactive oxygen species (ROS; i.e. superoxide
1179 anions and hydrogen peroxide) are produced by *C. polykrikoides* cells and may be one of the
1180 factors inducing fish kills (Kim et al. 1999). Interestingly, Kim et al. (2002) found that O_2^-
1181 and H_2O_2 production by *C. polykrikoides* was much lower than that by *Chattonella marina*, a
1182 species well-known for ROS production and that cell-free aqueous extract of *C. polykrikoides*
1183 cultures showed toxic effect on cervical cancer cells (cell line HeLa). Based on the
1184 observation of a gradual accumulation of polysaccharides in the culture medium of *C.*
1185 *polykrikoides*, Kim et al (2002) suggested that biologically active multiple metabolites
1186 secreted by *C. polykrikoides* such as cytotoxic agents and mucus substances may contribute
1187 to the fish kill mechanism of *C. polykrikoides*. This hypothesis is consistent with the
1188 previously proposed suffocation of fish caused by secretion of mucus-like materials from
1189 algal cells as a possible mechanism of fish kills by *C. polykrikoides* (Hallegraeff 1992; Lee
1190 1996).

1191 Harmful algal blooms caused by *Cochlodinium* species have been reported from both
1192 the west and east coasts of North America (Silva 1967; Whyte et al. 2001; Gárrate-Lizárraga
1193 et al., 2004; Gobler et al. 2008; Tomas and Smayda 2008; Curtiss et al. 2008), with the most
1194 recent phylogenetic study indicating that the species blooming on the west coast is *C.*
1195 *fulvescens* and the one in the east is *C. polykrikoides* (Iwataki et al. 2008; Gobler et al. 2008).
1196 From 2002-2008, blooms of *C. polykrikoides* have emerged as annual events during late

summer in the Peconic Estuary and Shinnecock Bay in Long Island, New York, USA (Nuzzi 2004; Gobler et al. 2008; unpublished data on 2007 and 2008 blooms). Here we report on the ichthyotoxicity of *C. polykrikoides* clonal isolates from Long Island and Cotuit Bay, MA, and on possible mechanisms of ichthyotoxicity. Our results demonstrate these cultures are substantially more toxic to fish and shellfish than raw bloom water. Results further suggest that the toxicity of these *C. polykrikoides* isolates may be attributable to non-hydrogen peroxide, highly reactive oxygen species (hROS) or organic hydroperoxides that are produced continuously by actively growing *Cochlodinium polykrikoides* cultures.

2. Methods

2.1. Culture establishment and maintenance

The culture isolate of *Cochlodinium polykrikoides*, strain CP1, was obtained by pipetting single cells to polystyrene cell culture plates containing sterile GSe culture medium (see below) under an inverted microscope. The bloom water sample from which the culture was established was collected on August 31, 2006 from the most western basin of the Peconic Estuary, Flanders Bay (40.923°N, 72.587°W). Molecular and microscopic identification of strain CP1 was reported previously (Gobler et al. 2008). The *Cochlodinium polykrikoides* strain CPCB-10 was isolated from Cotuit Bay, Massachusetts by Donald Anderson's laboratory and was generously provided by Gregory J. Doucette from NOAA/National Ocean Service, SC, US. Identification of both isolates as *Cochlodinium polykrikoides* has been confirmed with large subunit (LSU) rDNA sequencing (Iwataki et al. 2008, Gobler et al 2008). Cells were cultured in sterile GSe medium with a salinity of 31-32 PSU, made with autoclaved and 0.2 μm-filtered seawater (Doblin et al. 1999), at 21° C in an incubator with a 12:12h light:dark cycle, illuminated by a bank of fluorescent lights that provided a light intensity of ~100 μmol quanta m^{-2} s^{-1} to cultures. For all cultures, an antibiotic-antimycotic

1222 solution (a mixture of 10, 000 I.U. penicillin, 10, 000 μg mL^{-1} streptomycin, and 25 μg mL^{-1}
1223 amphotericin B; Mediatech. Inc., Hemdon, VA) was added into the medium immediately
1224 before inoculation, with a final concentration of 1-2% to minimize contamination by bacteria
1225 and fungus. Periodic DAPI-staining of cultures has indicated the absence of bacteria within
1226 cultures. Two other microalgae used in the study, *Prorocentrum minimum* CCMP696 and
1227 *Rhodomonas salina* CCMP1319, were kindly provided by Dr. N. Fisher's laboratory at Stony
1228 Brook University and cultured under the same conditions as *C. polykrikoides* strains. The
1229 culture of *Gymnodinium aureolum* (Hulburt) G. Hansen, used as a negative control, was
1230 isolated from a tidal tributary of the Chesapeake Bay, USA, identified with LSU rDNA
1231 sequencing (Tang et al. 2008), and cultured at conditions same to the other three cultures.
1232 Bioassays were conducted using the culture of the strain CP1 (referred as CP1 hereafter)
1233 unless otherwise indicated.
1234
1235 *2.2. Fish and shellfish bioassay using cultures and different culture components*
1236 Since bloom water of *C. polykrikoides* has been previously shown to be ichthyotoxic (Gobler
1237 et al. 2008), the experiments of this study were conducted using laboratory cultures to
1238 compare the toxicity of bloom water and clonal cultures and to explore the fish-killing
1239 mechanisms or chemical nature of *C. polykrikoides* toxicity. Ichthyotoxic experiments were
1240 conducted with juvenile (~ 2 weeks old, ~6 mm in length, unless otherwise indicated)
1241 sheepshead minnows (*Cyprinodon variegates*), a culture line that has been laboratory reared
1242 for more than a decade (Cosper Environmental Services, Bohemia, NY, USA), feed with live
1243 brine shrimp twice a day, and cultured in filtered seawater with a salinity of 24-26 PSU at 20-
1244 26˚C. Shellfish bioassays were conducted with juvenile (~1.1 cm in length) and sub-adult (~
1245 3 cm in length) bay scallops (*Argopecten irradians*) that were obtained from the Cornell
1246 Cooperative Extension shellfish hatchery facility in Southold, NY.

For the first set of experiments, individual minnows were gently transferred using a modified 200 µL transfer pipette into 8 mL of culture or culture components held in 6-well sterile, polystyrene plates, with one fish in each well (n = 6 per treatment). For experiments, cultures of CP1 and CPCB-10 were added into the plates at cell densities ranging from 3.5×10^2 to 4.3×10^3 cells mL^{-1} with the upper range representing the maximal cell densities achieved in culture. For other experiments, cultures were subjected to the following treatments: boiling (15 min) and cooling to room temperature; freezing (-20˚C) and thawing at room temperature; and sonicatation on ice for 3×30 s with a high energy sonicator (Ultrasonic Power Corporation, Freeport, Illinois. Model 1000L), with microscopic examination showing no intact cells in cultures after treatment. The concentrated and resuspended biomasses (the cells lost viability during the processing) were used for a fish bioassay, with or without further treatment of sonication or freezing. For all experiments, GSe medium was used as negative controls and no aeration was provided (fish survive > 7 days in GSe medium without aeration). A culture of *Gymnodinium aureolum* (Hulburt) G. Hansen was also used as a negative control to examine the impacts non-toxic algal biomass levels similar to those used in the *C. polykrikoides*. Fish in experiments were monitored continuously in the first hour, every 10 min in the first 5 - 8 hours, and several more times before 24 h and 48 h (sometimes up to 120 h). Fish were considered to be dead if their gills ceased to move, and/or they were lying on their sides, and/or there was no response to stimulation. The concentrations of *C. polykrikoides* were determined by counting cells in 0.1 - 1.0 mL of culture added in a Sedgewick-Rafter counting chamber after fixation with Lugol's solution. Dead fish were measured for length immediately following death. All fish bioassays were conducted with ~10 µmol photons m^{-2} s^{-1} at a temperature of 20-25˚C.

Experiments were conducted to establish the dose-response relationship between the death time of fish and the dosage of *C. polykrikoides* CP1 cells, which was defined as the

ratio of algal cell density to length of fish. Experiments were conducted with three batches of cultures in exponential growth stage (with initial cell densities of 1.6×10^3, 2.5×10^3, and 3.0×10^3 cells mL^{-1}) and sheepshead minnows with lengths ranging from 0.6 to 2.1 cm (1.14 ± 0.41 cm, n=30).

Another series of fish bioassays was conducted using CP1 cultures at different growth stages and densities to observe the differences in the ichthyotoxic activity of CP1 at different growth stages. Growth stages were determined from cell counts and with a fluorometer (Turner Designs Co., CA, USA, model TD-700). CP1 cultures in early exponential (with cell densities of 0.33×10^3 and 1.33×10^3 cells mL^{-1}), late exponential (2.71 - 4.32×10^3 cells mL^{-1}), and late stationary (2.71×10^3 cells mL^{-1}) growth were serially diluted with GSe medium, and were used in bioassays with 2-week old fish in the same procedure described as above. The average death time of fish for those treatments with 100% mortality in 24 h of the bioassay was used as the parameter to evaluate the ichthyotoxic activity of CP1 at different growth stages.

For shellfish bioassays, CP1 cultures of different cell concentrations were added into replicated 1-L glass beakers with five juvenile or one adult scallop (*Argopecten irradians*). Beakers were aerated via mild bubbling during the experiment. Experiments were conducted with juveniles (1.1 ± 0.01 cm in length) and sub-adults (~3.0 cm) and lasted 96 h. For each experiment, the same volume of filtered seawater was used as control.

2.3. Effect of co-occurring microalgae on the ichthyotoxicity of C. polykrikoides CP1

An obvious difference in the intensity of ichthyotoxicity of *C. polykrikoides* was observed between the bloom water (Gobler et al 2008) and laboratory culture. Therefore, cultures of *Rhodomonas salina* CCMP1319 and *Prorocentrum minimum* CCMP696 (the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Maine, USA) were added at

varying densities to CP1 cultures to assess the extent to which co-occurring plankton may affect the ichthyotoxicity of *C. polykrikoides*. Fish were exposed to 10 mL of mixed-cultures prepared by adding 2 mL of *R. salina* or *P. minimum* at varying densities into 8 mL each of *C. polykrikoides* culture (final concentrations of 1.2 - 12 $\times$ 10^4 cells mL^{-1} for *P. minimum* CCMP696 and 5.1 - 51 $\times$ 10^4 cells mL^{-1} for *R. salina* CCMP1319) while 10 mL GSe medium and 2 mL GSe medium plus 8 mL of *C. polykrikoides* culture served as negative and positive controls, respectively. An aliquot of the mixed suspensions was withdrawn after mixing, fixed with Lugol's solution, and used to enumerate cell densities of each species. The fish bioassays were performed as described above.

2.4. Fish bioassays with cell-free medium

To better understand mortality mechanisms associated with North American isolates of *C. polykrikoides*, experiments were conducted using cell-free medium prepared with filtration or via connection to an active culture through a 5μm mesh nylon barrier. For experiments using cell-free medium, cultures of CP1 were filtered through a 5μm-mesh nylon membrane (gravity filtration). After filtration, 8 mL of the filtrate was added into each well of the 6-well culture plate and fish were added immediately, or 10 and 60 minutes after. The experiments using cell-free culture medium connected to active cultures were conducted with an ice cube tray with half of the partitions replaced with 5μm nylon mesh fastened with silicon. The survival of fish in the tray for 96 h without cells demonstrated the silicon was not toxic to the fish. A volume of 40 mL culture of *C. polykrikoides* CP1 (or 40 mL GSe medium for control) was added into one side of the partitioned compartments and the cell-free medium was allowed to seep through the 5um barrier to the other side (containing less than 10 cells mL^{-1}). After the water levels of the two sides were balanced, three fish were added into either side of the compartments for two sets of the trays. No aeration was provided and

1322 periodicity of fish monitoring were as described above. Expired fish were removed
1323 immediately after death.
1324
1325 *2.5. Fish bioassays with chemical compounds to mitigate or cause toxicity: peroxidase,*
1326 *catalase, superoxide dismutase (SOD), trypsin, and hydrogen peroxide*
1327 Since previous reports suggested that reactive oxygen species (ROS) may be responsible for
1328 toxicity of *C. polykrikoides* strains from Korea (Kim et al. 1999), and because our results
1329 demonstrated that the toxicity was dependent on the viability and growth stage of *C.*
1330 *polykrikoides* CP1 but not physical contact with cells, experiments were designed to observe
1331 the effects of the scavengers of reactive oxygen species and organic hydroperoxides
1332 (peroxidase, catalase, and superoxide dismutase (SOD) on the toxicity of cultures and cell-
1333 free filtrate. To determine whether any protein or protein-like component is involved in the
1334 ichthyotoxicity of *C. polykrikoides*, the protelytic enzyme, trypsin, was added into CP1
1335 culture, since is has been previously shown to mitigate toxicity of protein-like toxins from
1336 harmful dinoflagellates (Matsuyama et al. 1997). Finally, a dose-response experiment was
1337 conducted with hydrogen peroxide and the juvenile sheepshead minnows of the same age and
1338 size are those used for experiments with *C. polykrikoides*.
1339 All solutions for experiments were used within 1 h of preparation or immediately
1340 stored frozen (-20ºC). Peroxidase (MP Biomedicals, LLC., Aurora, Ohio), catalase, and
1341 superoxide dismutase (SOD) (MP Biomedicals, LLC., Solon, Ohio) were prepared as
1342 aqueous solutions according to manufactures' guidelines and added into cultures and cell-free
1343 5μm-mesh filtrate at different concentrations (0.5 – 1.25 μg mL^{-1} for peroxidase, 1.0 – 10.0 U
1344 mL^{-1} for SOD, and 0.1 – 2.0 U mL^{-1} for catalase). Peroxidase-Avidin was dissolved in 10
1345 mM phosphate buffer, 0.15 M NaCl, pH 7.4, containing 1.0% BSA and 0.1% proclin as a
1346 preservative, with a concentration of 2.5 mg mL^{-1}. SOD was dissolved in 5 mM Tris-HCl

containing 10 mg mL^{-1} lactose, pH 7.5, 12,500-16,000 U mL^{-1} (one unit of activity converts 1.0 μmole of superoxide anion to H_2O_2 per minute at pH 7.5 and 30˚ C). 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). An aliquot of cultures exposed to each of the chemicals was fixed with Lugol's solution approximately half an hour after additions and were quantified. HPLC grade trypsin (MP Biomedicals, LLC., Solon, Ohio) stock solution was prepared with distilled water, and the final concentration added into CP1 culture were 100, 300, and 500 μg mL^{-1}, but the volume of trypsin solution added into test culture was not more than 0.2% (v/v) of the culture so as to avoid cell damaging by salinity shock. GSe medium with addition of the highest concentrations of trypsin (500 μg mL^{-1}) was used as control. To determine any effects of these compounds on cell activity or cell viability, peroxidase and catalase were added into CP1 culture at their highest concentrations used in the fish bioassays (2.5 μg mL^{-1} final concentration for peroxidase and 2.0 U mL^{-1} for catalase) and growth rates were compared to cultures without addition of these enzymes. After addition of the enzymes, CP1 cultures were monitored for one week and growth rates were quantified.

2.6 Statistics.

Differences among treatments were generally assessed using one-way or two-way ANOVAs followed by Holm-Sidak methods for multiple pairwise comparison or appropriate t-tests 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 $p < 0.05$ unless otherwise indicated.

3. Results

3.1 Toxicity of C. polykrikoides cultures and its different components to fish and shellfish

Live cultures of *C. polykrikoides* CP1 and CPCB-10 exhibited acute ichthyotoxicity to sheephead minnows (*Cyprinodon variegates*; Table 1). Using a CP1 culture with an initial cell concentration of 4.3 x 10^3 cells mL^{-1}, 100% mortality was observed within 24 h down to its 40% dilution (1.7 x 10^3 cells ml^{-1}; Table 1). The culture of strain CPCB-10 was not able to kill fish at an initial concentration of 3.5 x 10^2 cells mL^{-1} but caused 100% mortality of fish in < 3 h at a density of 1.0 x 10^3 cells mL^{-1} and 50% mortality in 8-24 h at an initial cell density of 7.1 x 10^2 cells mL^{-1} (Table 1). Three batches of fish bioassays using serial dilutions of CP1 cultures with the initial cell concentrations of 4.3 x 10^3, 3.5 x 10^3, and 2.7 x 10^3 cells mL^{-1} displayed a hyperbolic relationship between the cell density and the mortality (%) of sheepshead minnows within 24 h (Fig. 1). All fish died in 24 h at and above cell densities of 1.7 x 10^3 cells mL^{-1}, and 50% mortality in 24 h observed at 1.3 x 10^3 cells mL^{-1}. For those dilutions with 100% mortality, longer death time of fish corresponded to lower cell concentrations (Table 1, also see below).

Within the ranges of CP1 cell density (1.0 - 3.0×10^3 cells mL^{-1}) and fish size (0.6 to 2.1 cm) used for experiments, the time to death of fish depended on both the algal cell concentrations and the size of fish used in the tests (Fig. 2). It was observed that there was a significant negative correlation ($R^2 = 0.82$, $n = 30$, $p < 0.001$) between the time to death of fish and the ratio of algal cell concentration to the length of fish (Fig. 2), i.e. smaller fish were more susceptible to the CP1 toxicity, given that they are exposed to CP1 cultures with same cell density.

The CP1 culture caused 100% mortality (n=10) in juvenile bay scallops (*Argopecten irradians*) with an average length 1.1 ± 0.1 cm at a cell density of 3.1 x 10^3 cells mL^{-1} within 24 to 69 h (experiment 19 in Table 1) while larger scallops (2.9-3.2 cm) exposed to concentrations between 0.9 and 4.5 x 10^3 cells mL^{-1} died within 28-72 h (experiments 20-22

in Table 1). All parallel control treatment juvenile and sub-adult scallops survived during the time frame of the experiments.

The CP1 culture, which caused 100% mortality in fish when alive, lost its fish-killing ability after the treatments of freezing (-20˚C), heating (boiling for 15 min), and high-power sonication (Experiments 12-14 in Table 1). The concentrated biomass of CP1 (concentrated by filtration onto a 0.22μm-mesh filter and resuspended in GSe medium and then sonicated or frozen) also displayed no fish-killing ability even at an equivalent cell concentrations dramatically higher than those of live cultures that exhibited 100% mortality in 0.5 h (experiments 15, 17-18 in Table 1).

3.2 Effect of growth stages on the toxicity of C. polykrikoides CP1

The toxic activity of CP1 cultures changed with growth stages as early exponential or exponential cultures displayed higher toxic activity per cell than late exponential and stationary stages (Fig. 3). The plots of fish death time versus cell density of CP1 for six cultures at different growth stages did not form a unique curve, but rather formed discrete and generally parallel curves unique to the individual cultures from which the dilutions of cultures were prepared. For example, although most cultures yielded a mean death time of 1 – 3 h for the fish, the early exponential phase culture required a cell density of only 3×10^2 cells mL^{-1}, the mid-exponential phase culture required densities of $\sim 1 \times 10^3$ cells mL^{-1}, and the late exponential phase cultures required densities of $2 – 4 \times 10^3$ cells mL^{-1}, while fish survived 4 – 6 h in the late stationary phase culture despite cell densities of $2 – 3 \times 10^3$ cells mL^{-1} (Fig 3). Using the individually-obtained and best-fitted logarithmic regressions in Fig. 3, the corresponding cell densities required for killing fish in 1 h were 1.2×10^3 cells mL^{-1} in mid-exponential phase, $2.4 - 3.3 \times 10^3$ cells mL^{-1} in late exponential phase, and 4.2×10^3 cells mL^{-1} for late stationary phase cultures, demonstrating the lower ichthyotoxic activity per cell

in cultures in later growth stages. The toxic activity of CPCB-10 also appeared to be dependent on growth stage of culture as a late exponential phase culture of 9.0×10^2 cells mL^{-1} was non-toxic to fish whereas an early exponential phase culture with fewer cells (7.1×10^2 cells mL^{-1}) killed 50% of fish in 24 h (experiments 25 and 26 in Table 1).

3.3 Effect of co-occurring microalgae on the ichthyotoxicity of C. polykrikoides CP1

The addition of the microalgae, *Prorocentrum minimum* CCMP696 and *Rhodomonas salina* CCMP1319, into CP1 cultures reduced the toxicity of CP1 to sheepshead minnows in a density-dependent manner (Fig. 4A&B). Compared with the control CP1 culture, treatments with the addition of either *P. minimum* or *R. salina* had a fish death time that was significantly longer than that of the control (ANOVA, $p<0.001$), except for the sample with lowest concentration of *P. minimum* (1.2×10^4 cells mL^{-1}). Higher densities of *P. minimum* and *R. salina* had a more significant mitigation effect on the toxicity of *C. polykrikoides* than lower densities (ANOVA, $p=0.005$ for *P. minimum* and $p=0.02$ for *R. salina*; Fig. 4). Comparing between *P. minimum* and *R. salina* with similar cell densities, the mitigation effect of *P. minimum* was less significant than that of *R. salina* (paired t-test: $p<0.01$), which may be due to the larger total cell surface area for *R. salina* than for *P. minimum* (based on cell densities and the calculated cell surface area of 155 μm^2 for *R. salina* and 531 μm^2 $cell^{-1}$ for *P. minimum*, respectively). All fish survived the complete 96 h experiment in the presence of the highest density of *R. salina* (5.2×10^5 cells mL^{-1}), while fish experienced mortality in all other treatments.

3.4 Fish mortality in cell-free culture medium

Cell-free medium prepared by gravity filtrating CP1 through a 5μm-mesh nylon membrane exhibited fish-killing ability, although it was significantly less potent than the culture with

cells (ANOVA for death time, $p<0.001$). The toxicity of the 5μm-mesh-filtered fraction decreased with time after filtration; the later the fish were added to the cell-free medium, the longer they survived (ANOVA for death time, $p<0.001$, Fig.5). It is noteworthy that intoxication effect could be observed, as reflected in fish's slow response to stimuli, in those wells where the toxicity was not high enough to kill the fish during the 72 h experiments.

For the experiments conducted with the 5μm-nylon mesh-partitioned chambers, the culture suspension that was free of CP1 cells (< 10 cells mL^{-1}) but was connected with the live culture through the membrane was also lethal to fish (Fig. 5). However, the toxicity of this treatment was less acute than that of the live culture at the other side of the nylon membrane, as reflected in the average death time of fish (t-test, $p<0.001$; Fig. 5). The average death time of fish in the active culture was 0.6 h, while the corresponding mean death time across the membrane was 10 h (Fig. 5).

3.5 Fish bioassay using CP1 cultures with additions of peroxidase, catalase, superoxide dismutase (SOD), trypsin, and H_2O_2

Additions of peroxidase (0.5-2.5 μg mL^{-1}) and catalase (0.1-2.0 U mL^{-1}) into the CP1 cultures demonstrated these two compounds significantly reduced the toxicity of CP1 cultures ($p<0.001$), and there was an obvious dose-response relationship between the concentrations of peroxidase and catalase and the death time of fish and death rate of fish (Fig. 6). Superoxide dismutase (SOD), a O_2^- scavenger that converts superoxide anion to H_2O_2, also reduced the toxicity ($p<0.001$) but not as significantly as peroxidase and catalase in the concentration range used, and there was no obvious dose-response trend for SOD (Fig. 6). The ichthyotoxicity of CPCB-10 culture was also significantly reduced by addition of peroxidase (1.25 μg mL^{-1}) and catalase (0.5 U mL^{-1}; Fig. 7). Toxicity of the filtrate of CP1 culture through 5μm membrane (<10 cells mL^{-1}) was completely eliminated by the additions

of peroxidase (final concentration 0.5 μg mL^{-1}) and catalase (0.2 U mL^{-1}), with a 24 h mortality of fish reduced from 100% to 0% (data not shown). Additions of these enzymes did not kill or alter the growth of *C. polykrikoides*. Repeated grow-out experiments for control CP1 cultures and CP1 cultures with 2.5 μg mL^{-1} of peroxidase and CP1 with 2.0 U mL^{-1} of catalase (the highest concentrations used in both bioassays) yielded statistically equal growth rates or cell yields (data not shown). The addition of trypsin in the final concentrations of 100, 300, and 500 μg mL^{-1} did not alter the toxicity of CP1 to fish (ANOVA, $p>0.05$; Fig 6). It is noteworthy that the GSe medium with addition of the highest concentrations of trypsin (500 μg mL^{-1}) also did not show any toxicity to the minnows in a 96 h observation. Finally, sheepshead minnows placed in a range of concentrations of hydrogen peroxide displayed complete mortality after 24 h at concentrations of 1 mM, but complete survival at concentrations ≤ 500 μM (data not shown).

4. Discussion

4.1 Toxicity of C. polykrikoides and the effects of growth stage and co-occurring microalgae on toxicity

C. polykrikoides is globally well-known for its fish killing ability and our previous studies in NY estuaries documented that *C. polykrikoides* bloom water killed multiple fish and shellfish species (Gobler et al. 2008). That study also established a hyperbolic relationship between the initial cell density in bloom waters and the fraction of sheepshead minnows surviving after 24 h; all fish survived 24 h at cell densities < 1.0×10^3 mL^{-1} and all experiments with ≥ 5×10^4 cells mL^{-1} displayed 100% mortality (Gobler et al. 2008). Our present study using laboratory cultures of *C. polykrikoides* CP1 and CPCB-10 confirmed the ichthyotoxic nature of US east coast isolates of *C. polykrikoides* to fish and shellfish. Our results also yielded a similar hyperbolic relationship between fish mortality and *C. polykrikoides* cell density (Fig.

1), and established a correlation between the death time of fish, the *C. polykrikoides* cell concentration, and the size of fish (Fig.2). The dependence of death time of fish on both the cell density and fish size has been also observed in the toxicity of the dinoflagellate *Alexandrium leei* to Asian sea bass (Tang et al. 2007) and demonstrates that smaller fish are most susceptible to the toxicity of *C. polykrikoides*. This suggests there may be a cryptic fish-killing effect on juvenile and larval fish during blooms of this species. More importantly, *C. polykrikoides* cultures exhibited dramatically more potent ichthyotoxicity to both finfish and shellfish compared to bloom water, with 100% mortality of sheepshead minnows at a cell density of only 3.3 x 10^2 cells mL^{-1} within 1.5 h, 100% mortality of juvenile bay scallops at 3.1 x 10^3 cells mL^{-1} within 70 h, and adult scallops at 9.4 x 10^2 cells mL^{-1} within 72 h (Table 1). In contrast, bloom water with > 10^4 cells mL^{-1} was needed to yield 100% mortality in sheepshead minnows and a 9-day exposure was needed to kill 66% of juvenile bay scallops exposed to bloom water with *C. polykrikoides* cell densities of ~3 x 10^4 cells mL^{-1} (Gobler et al. 2008). Several results of the current study provide evidence for mechanisms that may account for these differences.

We found that the toxicity of *C. polykrikoides* was highly dependent on the growth stage of CP1 and CPCB-10 cultures with early exponential growth cultures being significantly more toxic than late exponential or stationary stages (Fig. 3; Tests 23-28 in Table 1 for CPCB-10). This finding indicates the cells of a 'younger' culture actively produce more of the agents responsible for fish-killing. This may also partly account for the difference in the strength of toxicity between bloom water and the laboratory culture mentioned above, since the bloom water collected for fish bioassays was of a higher cell density of *C. polykrikoides* (> 10^4 cells mL^{-1}) and thus may have been a population at late growth stage (Gobler et al. 2008). The mitigation effect of co-occurring microalgae, *P. minimum* CCMP696 and *R. salina* CCMP1319, to the toxicity of *C. polykrikoides* CP1 (Fig. 4) may also partly explain

1522 the difference in toxicity between bloom water and cultures. As *C. polykrikoides* blooms
1523 develop and dissipate, other dinoflagellates or cryptophytes such as *P. minimum* and *R. salina*
1524 may co-dominate phytoplankton communities (Gobler et al 2007) and reduce the toxicity of
1525 *C. polykrikoides* cells. Even during the peak of *C. polykrikoides* blooms when this species
1526 dominates the microalgal community to the exclusion of all other phytoplankton, co-
1527 occurring bacteria present at high densities ($> 10^7$ cells mL^{-1}; Gobler et al 2007) may be
1528 capable of similarly mitigating the toxicity of *C. polykrikoides*. Therefore, the ecological
1529 impacts of *C. polykrikoides* likely depend not only on the absolute cell densities achieved by
1530 blooms but also on the relative dominance of *C. polykrikoides* among the total plankton
1531 community.
1532
1533 *4.2 Mechanisms of ichthyotoxicity for northeast US strains of C. polykrikoides*
1534 The mechanism or chemical nature of *Cochlodinium* toxicity has been a controversial issue
1535 and may involve PSP toxins (Onoue and Nozawa 1989b), neurotoxic, hemolytic, and
1536 hemagglutinative toxic fractions (Onoue and Nozawa 1989a), fatty acids (Lee 1996), reactive
1537 oxygen species (Kim et al 1999), extracellular polysaccharides and/or mucus (Hallegraeff
1538 1992; Lee 1996; Kim et al 2002). During our experiments, low oxygen should not have
1539 influenced fish survival as fish in the negative control of GSe medium, in which there was no
1540 aeration, and in the negative control comprised of a non-toxic alga (*Gymnodinium aureolum*)
1541 survived to the end of all experiments conducted (up to 5 days). In addition, cultures which
1542 were bubbled maintained their ichthyotoxicity (Table 1, bay scallop experiments). Oxygen
1543 supersaturation was also likely not responsible for the observed fish killing as no aeration was
1544 provided in most fish bioassays and fish did not perish in negative controls comprised of a *G.*
1545 *aureolum* culture with cell densities similar to those in live treatments of *C. polykrikoides*
1546 (Table 1).

1547 The inability of concentrated-, frozen-and-thawed-, sonicated-, heat-killed- *C.*
1548 *polykrikoides* biomass (with or without pre-concentration), to cause fish mortality (Table 1)
1549 suggests that the agent(s) responsible for toxicity are dependent on the viability of *C.*
1550 *polykrikoides* cells and/or are short-lived compounds, and/or are not stable at low and high
1551 temperature. Fish bioassays using the filtrate from a 5.0μm nylon mesh also demonstrated
1552 the short-lived feature of the toxic agent(s) (minutes; Fig. 5). Furthermore, the fish bioassays
1553 conducted with a 5.0μm mesh partition between live culture and cell-free medium
1554 demonstrated that the fish-killing ability of *C. polykrikoides* is not entirely dependent on
1555 direct contact between cells and fish but rather relies on the viability of *C. polykrikoides* (Fig.
1556 5).
1557 The significant mitigation of *C. polykrikoides* toxicity caused by the additions of
1558 peroxidase and catalase into cultures of both CP1 and CPCB-10 (Figs. 6 and 7) suggests
1559 compounds binding with these enzymes are likely involved in the ichthyotoxicity of *C.*
1560 *polykrikoides*. Because we observed that > 500 μM H_2O_2 (hydrogen peroxide) was required
1561 to cause rapid (< 24 h) morality in juvenile sheepshead minnows (data not shown), and
1562 because such levels are not likely produced by active reactive oxygen species (ROS)-
1563 producing algae (Tang et al 2005), we do not believe H_2O_2 is responsible for the fish-killing
1564 ability of *C. polykrikoides*. However, peroxidase and catalase react with multiple
1565 compounds including organic hydroperoxides and lipid peroxides. Moreover, these enzymes
1566 can increase the rates of dismutation and decomposition reactions of other highly ROS (e.g.
1567 $O_2\bullet^-$, $HO_2\bullet$, and OH•) into H_2O_2. Histopathological examination of the fish killed by *C.*
1568 *polykrikoides* cultures revealed acute gill epithelial lifting and hydropic degeneration of
1569 epithelial and chloride cells in the gills, causing the primary and secondary structure of gill
1570 lamellae to be lost (P.R. Bowser, Aquatic Animal Health Program, College of Veterinary
1571 Medicine, Cornell University, pers. comm.). Such degradation of the gill lamellae would

prohibit gill function (i.e. gas exchange) and is nearly identical to the histopathological characteristics of fish killed by raw bloom water (Gobler et al 2008) and to the damage incurred to fish gills exposed to lethal levels of reactive oxygen species (Tort et al 2002). Importantly, *C. polykrikoides* cells exposed to peroxidase and catalase at the highest levels used in the bioassay remained viable and grew at rates equal to those of unamended cultures, suggesting compromised algal cells were not responsible for the significant decrease in toxicity of cultures. The absence of an effect of trypsin additions (Fig 6), which hydrolyzes various types of protein and polypeptides, at levels that have previously mitigated the toxicity of other dinoflagellate cultures (Matsuyama et al 1997) suggests *C. polykrikoides* toxicity is not associated with protein-like toxins.

The toxicity of *C. polykrikoides* was dependent on the growth stage of CP1 and CPCB-10 cultures (Fig. 3 and Table 1). The nature of this toxicity contrasts with many HAB toxins such as PSP toxins (Touzeta et al. 2007), domoic acid (Howard et al. 2007) and even polysaccharides (Bricelj et al 2001), which tend to be maximal and accumulate when cell growth declines (Sunda et al 2006). The higher level of toxicity observed during exponential growth suggests the toxic principle from *C. polykrikoides* is continually produced by actively growing cells as has been reported for ROS production (Kim et al. 1999). Further, the inability of highly concentrated but killed-, frozen and thawed-, sonicated-, heat-killed- CP1 biomass to kill fish coupled with the ability of live cells separated from fish by a 5µm mesh is consistent with a highly labile, extracellular compound contributing to ichthyotoxicity. Finally, the ability of co-occurring microalgae to mitigate *C. polykrikoides* ichthyotoxicity could have been due to the presence of additional adsorptive surfaces or reactive agents that reduced the toxicity of a highly labile, extracellular, toxic principle.

Kim et al. (1999) proposed that ROS are possibly one of the factors inducing fish kills caused by *C. polykrikoides*. On the contrary, another group of researchers proposed different

1597 mechanisms of fish killing by *C. polykrikoides* (Kim et al. 2002), based on their observations
1598 that O_2^- and H_2O_2 production by *C. polykrikoides* was less than that obtained by Kim et al.
1599 (1999) and also much lower than that by *Chattonella marina*, a species well-known for ROS
1600 production. In addition, according to their observation of a gradual accumulation of
1601 polysaccharides in the culture medium of *C. polykrikoides*, Kim et al. (2002) suggested that
1602 multiple biologically active metabolites such as cytotoxic agents and mucus substances may
1603 contribute to the fish killing mechanism of *C. polykrikoides*. The different observations by
1604 Kim et al. (1999) and Kim et al. (2002) could be due to differences inherent from different
1605 strains of *C. polykrikoides* (Iwataki et al 2008), involvement of compounds or ROS species
1606 beyond O_2^- and H_2O_2 in toxicity, and/or variations in ROS production during different growth
1607 phases. For example, Kim et al. (2002) used a culture of *C. polykrikoides* with a cell density
1608 more than 8,000 cells mL^{-1} for the detection of ROS, which could have been in stationary
1609 growth phase and thus may not have been actively generating ROS, as seen in Kim et al.
1610 (1999). Regarding polysaccharides, our cultures were most toxic in the earliest stage of
1611 growth (Fig. 3) and were toxic to fish across a 5 μm mesh on a short time scale (hours; Fig.
1612 5). Since polysaccharides tend to accumulate as cultures age (Mague et al. 1980) and
1613 because mucus-like aggregates may not quickly and passively penetrate a fine mesh barrier,
1614 polysaccharides themselves may not be the primary toxic agent in *C. polykrikoides* strains
1615 CP1 and CPCB-10.

1616 In conclusion, our observations of *C. polykrikoides* strains CP1 and CPCB-10
1617 demonstrate that ichthyotoxicity is dependent on growth stage of cultures and can be
1618 mitigated by the presence of other microalgae. Therefore, the toxicity of *C. polykrikoides*
1619 blooms may vary as a function of cell densities, relative abundance of *C. polykrikoides*
1620 among the plankton community, and the progression / nutrient limitation of blooms. The
1621 ichthyotoxicity of *C. polykrikoides* was mitigated by the enzymes peroxidase and catalase

and was dependent on cell viability but not on physical contact between cells and fish. Therefore, extracellular, non-H_2O_2, labile toxins such as ROS-like compounds may play a role in bloom toxicity.

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Table **1**. Fish and shellfish bioassays examining the toxicity of *C. polykrikoides* strains CP1 and CPCB-10. The GSe medium and a non-toxic culture of *Gymnodinium aureolum* were used as negative controls.

Exp. No.	Culture and treatments[a]	Cell conc. (or Equiv. conc) (cells mL^{-1})	No. of fish used	No. of fish dead in 24 h	Average time of fish death (h)	Length of fish (Mean.±Stdev) (cm)
1	GSe medium[b]	0	6	0	—	0.7±0.2
2	*Gymnodinium aureolum* culture	11,684	8	0	—	0.7±0.2
3	*C. polykrikoides* CP1 100%	4,320	6	6	0.25±0.0	0.7±0.2
4	CP1 dilution (90% of Exp. 3)	3,890	6	6	0.69±0.2	0.7±0.2
5	CP1 dilution (80% of Exp. 3)	3,460	6	6	0.76±0.2	0.7±0.2
6	CP1 dilution (70% of Exp. 3)	3,020	6	6	1.28±0.07	0.7±0.2
7	CP1 dilution (60% of Exp. 3)	2,590	6	6	1.47±0.16	0.7±0.2
8	CP1 dilution (50% of Exp. 3)	2,160	6	6	2.54±0.74	0.7±0.2
9	CP1 dilution (40% of Exp. 3)	1,730	6	6	3< t <15	0.7±0.2
10	CP1 dilution (30% of Exp. 3)	1,300	6	3	3< t <72	0.7±0.2
11	CP1 dilution (20% of Exp. 3)	860	6	0	47< t <72	0.7±0.2
12	CP1 sonicated (from Exp. 3)	Eq. 4,320	6	0	—	0.7±0.2
13	CP1 boiled for 15min (from Exp. 3)	Eq. 4,320	6	0	—	0.7±0.2
14	CP1 frozen (-20˚C) and thawed (from Exp. 3)	Eq. 4,320	6	0	—	0.7±0.2
15	CP1 concentrated biomass and resuspended in GSe medium (from Exp. 3)	Eq. 8,640	6	0	—	0.7±0.2
16	CP1 culture	2,220	6	6	0.5±0.08	0.7±0.2
17	CP1 concentrated and sonicated biomass, resuspended in GSe (from Exp. 16)	Eq. 17,620	6	0	>96	0.8±0.1
18	CP1 concentrated and frozen biomass, resuspended in GSe (from Exp. 16)	Eq. 17,620	6	0	>96	0.8±0.1

19	CP1 vs. scallops (*Argopecten irradians*)[c]	3,060	10	10	24< t <69h	1.1±0.1
20	CP1 vs. scallops (*Argopecten irradians*)[c]	940	2	2	58-72	3.0±0.0
21	CP1 vs. scallops (*Argopecten irradians*)[c]	4,565	2	2	31-47	3.0±0.0
22	CP1 vs. scallops (*Argopecten irradians*)[c]	1,395	2	2	28, 39	3.1±0.6
23	*C. polykrikoides* CPCB-10	350	6	0	Alive for >4d	0.6±0.2
24	CPCB-10 100%[d]	1,000	6	6	3.0±0.6	0.6±0.1
25	CPCB-10 dilution (90% of Exp. 24) [d]	900	6	0	>72	0.7±0.2
26	CPCB-10 100%[e]	710	6	3	8< t <24	0.5±0.1
27	CPCB-10 dilution (75% of Exp. 26) [e]	530	6	1	4.0	0.5±0.1
28	CPCB-10 dilution (50% of Exp. 26) [e]	350	6	0	>96	0.5±0.1

[a] All Experiments were against the sheepshead minnows (*Cyprinodon variegates*) unless otherwise indicated.
[b] Same control used in each experiment for both fish and shellfish; no control fish or shellfish died during experiments
[c] Five scallops into each of two 1-L flasks containing CP1 culture. Air bubbling was used for aeration. Seawater from which GSe medium was made was used as control; all control animals survived.
[d] Culture was in late exponential phase growth.
[e] Culture was in exponential phase growth.

Figure Legends

Fig. 1. Sheepshead minnow (*Cyprinodon variegates*) bioassays using *C. polykrikoides* CP1 culture and its dilutions, showing the relationship between cell concentration of *C. polykrikoides* CP1 and the mortality of (2-wk old) sheepshead minnows in 24h. The dilutions of CP1 were prepared with GSe medium that was used for culturing CP1. The initial cell concentrations (100%) of CP1 for each of the three experiments were 4,320, 3,460, and 2,710 cells mL^{-1}, respectively.

Fig. 2. Sheepshead minnow (*Cyprinodon variegates*) bioassays using *C. polykrikoides* CP1 culture and its dilutions, showing a correlation between death time of fish and dose of *C. polykrikoides* CP1 cells (defined as the ratio of algal cell density of CP1 to the average length of fish). Experiments were conducted with three batches of cultures at exponential stage (with cell densities of 1.6×10^3, 2.5×10^3, and 3.0×10^3 cells mL^{-1}) and fish with lengths ranged from 0.6 to 2.1 cm. The regression equation and its significance are shown.

Fig. 3. Sheepshead minnow (*Cyprinodon variegates*) bioassays using *C. polykrikoides* CP1 culture and its dilutions, showing the effect of growth stage on the ichthyotoxicity of *C. polykrikoides* CP1 in terms of the death time of fish within 24h. *C. polykrikoides* CP1 cultures at growth stages from early and middle exponential, late exponential, to late stationary phases, with initial cell densities of 0.33×10^3, 1.33×10^3, 2.96×10^3, 3.0×10^3, 3.69×10^3, 4.32×10^3, and 2.71×10^3 cells mL^{-1}, respectively. Six fish of ~ two weeks old (length range 0.5 – 0.8 cm, 0.6±0.1cm) were used for each treatment. The error bars indicate ±1 SD for the death time of fish.

Fig. 4. Effects of the co-occurring microalgae (*Prorocentrum minimum* CCMP696 and *Rhodomonas salina* CCMP1319) on the toxicity of *C. polykrikoides* CP1 to 2 week-old juvenile, sheepshead minnow (C. *variegates*; 0.6 ± 0.1 cm, n=54) as reflected in (A) the average death time of fish and (B) the percent mortality of fish population. No fish died in the GSe medium negative controls until the test was stopped at 96h. The CP1 cell density in all treatments was 1.34 x 10^3 cells mL^{-1}. The error bars indicate ± 1 standard deviation, and * indicates a significant increase in death time as compared with the control ($p < 0.05$).

Fig. 5. Sheepshead minnow (*Cyprinodon variegates*) bioassay using cell-free medium of *C. polykrikoides* CP1, prepared with gravity flow through 5µm-mesh nylon membrane (closed bars) and 5-µm partition chamber (open bars), showing the difference in mortality in 24h (percentages above each bar) and the death time of fish between the positive control and cell-free medium (bars). GSe medium was used as negative control and no fish died until the tests were stopped. Six fish were used for each treatment. For the test using cell-free filtrate through 5µm-mesh nylon membrane (closed bars), the cell density of *C. polykrikoides* in the initial culture (CP control) was 1.0 x10^3 cell mL^{-1}, and fish sizes were 0.6±0.1 cm. For the test using the ice-cube tray, the cell densities of *C. polykrikoides* in the culture side (doubled from initial culture) and the other side were 3.36 x10^3 and <2 cell mL^{-1}, respectively, and the fish sizes for the experiment were 0.5±0.1 cm. The error bars indicate ± 1 standard deviation, and * indicates a significant increase in death time as compared with the positive control ($p < 0.05$). The average death time of fish was calculated based on the 24h mortality shown above each bar.

Fig. 6. Effects of the addition of peroxidase (Pero.), superoxide dismutase (SOD), catalase (Cata.), and trypsin (Tryp.) to *C. polykrikoides* cultures (CP1) on the ichthyotoxicity of *C. polykrikoides* to the 2 week-old sheepshead minnow (*Cyprinodon variegates*). For all batches

of experiments, GSe medium alone or the medium with additions of the above enzymes and chemicals in their highest concentrations as added into cultures were used as negative controls, while the initial cultures of CP1 (100%) were used as positive controls. No fish died in the GSe medium and all other negative controls. Six fish were used per treatment. The cell densities of *C. polykrikoides* and fish lengths for the three Experiments (as separated by the space) were 2.67 $x10^3$, 1.70 $x10^3$, and 1.33 $x10^3$ cells mL^{-1}, and 0.7±0.2, 0.5±0.1, and 0.5±0.1 cm, respectively. The error bars indicate ± 1 standard deviation, and * indicates a significant increase in death time as compared with the CP control (100%; $p < 0.05$).

Fig. 7. Effects of the addition of peroxidase (Pero.) and catalase (Cata.) on the ichthyotoxicity of *C. polykrikoides* CPCB-10 to the 2 week-old sheepshead minnows (*Cyprinodon variegates*), as measured in the mortality of fish over 96 h. GSe medium was used as negative control. Six fish were used per treatment. The initial cell density of *C. polykrikoides* CPCB-10 (100%) and fish lengths were 7.1 $x10^2$ cells mL^{-1} and 0.5±0.1 cm, respectively. The final concentrations of peroxidase and catalase added into the initial culture of CPCB-10 were 1.25 μg mL^{-1} and 0.5 U mL^{-1}, respectively.

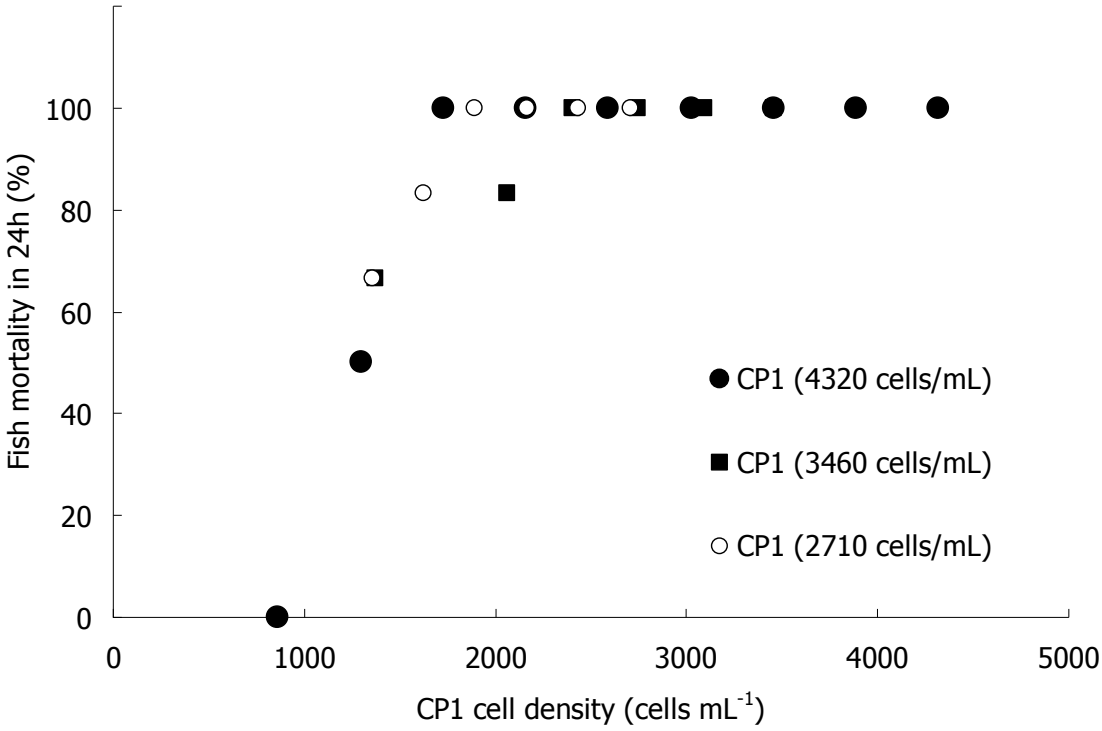
Fish mortality in 24h (%)
100
80
60
40
20
0
0
1000
2000
3000
4000
5000
CP1 cell density (cells mL-1)
CP1 (4320 cells/mL)
CP1 (3460 cells/mL)
CP1 (2710 cells/mL)

Fig.1

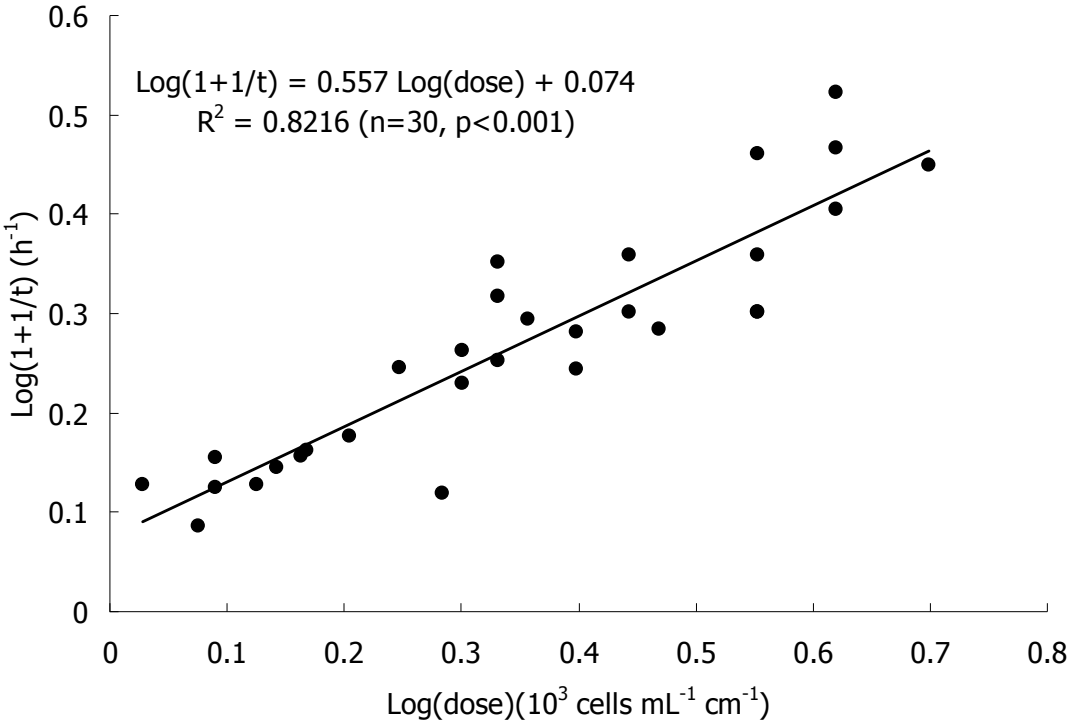
Log(1+1/t) = 0.557 Log(dose) + 0.074
R2 = 0.8216 (n=30, p<0.001)
Log(1+1/t) (h-1)
Log(dose)(10^3 cells mL-1 cm-1)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

Fig.2

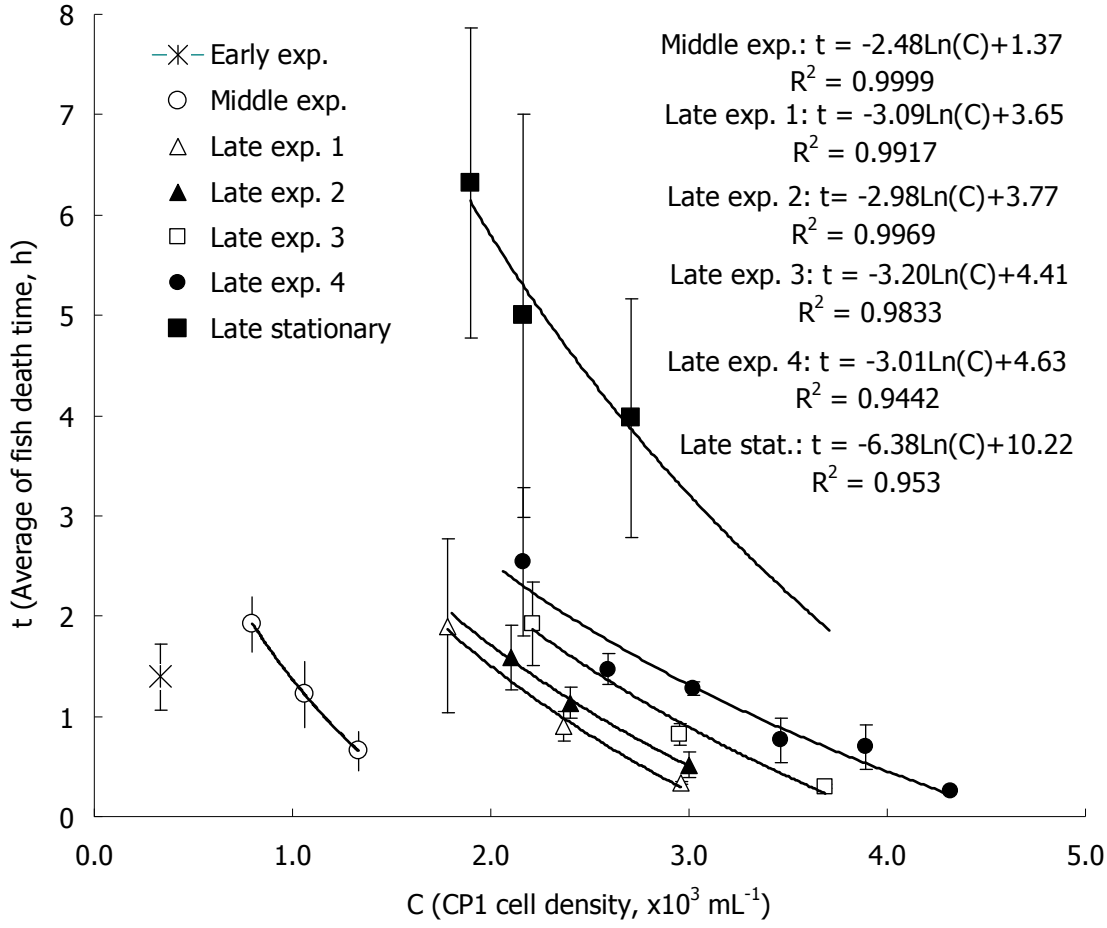
Early exp.
Middle exp.
Late exp. 1
Late exp. 2
Late exp. 3
Late exp. 4
Late stationary
Middle exp.: t = -2.48Ln(C)+1.37
R2 = 0.9999
Late exp. 1: t = -3.09Ln(C)+3.65
R2 = 0.9917
Late exp. 2: t= -2.98Ln(C)+3.77
R2 = 0.9969
Late exp. 3: t = -3.20Ln(C)+4.41
R2 = 0.9833
Late exp. 4: t = -3.01Ln(C)+4.63
R2 = 0.9442
Late stat.: t = -6.38Ln(C)+10.22
R2 = 0.953
t (Average of fish death time, h)
C (CP1 cell density, x10^3 mL^-1)
0
1
2
3
4
5
6
7
8
0.0
1.0
2.0
3.0
4.0
5.0

Fig.3

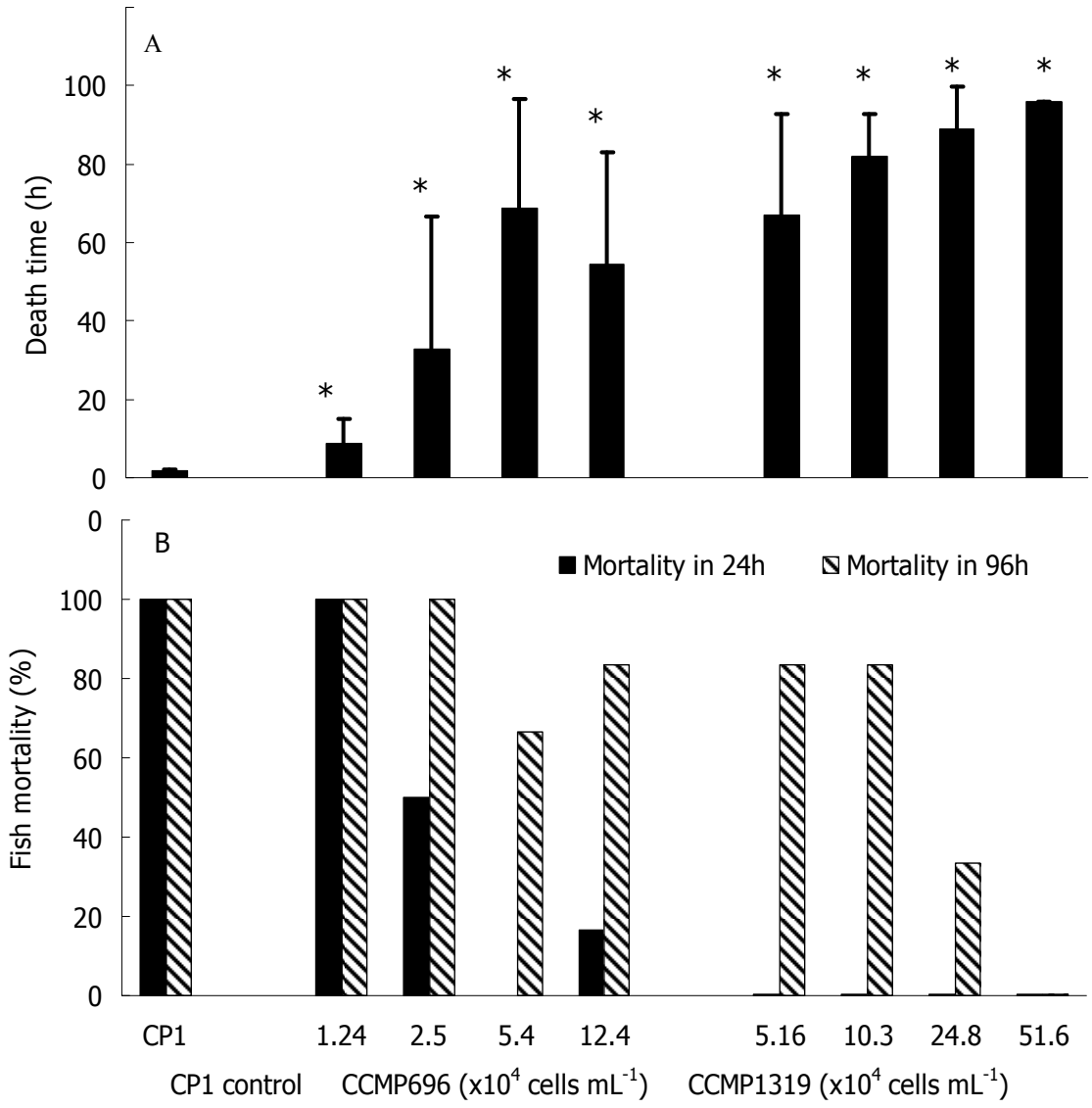
A
Death time (h)
0
20
40
60
80
100
*
B
Mortality in 24h
Mortality in 96h
Fish mortality (%)
0
20
40
60
80
100
CP1
1.24
2.5
5.4
12.4
5.16
10.3
24.8
51.6
CP1 control
CCMP696 (x10^4 cells mL^-1)
CCMP1319 (x10^4 cells mL^-1)

Fig.4

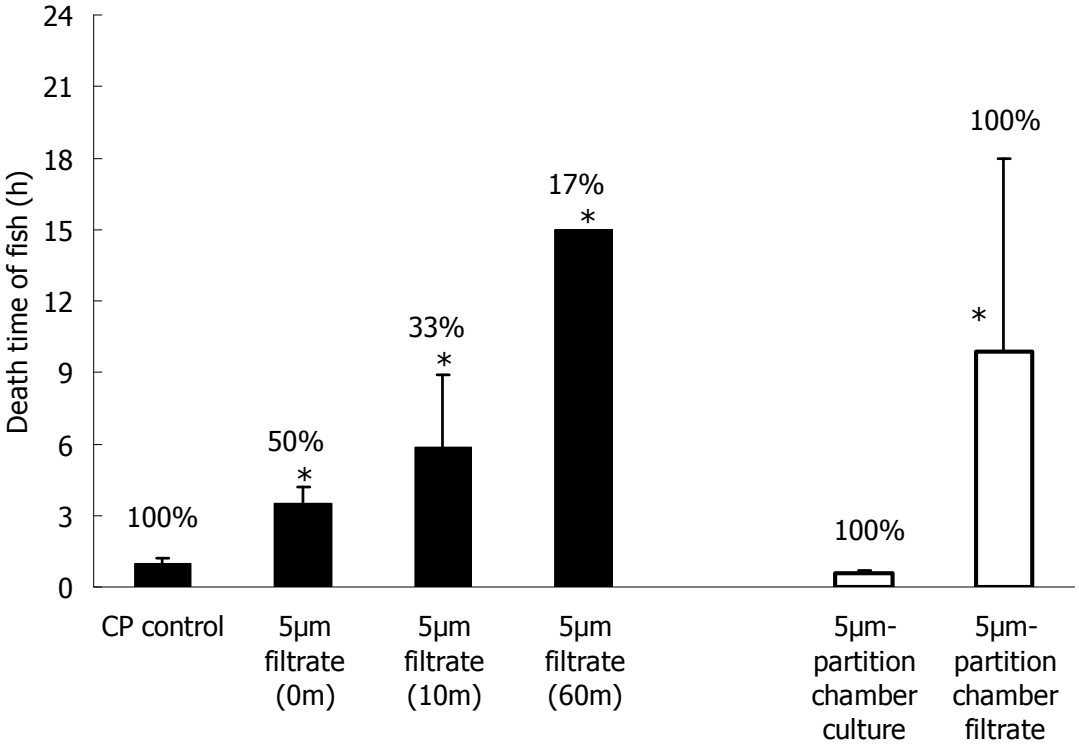
Death time of fish (h)
24
21
18
15
12
9
6
3
0
100%
50%
*
33%
*
17%
*
100%
*
100%
CP control
5μm filtrate (0m)
5μm filtrate (10m)
5μm filtrate (60m)
5μm-partition chamber culture
5μm-partition chamber filtrate

Fig. 5

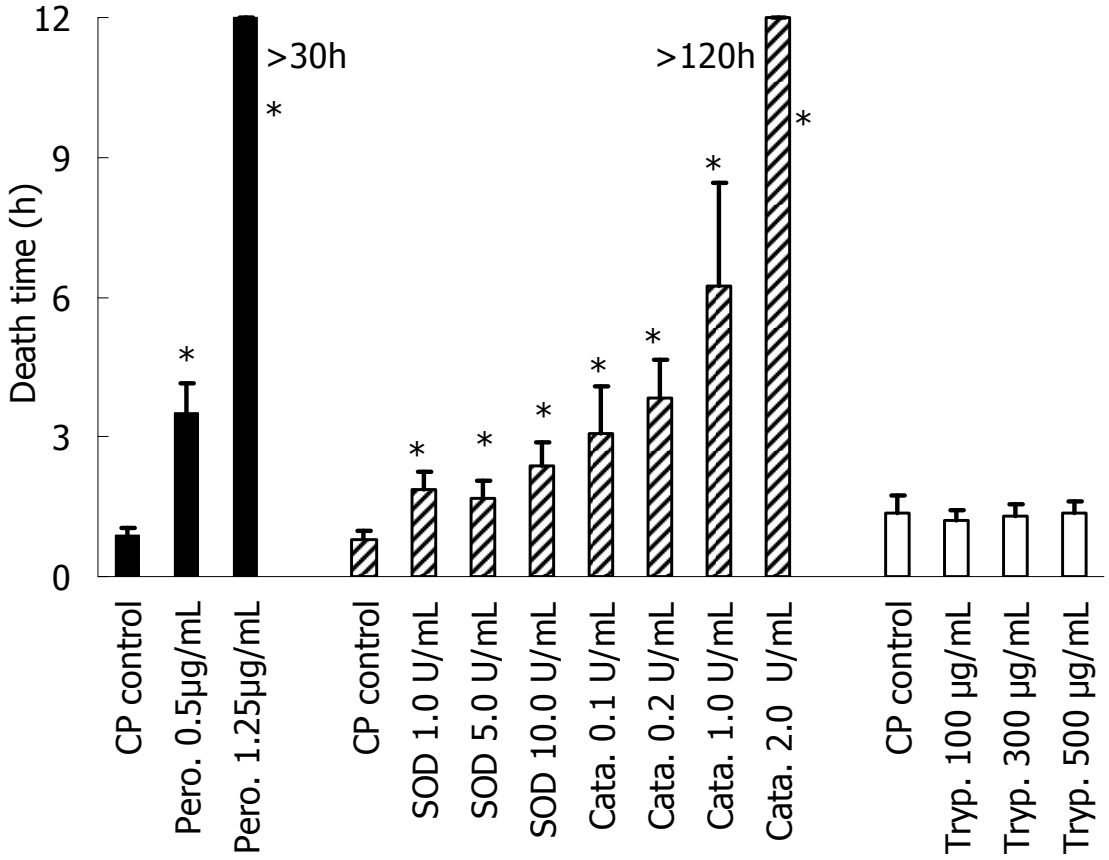
12
9
6
3
0
Death time (h)
>30h
>120h
*
CP control
Pero. 0.5μg/mL
Pero. 1.25μg/mL
CP control
SOD 1.0 U/mL
SOD 5.0 U/mL
SOD 10.0 U/mL
Cata. 0.1 U/mL
Cata. 0.2 U/mL
Cata. 1.0 U/mL
Cata. 2.0 U/mL
CP control
Tryp. 100 μg/mL
Tryp. 300 μg/mL
Tryp. 500 μg/mL

Fig. 6

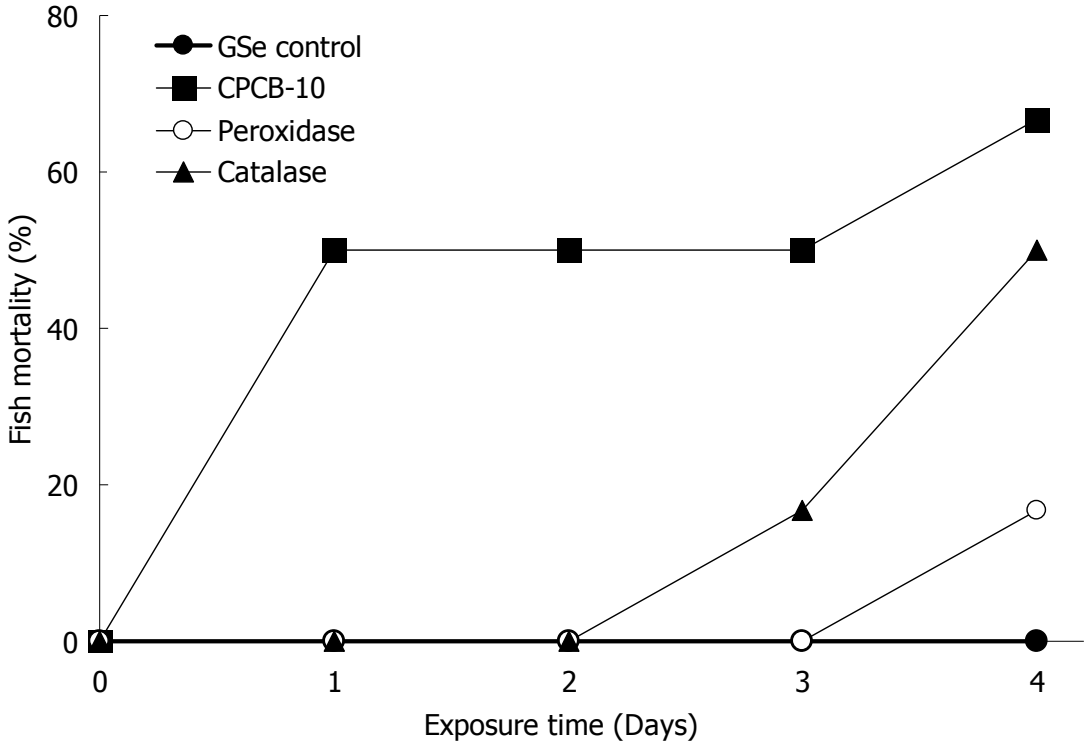
80
60
40
20
0
Fish mortality (%)
GSe control
CPCB-10
Peroxidase
Catalase
0
1
2
3
4
Exposure time (Days)

Fig. 7

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

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KEY WORDS: *Cochlodinium polykrikoides,* harmful algal blooms, bivalve larvae, toxicity, mortality of bivalve larvae, Eastern oyster, bay scallop, Northern quahog (hard clam), reactive oxygen species

189

190

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 x 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.

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 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; Bricelj 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* ($> 10^4$ cells ml^{-1}) from the York River, Virginia, USA demonstrated 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 $3x10^4$ 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 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 (see below) 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

290 of the Peconic Estuary, Flanders Bay (40.923°N, 72.587°W) and September 4, 2008 from
291 Great Peconic Bay (40.936°N, 72.512°W), New York, USA. Molecular and microscopic
292 identification of strain CP1 has been reported previously (Gobler et al. 2008). The *C.*
293 *polykrikoides* strain CPCB-10 was isolated from Cotuit Bay, Massachusetts, USA.
294 Identification of both isolates as *C. polykrikoides* was confirmed with large subunit (LSU)
295 rDNA sequencing (Gobler et al. 2008; Iwataki et al. 2008). Cultured *Isochrysis galbana*
296 (Tahitian strain; T-Iso) was used as a control since it fosters maximal growth and survival in
297 bivalve larvae (e.g. Padilla et al. 2006). All cultures were maintained in sterile GSe medium
298 with a salinity of 31-32, made with autoclaved and 0.2 μm-filtered seawater (Doblin et al.
299 1999), at 21° C in an incubator with a 12 h light: 12 h dark photoperiod, illuminated by a
300 bank of fluorescent lights providing a light intensity of ~100 μmol quanta m^{-2} s^{-1}. For all
301 cultures, an antibiotic-antimycotic solution (a mixture of 10,000 I.U. penicillin, 10,000 μg ml^{-1}
302 streptomycin, and 25 μg ml^{-1} amphotericin B; Mediatech. Inc., Hemdon, Virginia) was
303 added into the medium immediately before inoculation, with a final concentration of 1% to
304 minimize contamination by bacteria and fungus. This antibiotic mixture has no negative
305 effects on the growth and survivorship of bivalve larvae (Padilla et al. 2006). Periodic DAPI-
306 staining of cultures during the study indicated the general absence of bacteria in the cultures
307 most of the time.
308
309 Bivalve larvae
310 Eastern oyster larvae (*Crassostrea virginica*) were provided by Dave Veilleux and Dr. Gary
311 Wikfors of Milford laboratory, NOAA Fisheries Northeast Fisheries Science Center
312 (Connecticut, USA); bay scallop larvae (*Argopecten irradians*) were obtained from the East
313 Hampton Shellfish Hatchery (New York, USA), and Northern quahog larvae (*Mercenaria*
314 *mercenaria*) were obtained from the Cornell Cooperative Extension shellfish hatchery facility

315 in Southold (New York, USA) as well as from the East Hampton Town Shellfish Hatchery
316 (New York, USA). Before experiments, all larvae were maintained in filtered seawater with
317 daily feeding of T-Iso cultures at ~5 x 10^4 cells ml^{-1} and mild aeration at ~22ºC.
318
319 Experiments
320 All experiments were conducted during June through September of 2008 with sterile, 6-well
321 polystyrene culture plates (*n*=6 for each treatment). For each treatment of each experiment,
322 10-ml experimental cultures of (CP1, CPPB17, or CPCB-10 or bloom water) of
323 *Cochlodinium polykrikoides* were added to each well. Unless otherwise indicated, all cultures
324 were within exponential stage growth. Approximately 20 larvae (i.e. ~ 2 larvae ml^{-1}) were
325 gently transferred into each well with a modified 1.0-ml pipette, yielding a final density
326 consistent with those used for prior experiments investigating the toxicity of harmful algae to
327 bivalve larvae (Leverone et al. 2006; Bricelj and MacQuarrie 2007; Stoecker et al. 2008). At
328 24 and 72 h (except for the first scallop larva bioassay, which was 10 h), the number of dead
329 larvae in each well was enumerated under an inverted light microscope (total magnification
330 x40, occasionally x100) by scanning the entire well. The total number of larvae was
331 determined the same way after all samples were fixed with Lugol's solution (final
332 concentration 1%) at the end of each experiment (immediately after the final enumeration of
333 dead larvae). A larva was considered dead when swimming and movements of the velum and,
334 when visible, internal organs, ceased. Experiments were conducted using 24-h old veligers
335 (D-stage) and with older larvae (8 – 11 d) which were also veligers. The single exception
336 was an experiment conducted with 10 d-old bay scallop larvae which were a mix of veligers
337 and a small portion of pediveligers. All plates (with covers) were maintained with a salinity
338 of 31-32 at light intensity of 10 μE m^{-2} s^{-1} and ~24ºC, a temperature yielding maximal growth

339 rates for *Crassostrea virginica, Argopecten irradians*, and *Mercenaria mercenaria* larvae
340 (Carriker 2001; Matthiessen 2001; Cragg 2006; Padilla et al. 2006).
341 For all experiments, a treatment of T-Iso at 1 - 4 x 10^4 cells ml^{-1} was used as the
342 control, densities that yield maximal growth rates for shellfish larvae (Padilla et al. 2006).
343 Dilution series of *C. polykrikoides* cultures (CP1, CPPB17, and CPCB-10) or bloom water
344 were prepared by diluting treatment water with GSe medium with a salinity similar to culture
345 and bloom water. For several experiments, a parallel series of dilutions was prepared with 1.0
346 to 4.0 x 10^4 cells ml^{-1} of T-Iso (identical density to the control) added into each well with the
347 CP1 and CPCB-10 cultures. For one experiment (24-h Northern quahog larvae), 0.22µm-
348 filtered CP1 cultures, with and without the addition of T-Iso, were used as treatments to
349 assess the importance of intracellular and extracellular toxicity of *C. polykrikoides*.
350 An experiment with Northern quahog larvae was conducted using *Cochlodinium*
351 *polykrikoides* bloom water collected from Old Fort Pond, (40.868°N, 72.446°W), a tidal
352 tributary in Eastern Shinnecock Bay, and Great Peconic Bay (40.936°N, 72.512°W), on 5
353 September 2008, both dominated by *C. polykrikoides*. Dilutions of bloom water were made
354 with GSe medium as described above. Initial concentrations of *C. polykrikoides* and other
355 microalgae > 10 µm diameter were determined by counting cells in 1.0 ml of triplicate bloom
356 water samples added to a Sedgewick-Rafter counting chamber after fixation with Lugol's
357 solution. *Cochlodinium polykrikoides* abundances in the bloom water were 1.8 x10^3 cells ml^{-1}
358 and 0.8 x10^3 cells ml^{-1} for Shinnecock Bay (Old Fort Pond) and the Peconic Estuary (Great
359 Peconic Bay), respectively, while the abundances of other phytoplankton >10 µm were 1.6
360 x10^3 cells ml^{-1} and 0.2x10^3 cells ml^{-1}, respectively.
361 Since prior research has demonstrated that reactive oxygen species (ROS)-scavenging
362 enzymes can mitigate or reduce the toxicity of *C. polykrikoides* to fish (Kim et al. 1999; Tang
363 and Gobler 2009), additions of peroxidase and catalase to CP1 cultures were used as

364 treatments during experiments with bay scallop and Northern quahog larvae. The stock
365 solutions of horseradish peroxidase (MP Biomedicals, LLC., Aurora, Ohio) and catalase (MP
366 Biomedicals, LLC., Solon, Ohio) were added to cultures of CP1 in a final concentration of
367 1.25 μg ml^{-1} for peroxidase and 0.5 U ml^{-1} for catalase. In the stock solutions of enzymes,
368 horseradish peroxidase was dissolved in 10 mM phosphate buffer, 0.15 M NaCl, pH 7.4,
369 containing 1.0% BSA and 0.1% proclin as a preservative, with a concentration of 2.5 mg ml^{-1}
370, while catalase was dissolved in an aqueous solution with a concentration of 1,108 U ml^{-1}
371 (one unit decomposes 1.0 μmole of hydrogen peroxide per minute at pH 7.0, 25° C). We
372 have previously demonstrated that these compounds at the concentrations used here do not
373 negatively affect the growth or viability of *C. polykrikoides* (Tang and Gobler 2009).
374 Since the ichthyotoxicity of *C. polykrikoides* can also be affected by the growth stages
375 of cultures (Tang and Gobler 2009), three batches of CP1 culture in exponential, stationary,
376 and late-stationary growth phases at equal cell densities were used as treatments for a
377 Northern quahog larvae bioassay. The cell densities of CP1, CPCB-10, and *C. polykrikoides*
378 in bloom water, cell densities of T-Iso, species and ages of larvae, average numbers of larvae
379 in each well, and final concentrations of peroxidase and catalase are summarized in Table 1.
380
381 Statistics
382 All percentage data (percent mortality) were arcsine square root-transformed before
383 ANOVAs were performed. One-, two-, or three-way ANOVAs were performed to assess the
384 effects of the source of *C. polykrikoides* cells (strain CP1, strain CBCP-10, or bloom water),
385 density of *C. polykrikoides* cells, the duration of larval exposure to *C. polykrikoides* cells (24
386 h or 72 h), the addition of other microalgae (T-Iso), the addition of enzymes, and/or culture
387 filtrate on larval survival. Only subsets of these factors were part of each experiment.
388 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 ($0.39\text{-}0.78 \times 10^3$ 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 in the control (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 the control (post hoc comparison; $p<0.001$; Fig 2A). Eight-day old larvae exposed to CP1 for 24 h

414 experienced mortalities of 60 to 100% at cell densities from 0.88 to 2.19 x10^3 cells ml^{-1}, all of
415 which were significantly higher than mortalities in the control (post hoc comparison; Fig 2B).
416 The addition of T-Iso to CP1 significantly lessened its toxic effect on 8-d larvae at cell
417 densities > 0.88 x10^3 cells ml^{-1} ($p<0.001$, two-way ANOVA; Fig. 2B).
418 Ten-day larvae were more resistant to *C. polykrikoides* cultures than the younger
419 larvae (Fig. 3). For example, 10-d larvae exposed to 1.7 x10^3 CP1 cells ml^{-1} displayed only
420 9.6% mortality in 24 h (Fig. 3) whereas 24-h larvae exposed to 1.5 x10^3 CP1 cells ml^{-1} for 10
421 h experienced 83% mortality (Fig. 1A) . In general, the mortality of 10-d larvae was
422 dependent on *C. polykrikoides* cell densities ($p<0.001$; Three-way ANOVA) but did not differ
423 between strains CP1 and CB-CP10 ($p>0.05$; Three-way ANOVA). For example, strain
424 CPCB-10 caused larval mortalities of 54% to 80% at cell densities of 1.07 to 1.78 x10^3 cells
425 ml^{-1} in 72 h while CP1 caused similar mortalities (50% and 89% at cell densities of 0.87 and
426 1.74 x10^3 cells ml^{-1}; Fig. 3). Finally, exposure time had a significant treatment effect, as 72-h
427 exposure yielded three- to ten-times greater mortalities than those observed at 24 h at
428 densities $\geq$ 8.7 x 10^2 cells ml^{-1} ($p < 0.05$; Three-way ANOVA; post hoc comparison).
429
430 Toxicity of *C. polykrikoides* to the larvae of Northern quahog (*Mercenaria mercenaria*)
431 One day-old Northern quahog larvae were exposed to cultures of T-Iso (control), CP1 (with
432 equal level of T-Iso), and *C. polykrikoides* bloom water from Peconic Bay (PB) and Old Fort
433 Pond (OFP) for 24 h and 72 h (Fig. 4A). The CP1 culture caused mortalities in larvae from
434 9% to 59% in 24 h and 14% to 84% at 72 h at cell densities from 0.35 to 1.39 x10^3 cells ml^{-1}
435 (Fig. 4A). Bloom water from PB (*C. polykrikoides* cell density 0.79 x10^3 ml^{-1}) caused larval
436 mortalities of 5% to 23% in 24 h and 27% to 97% in a 72-h exposure with different dilutions
437 (Fig. 4A), while bloom water from OFP (*C. polykrikoides* cell density 1.79 x10^3 ml^{-1}) was
438 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 were 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 $x10^3$ 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 x 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 $x10^3$ *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 compared to the control (post hoc comparison; $p<0.001$; Table 2) and mortalities were significantly

464 higher after 72 h compared to the 24-h exposure (post hoc comparison; $p<0.001$; Table 2).
465 Older larvae (11 d) were similarly susceptible to CPCB-10 as cell densities of 0.86 $x10^3$ cells
466 ml^{-1} caused 59% and 54% mortalities without and with the addition of T-Iso after a 24-h
467 exposure and significantly higher mortalities (97% and 80%) after the 72-h exposure (post
468 hoc comparison; $p<0.001$; Table 2). The mortality of the 11-d larvae was significantly higher
469 in all treatments with *C. polykrikoides* CPCB-10 compared to the control (post hoc
470 comparison; $p<0.001$; Table 2). Finally, *C. polykrikoides* strain CPPB17 caused significantly
471 greater mortality in 24-h Northern quahog larvae compared to control treatments (post hoc
472 comparison; $p<0.001$; Table 2).
473
474 Effects of growth stage and enzymes on toxicity of *C. polykrikoides* to bivalve larvae
475 *Cochlodinium polykrikoides* cultures in exponential growth were significantly more toxic to
476 the 24-h Northern quahog larvae after a 72-h exposure (96% mortality) than late exponential
477 (39% mortality) and stationary stages (31% mortality; post hoc comparison, $p<0.001$; Fig. 5),
478 while the later two showed similar toxicity (post hoc comparison, $p>0.05$). Additions of the
479 enzymes peroxidase (final concentration 1.25 μg ml^{-1}) and catalase (final concentration 0.5 U
480 ml^{-1}) to the culture of CP1 with a cell density of 1.74 $x10^3$ cells ml^{-1} significantly reduced the
481 mortality of 10-d bay scallop larvae from 85% to 27% and 61%, respectively, during a 72-h
482 exposure (post hoc comparison, $p<0.001$; Fig. 6A). The addition of peroxidase was more
483 effective in mitigating the toxicity of CP1 to scallop larvae than catalase (post hoc
484 comparison; $p<0.05$). Peroxidase (1.25 μg ml^{-1}) and catalase (0.5 U ml^{-1}) also significantly
485 reduced the mortality of 24-h Northern quahog larvae in 24-h and 72-h exposures to CP1
486 (Two-way ANOVA, post-hoc comparison, $p<0.001$; Fig. 6B), with a more significant
487 mitigation effect observed during the 24-h exposure compared to the 72-h exposure (Two-
488 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-h and 72-h exposures, respectively) was nearly identical to that in the T-Iso control (4% and 8% mortalities in 24-h 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-h 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* (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

514 investigated Asian strains of *C. polykrikoides*. The greater resistance of older larvae of
515 scallops and Northern quahog to the toxicity of *C. polykrikoides* may be due to their more
516 calcified shells (Carriker 2001) and/or ingestion of *C. polykrikoides* cells during incubations
517 (thus reducing the density of toxic cells; Jeong et al. 2004). However, this finding differs
518 from the toxic effect of *Karlodinium veneficum* to Eastern oyster larvae which were more
519 sensitive at later stages (Stoecker et al. 2008). Since older larvae consume more prey, the
520 differences in toxicity between *C. polykrikoides* and *K. veneficum* to older larvae suggests
521 that *K. veneficum* toxicity involves an intracellular toxin which is consumed in higher
522 quantities by older individuals (i.e. karlotoxin; Stoecker et al. 2008) whereas *C. polykrikoides*
523 toxicity does not. The significant reduction in mortalities of bay scallop and Northern
524 quahog larvae by the removal of *C. polykrikoides* cells (i.e. culture filtrate) or the addition of
525 the enzymes peroxidase and catalase to a CP1 culture was also consistent with previous
526 investigations of juvenile finfish (Tang and Gobler 2009). These findings suggest a similar
527 toxic mechanism is responsible for killing finfish and bivalve larvae and that toxicity is
528 maximized by close contact between viable cells and victim organisms and may involve
529 highly labile toxins, such as reactive oxygen species which can be scavenged by peroxidase
530 and catalase (Kim et al. 1999; Tang and Gobler 2009). However, further study is warranted to
531 establish the mortality mechanism associated with *C. polykrikoides*.
532 Previously, a field study using seawater containing high abundances of *C.*
533 *polykrikoides* cells ($> 10^4$ cells ml^{-1}) demonstrated this bloom water could rapidly kill Eastern
534 oyster larvae (*Crassostrea virginica*; Ho and Zubkoff 1979). However, the levels of mortality
535 of oyster larvae (~90%) exposed to 10^4 cells ml^{-1} were similar to our observations of cultures
536 and some blooms at an order of magnitude lower densities. Ho and Zubkoff (1979)
537 suggested that spatial competition was the cause of adverse effects that the bloom water
538 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 $x10^3$ 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 $x10^3$ 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$x10^3$ cells ml^{-1}) present compared to Old Fort Pond (1.6 $x10^3$ 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 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 $x10^3$ 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 $x10^4$ 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 of the toxicity of *C. polykrikoides* can vary widely depending on the

564 viability and physiological status of cultures (Fig 5; Tang and Gobler 2009), cell dosage and
565 concepts such as median lethal concentration (LC50) have are not ideal indices of *C.*
566 *polykrikoides*.toxicity.

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

575 The cell abundances of *C. polykrikoides* used in our experiments ($\leq$ 2.2 x10^3 cells ml^{-1})
576 were equivalent to levels found in blooms in US estuaries (Gobler et al. 2008). Since the
577 bivalve species used in our study spawn in months (June to October; Barber and Blake 1983;
578 Hesselman et al. 1989; Thompson et al. 1996; Tettelbach et al. 2002) overlapping with the
579 occurrence of *C. polykrikoides* blooms on the east coast of North America (July to October;
580 Nuzzi 2004; Gobler et al. 2008), our results suggest that blooms of *C. polykrikoides* may have
581 severe negative impacts on Eastern oyster, bay scallop, and Northern quahog populations.
582 Since many wild populations of bivalves, including the species studied here, have
583 experienced precipitous declines (Jackson et al. 2001; Kemp et al. 2005; Lotze et al. 2006;
584 Myers et al. 2007), future management and restoration planning should consider the temporal
585 and spatial range of *C. polykrikoides* and other harmful algal blooms.

586

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597
598

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819 **Table 1** Experimental conditions for bioassays using cultures of CP1 and CPCB-10 or bloom water with *C. polykrikoides*, and larvae of Eastern
820 oyster (*Crassostrea virginica*), bay scallop (*Argopecten irradians*), and Northern quahog (*Mercenaria mercenaria*). Concentrations in columns
821 5-7 refer to ranges of series dilutions.
822

Test	Species	Larval Age (d)	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 No. 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)	-

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825 **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

826 Northern quahog (*Mercenaria mercenaria*) of different ages. Exposure time of larvae to cultures was 24 h and 72 h for all three experiments

Larval Age (d)	Cell Density of *C. polykrikoides* (cells ml^{-1})	Cell Density of T-Iso (cells ml^{-1})	Larval Mortality (%) in 24 h (Mean ± 1 SD, *n*=6)	Larval Mortality (%) in 72 h (Mean ± 1 SD, *n*=6)
1	0	11 $x10^3$ (control)	0 ± 0	2 ± 4
1	CPCB-10: 0.4 $x10^3$	0	17 ± 6	20 ± 6
1	CPCB-10: 0.4 $x10^3$	11 $x10^3$	11± 2	24 ± 18
11	0	16 $x10^3$ (control)	29± 20	34± 23
11	CPCB-10: 0.86 $x10^3$	0	59 ± 27	97 ± 7
11	CPCB-10: 0.86 $x10^3$	16 $x10^3$	54± 35	79 ± 20
1	0	1.65 $x10^4$ (control)	0	0
1	CPPB-17: 350	16 $x10^4$	13±5	27±14
1	CPPB-17: 350	0	7.5±4	35±12

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Table 3 Comparison of toxicity of *C. polykrikoides* and other harmful dinoflagellate species to bivalve larvae. *Karlodinium veneficum* experiments used strain CCMP1974 and *Karenia brevis* experiments used the Wilson clone

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

Figure Legends

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 d old, the final concentration of *Isochrysis galbana* (T-Iso) in negative control and treatments was 2.2 $x10^4$ cells ml^{-1}. Error bars indicate SD of 6 replicates.

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 $x10^4$ cells ml^{-1}; (B) Experiment using larvae of 8 d 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 $x10^4$ 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 d old larvae in 24 h and 72 h. Cell density of T-Iso control was 7.8 $x10^3$ cells ml^{-1}. Error bars indicate SD of 6 replicates.

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 5 September, 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 $x10^4$ cells ml^{-1}; (B) Experiment using larvae of 11 d 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 $x10^4$. cells ml^{-1}. Error bars indicate SD of 6 replicates.

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 $x10^3$ cells ml^{-1}. Error bars indicate SD of 6 replicates.

Fig. 6. (A) Bay scallop (*Argopecten irradians*) (10 d 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 $x10^3$ and 7.95 $x10^3$ 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.

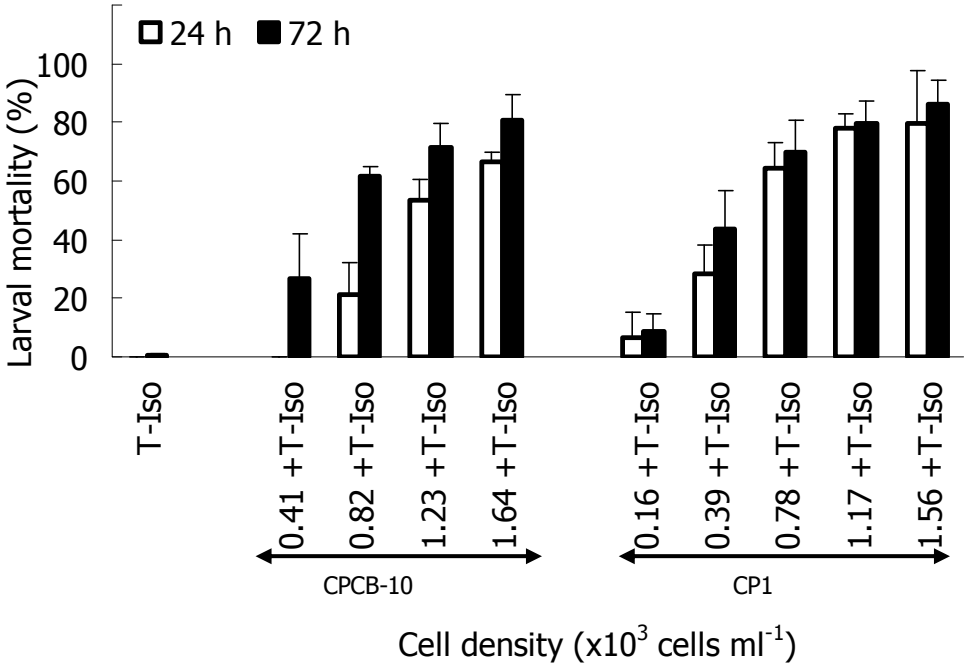

24 h
72 h
Larval mortality (%)
100
80
60
40
20
0
T-Iso
0.41 +T-Iso
0.82 +T-Iso
1.23 +T-Iso
1.64 +T-Iso
0.16 +T-Iso
0.39 +T-Iso
0.78 +T-Iso
1.17 +T-Iso
1.56 +T-Iso
CPCB-10
CP1
Cell density (x10^3 cells ml^{-1})

Fig. 1

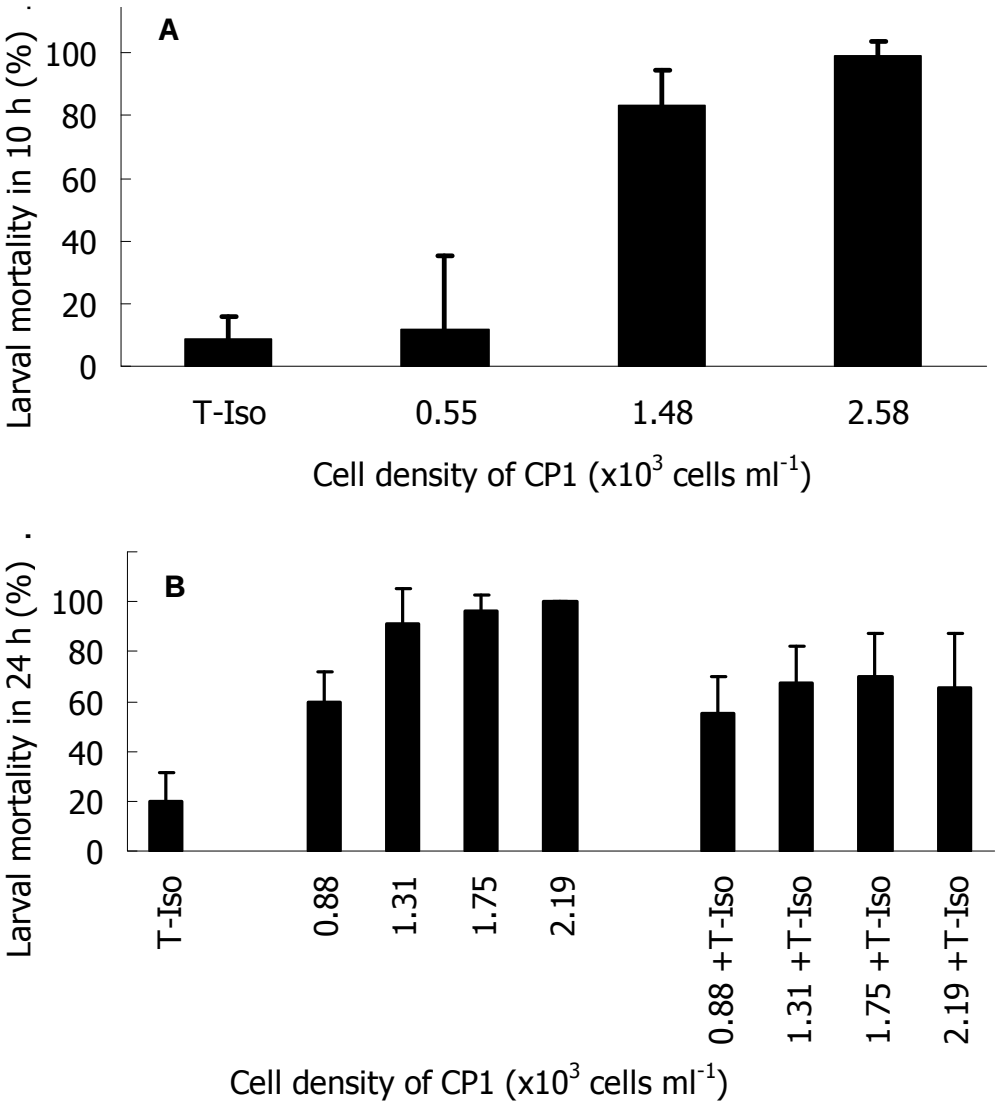

A
Larval mortality in 10 h (%)
100
80
60
40
20
0
T-Iso
0.55
1.48
2.58
Cell density of CP1 (x10^3 cells ml^{-1})
B
Larval mortality in 24 h (%)
100
80
60
40
20
0
T-Iso
0.88
1.31
1.75
2.19
0.88 +T-Iso
1.31 +T-Iso
1.75 +T-Iso
2.19 +T-Iso
Cell density of CP1 (x10^3 cells ml^{-1})

Fig. 2

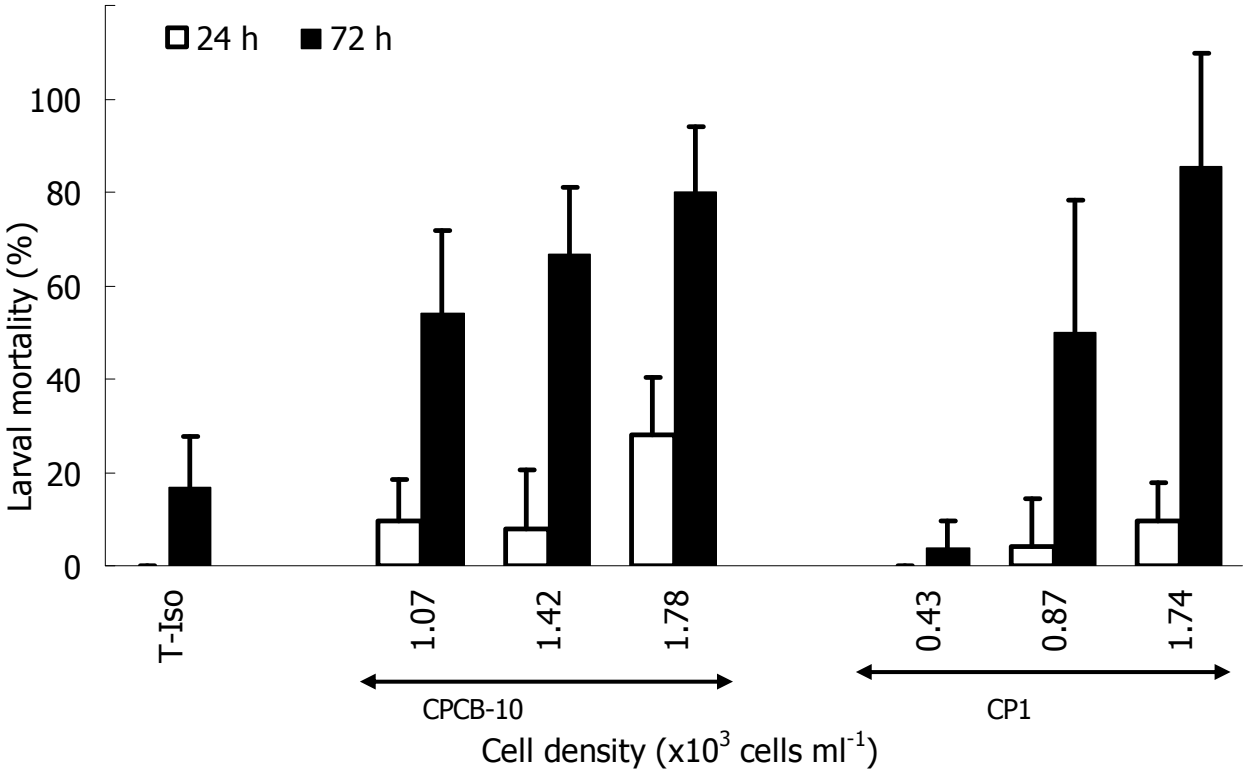
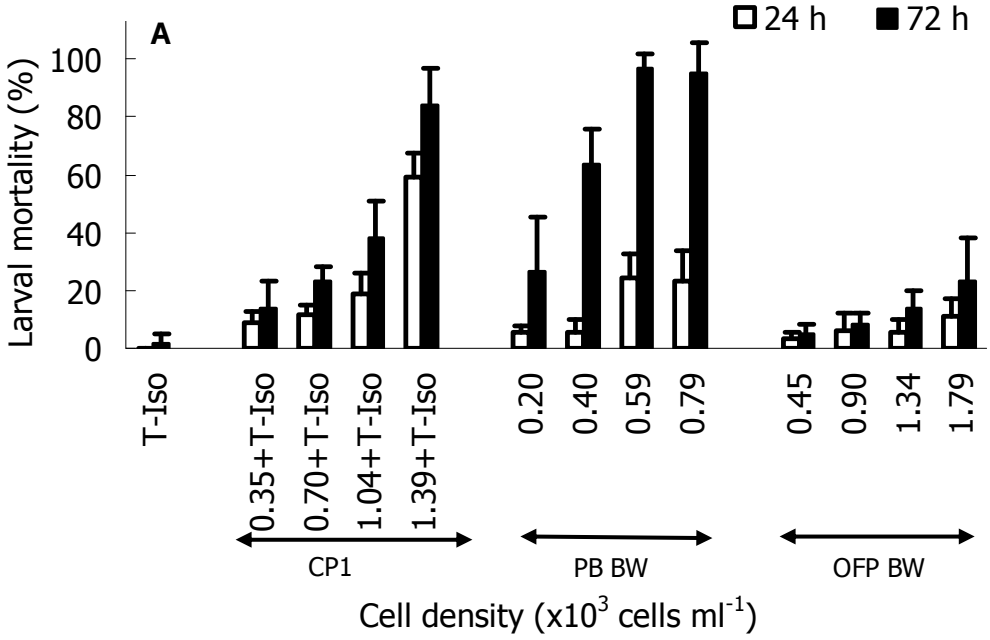

24 h
72 h
Larval mortality (%)
0
20
40
60
80
100
T-Iso
1.07
1.42
1.78
0.43
0.87
1.74
CPCB-10
CP1
Cell density (x10^3 cells ml^{-1})

Fig. 3


A
24 h
72 h
Larval mortality (%)
100
80
60
40
20
0
T-Iso
0.35+T-Iso
0.70+T-Iso
1.04+T-Iso
1.39+T-Iso
0.20
0.40
0.59
0.79
0.45
0.90
1.34
1.79
CP1
PB BW
OFP BW
Cell density (x10^3 cells ml^{-1})

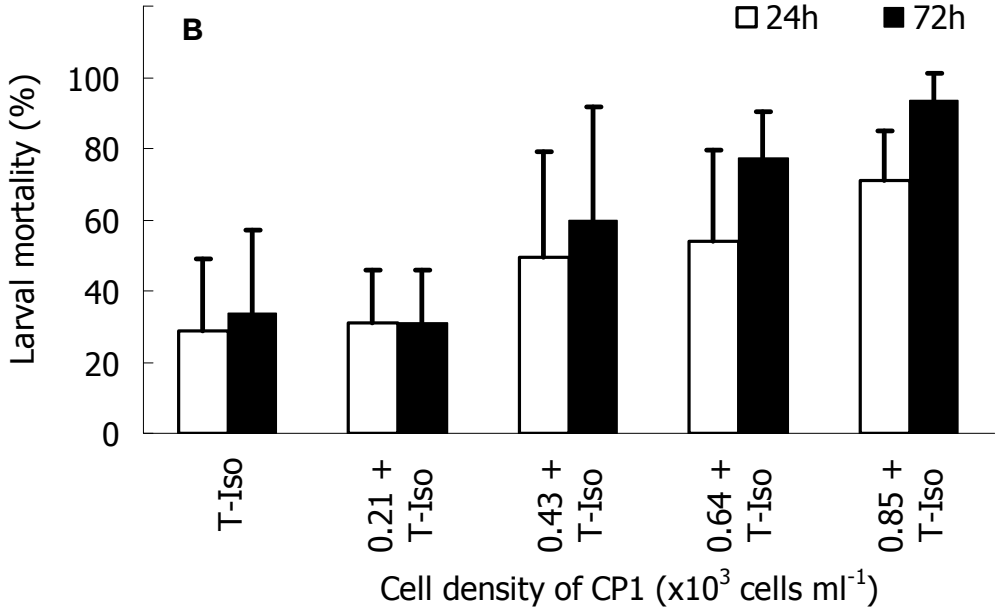

B
24h
72h
Larval mortality (%)
100
80
60
40
20
0
T-Iso
0.21 + T-Iso
0.43 + T-Iso
0.64 + T-Iso
0.85 + T-Iso
Cell density of CP1 (x10^3 cells ml^{-1})

Fig. 4

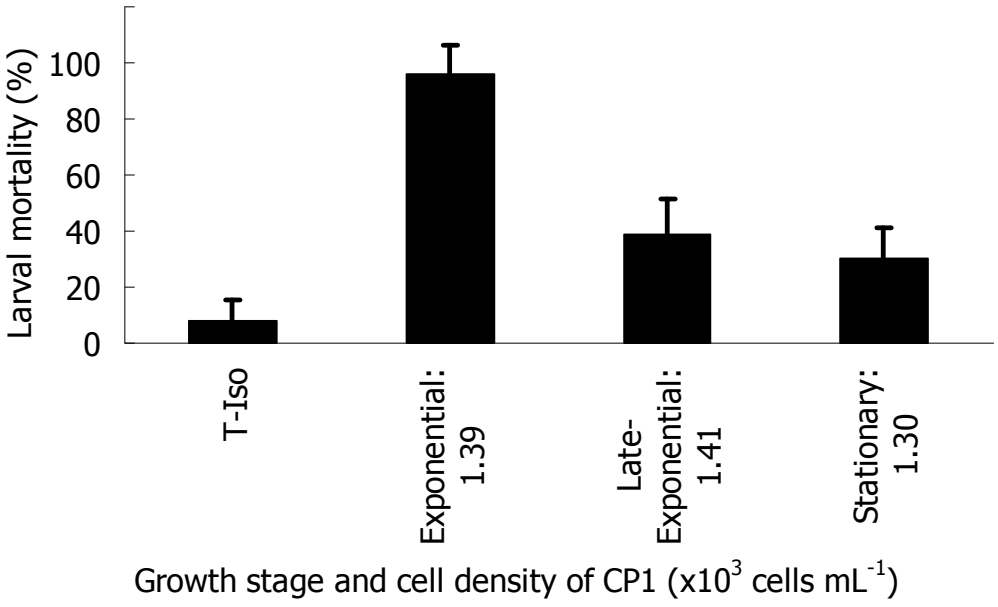
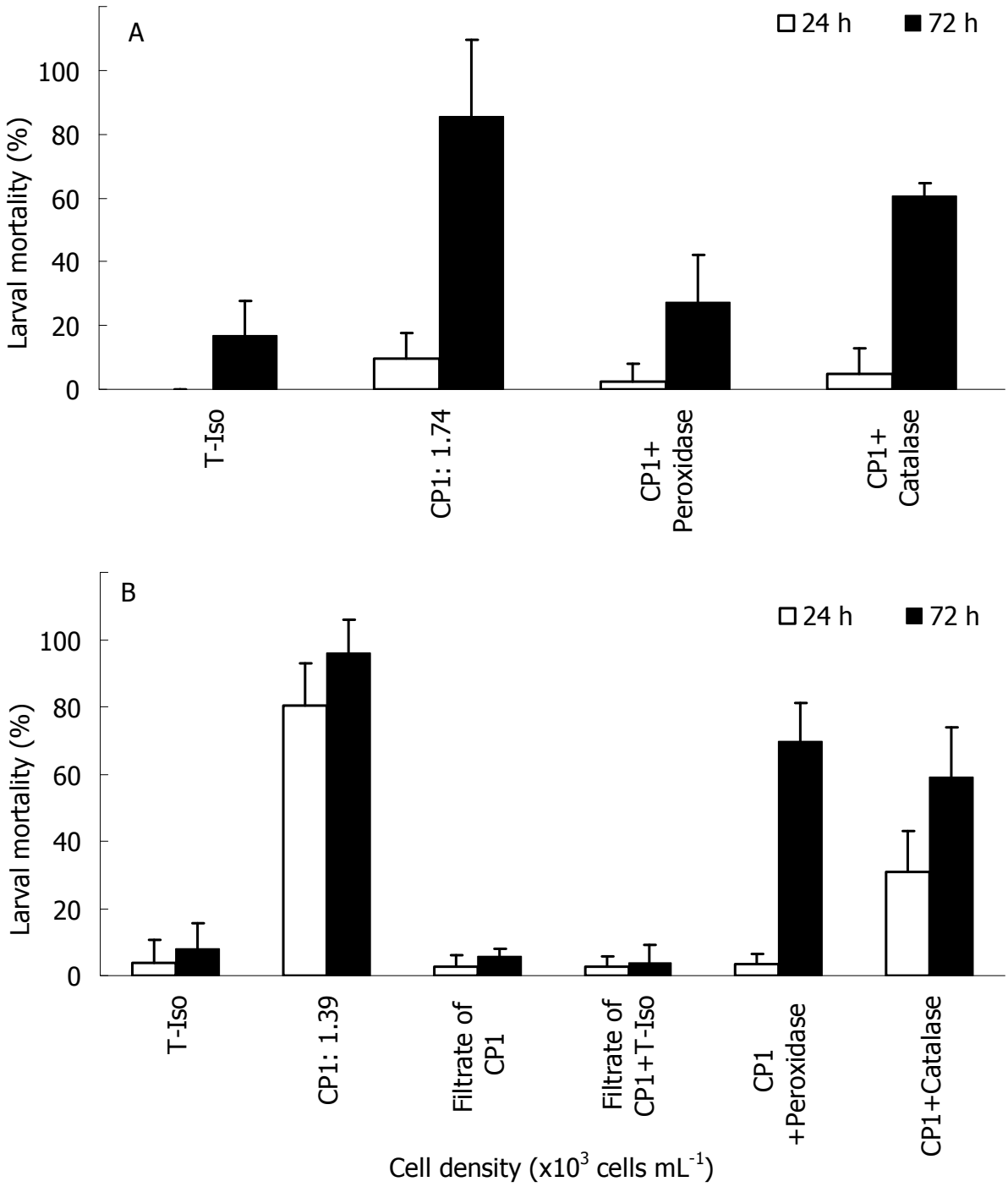

Larval mortality (%)
100
80
60
40
20
0
T-Iso
Exponential: 1.39
Late-Exponential: 1.41
Stationary: 1.30
Growth stage and cell density of CP1 (x10^3 cells mL^{-1})

Fig. 5



991

992

993 Fig. 6

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

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ABSTRACT: The toxic dinoflagellate, *Cochlodinium polykrikoides*, forms harmful algal blooms (HAB) 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 the estuaries of New York, USA, on natural communities and cultured phytoplankton, including *Rhodomonas salina*, *Isochrysis galbana, Aureococcus anophagefferens, Thalassiosira weissflogii, Chattonella marina, Heterocapsa rotundata, Scrippsiella* cf. *trochoidea, Akashiwo sanguinea, Gymnodinium aureolum,* and *Gymnodinium instriatum*. The dramatic allelopathic effects of *C. polykrikoides* cultures and blooms on the target microalgal cells included the loss of motility, changes in cell morphology, and 60 – 100% cell mortality within time periods of minutes to 24 h. The allelopathic effects of *C. polykrikoides* on target microalgae were 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 5μm-mesh nylon membrane indicated that the allelopathic agents were extracellular and that direct cellular contact between the donor and 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 agents responsible for allelopathy 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 reactive oxygen species (ROS)-like chemical(s) were responsible

for the algae-killing effect of this alga. These results are consistent with fish and shellfish larvae bioassays with cultures of *C. polykrikoides*, suggesting that its toxicity to fish, shellfish, and shellfish larvae and its allelopathic effects on algae were caused by the same agents. The rapid and strong allelopathic effects exhibited by *C. polykrikoides* suggest that this species may utilize allelopathy to eliminate competing species and form mono-specific blooms.

KEY WORDS: Allelopathy, *Cochlodinium polykrikoides,* harmful algal blooms (HAB), reactive oxygen species (ROS), toxicity, physiology, ecology, phytoplankton

1051 **1. Introduction**

1052 Harmful algal blooms (HABs) represent a serious threat to the inhabitants of coastal ecosystems
1053 across the globe as they negatively impact marine life, habitats, and economies. Anthropogenic
1054 nutrient loading and physical oceanographic processes are known to play a central role in the
1055 outbreak of HABs (Paerl 1988, Hallegraeff 1993, Pitcher & Calder 2000, McGillicuddy et al.
1056 2005, Anderson et al. 2008, Heisler et al. 2008). Biological interactions among the plankton are
1057 also important for the development and sustenance of HABs. Among these, the ability or
1058 inability of pelagic (Turner & Tester 1997, Gobler et al. 2004, Buskey 2008) and benthic grazers
1059 (Bricelj & Shumway 1998, Cerrato et al. 2004, Hegaret et al. 2007) to consume bloom-forming
1060 microalgae has been most commonly investigated. Another biological factor which may
1061 strongly influence the dynamics of HABs but has been less frequently considered is allelopathy.

1062 Allelopathy has long been known to play a crucial role in the ecology of terrestrial plants
1063 (Muller 1966, Putnam & Duke 1974, Rice 1984, Siqueria et al. 1991). Phytoplankton showing
1064 allelopathic effects on other algae, bacteria, and grazers include both freshwater and marine
1065 species and include diatoms, cyanobacteria, haptophytes, and dinoflagellates in particular
1066 (Legrand et al. 2003, Granéli & Hansen 2006, Tillmann et al. 2008b). More recently, several
1067 phytoplankton species which form harmful algal blooms have been found to have allelopathic
1068 effects on other microalgae (Legrand et al. 2003, Granéli & Hansen 2006, Tillmann et al. 2008b).
1069 Allelopathy has been purported to regulate both phytoplankton community composition and
1070 HAB dynamics (Legrand et al. 2003, Granéli & Hansen 2006, Tillmann et al. 2008b), although
1071 in many cases, the responsible allelochemical agents have not been identified (Legrand et al.
1072 2003, Granéli & Hansen 2006, Tillmann et al. 2008b). Others suggest allelopathy may be
1073 important for HAB maintenance but not bloom initiation (e.g. Solé et al. 2006, Jonsson et al.

2009). The allelopathic effects of HAB are often caused by extracellular chemical agents that are different from the chemicals responsible for poisoning human 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), Philippines (Azanza et al. 2008), Malaysia (Anton et al. 2008), Indonesia (Anton et al. 2008), the Arabian Gulf (Richlen et al. in press), 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 two species of *Cochlodinium* including *C. polykrikoides* have occurred in the US (Silva 1967, Marshall 1995, Tomas & Smayda 2008, Kudela et al. 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, Tang & Gobler 2009a and b, Jiang et al. 2009). 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, Tang & Gobler 2009a and b, Jiang et al. 2009). 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 which are ichthyotoxic are also known to have allelopathic impacts on other algae including *Karenia brevis* (Kubanek et al. 2005, Prince et al.

1097 2008), *Prymnesium parvum* (Fistarol et al. 2003, Granéli & Johansson 2003, Tillmann 2003),
1098 *Alexandrium* spp. (Arzul et al. 1999, Tillmann & John 2002, Fistarol et al. 2004),
1099 *Chrysochromulina polylepis* (Schmidt & Hansen 2001), and *Heterosigma akashiwo* (Yamasaki
1100 et al. 2009). However, to date, the allelopathic effects of isolates of *Cochlodinium* species on
1101 other microalgae have not been documented. Yamasaki et al. (2007) observed growth inhibition
1102 and formation of abnormal cells in *Akashiwo sanguinea* co-cultured with *C. polykrikoides*, but
1103 allelopathy was clearly excluded from their explanations of the possible causes, since a low cell
1104 density-culture of *C. polykrikoides* connected with a culture of *A. sanguinea* through a 3 μm-
1105 mesh membrane did not exhibit the inhibitory effect. Instead, the observed growth inhibition was
1106 interpreted with a mechanism requiring direct cell contact.

1107 Here we present our investigation on the allelopathic impacts of *C. polykrikoides* clones
1108 and blooms from Long Island, NY, USA, to various species of phytoplankton (target species)
1109 which are indigenous to most coastal waters. Cultures of the target species exposed to or co-
1110 cultured with *C. polykrikoides* and connected to *C. polykrikoides* cultures through a 5 μm mesh
1111 exhibited dramatic mortalities and declines in cell densities during short-term exposures (1 h – 4
1112 days). A series of experiments are also presented which suggest a possible mechanism of the
1113 allelopathic effects observed.

1114

1115 **2. Materials and Methods**

1116 *2.1. Cultures and culturing conditions*

1117 Three clones of *Cochlodinium polykrikoides*, CP1, CPPB12, and CPPB17 were examined for
1118 their allelopathic effects on other microalgae. The strains were obtained by pipetting single cells
1119 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 the Peconic Bay (40.9368N, 72.5128W; 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 *Cochlodinium polykrikoides* has been confirmed with large subunit (LSU) rDNA sequencing (Iwataki et al. 2008, Gobler et al. 2008). Cells were cultured in sterile GSe medium with a salinity of 32.5 PSU, 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 12:12 h light: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 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 *Chattonella marina* (ChatM1) from Singapore coastal waters was kindly provided by M. J. Holmes in 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 YZ Tang from Chesapeake Bay (Virginia, USA; AS2, KA2, L6), or Shinnecock Bay (New York, USA; MS3 and MS5). All the cultures were maintained under the same conditions as for *C. polykrikoides* cultures.

1142 Since *A. sanguinea* was highly sensitive to *C. polykrikoides* in preliminary experiments
1143 and easily identified in the co-culture with *C. polykrikoides*, it was used as a model target alga in
1144 multiple experiments. In addition, *Akashiwo sanguinea* AS2, and *Gymnodinium instriatum* L6
1145 were used for bi-algal culture experiments with *C. polykrikoides* because these species are
1146 commonly found co-occurring with *C. polykrikoides* in the estuaries of the region (Gobler et al.
1147 2007). All cultures used for experiments were in early or mid-exponential phase growth with
1148 high levels of ambient nutrients still present. Additions of GSe medium were made to all
1149 experiments, and measured nitrate concentrations were above 100 μM during all time points of
1150 all experiments. Given that these concentrations are two-orders of magnitude above the half
1151 saturation constant of nitrate for growth of *C. polykrikoides* (Gobler et al. unpublished data), all
1152 experiments were performed under nutrient replete conditions. In addition, pH levels were
1153 always ranged between 7.8 and 9.0 in both treatments and control cultures which were equivalent
1154 to or even smaller than the range of changes in pH in the cultures of *C. polykrikoides* and target
1155 species, indicating treatment effects were not associated with changes in pH.
1156
1157 *2.2. Generality of the allelopathic effects of C. polykrikoides*
1158 To examine whether *C. polykrikoides* (strain CP1) had allelopathic effects on the co-occurring
1159 microalgal species and whether this effects were observable from particular species, 10 species
1160 of microalgae from different classes were chosen as target organisms to be co-cultured with *C.*
1161 *polykrikoides* CP1 (final cell density 2,450 ± 390 cells mL^{-1}, n = triplicates cultures) in 6-well
1162 culture plates for 24 h under the same conditions used for maintaining cultures. The initial cell
1163 biomass of all the target species was biovolume-normalized via dilution with GSe medium.
1164 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 combined with CP1 culture or, for controls only, with GSe medium) in treatments and controls of *A. anophagefferens*, *R. salina*, *I. galbana*, *T. weissflogii*, *A. sanguinea*, *G. aureolum*, *G. instriatum*, *H. rotundata*, *S.* cf. *trochoidea*, and *C. marina* were 3.88 ±0.18 x 10^4, 2.9 ± 0.27 x 10^4 , 1.46 ± 0.08 x 10^4, 3.66 ± 0.01 x 10^3, 340 ± 60, 1.18 x ± 0.15^3, 250 ± 35, 1.24 ± 0.58 x 10^3, 2.59 ± 0.20 x 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, Japan) 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).

2.3. Allelopathic effects of multiple strains of C. polykrikoides

To determine whether the allelopathic effects observed from *C. polykrikoides* were a feature specific to strain CP1, experiments were conducted with two additional strains of *C. polykrikoides*: CPPB-12 (targeting *G. instriatum* L6) and CPPB-17 (targeting *A. sanguinea*), while the strain CP1 was used as 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 24h after which cultures were preserved with Lugol's solution (2% final concentration) for enumeration.

2.4. The dose-dependence of allelopathic effects of C. polykrikoides

Experiments examining how the allelopathic effects changed with 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 (*A. 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 and 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*, and 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 370 cells mL^{-1} *C. polykrikoides* with 720 cells mL^{-1} *G. instriatum*.

2.5. Allelopathic effects of different treatments and fractions of C. polykrikoides

To better understand the nature of the allelopathic effects *of C. polykrikoides* on other microalgae, *e*xperiments 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 cool to 21 ˚C); freezing (-80˚C for 30 min and thawed to 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, Illinois, USA; the intactness of cells was checked visually under microscope); addition of reactive oxygen species (ROS)-scavenging enzymes peroxidase (2.5 μg mL^{-1}) and catalase (1.0 U mL^{-1}) into the culture immediately before experiment (Tang & Gobler 2009a). *Akashiwo sanguinea* at a final cell density of 450 cells mL^{-1} was co-cultured with the CP1 cultures after these treatments (final CP1 cell density, or equivalent, 920 cells mL^{-1}) or *C. polykrikoides* CP1 culture without treatment (final cell density 920 cells mL^{-1}; positive control) in triplicate 200mL Erlenmeyer flasks. A *Akashiwo sanguinea* monoculture at the same final cell density as above was used as negative control. All flasks were incubated for 24 h under conditions as used for culture maintenance after which cultures were preserved with Lugol's solution (2% final concentration) and cell densities for both *Akashiwo sanguinea* and CP1 were enumerated using a Sedgewick rafter counting chamber. .

2.6. Allelopathic effects of cell-free medium connected to a live culture of C. 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 5μm-mesh nylon barrier (Sefar Filtration Inc., NY, USA). The experiments were performed in a specifically modified plastic (high density polyethylene, HDPE) chamber with 30, 4 x 4 x 4 cm compartments, in which pairs of two adjacent compartments were separated with 5μm-mesh nylon (Tang & Gobler 2009a). A snap-shut lid for the chamber allowed for UV-sterilization, simple closing, and access to all individual chambers at the start and end of all experiments.

1234 Three experiments were conducted with the target species *A. sanguinea* (exposure time
1235 72h), *G. instriatum* (exposure time 72h), and *C. marina* (exposure time 144h). For each
1236 experiment, each chamber contained 30 mL of either one of the following four cultures in
1237 triplicate: CP1 control (in both sides of the two membrane-partitioned chambers), the target
1238 species control (in both sides of the two membrane-partitioned chambers), a bi-algal mixture of
1239 CP1 and a target species with cell densities the same as controls (positive control), and the
1240 culture of a target species connected to the culture of CP1 through the 5μm membrane. The
1241 initial concentrations of *A. sanguinea*, *G. instriatum*, and *C. marina* in the bi-algal mixtures were
1242 640, 600, and 500 cells mL^{-1}, respectively, while the corresponding initial cell concentrations of
1243 *C. polykrikoides* CP1 were 1,740, 1,650, and 900 cells mL^{-1}, respectively. After the cultures
1244 were added, the chamber was closed and incubated under standard conditions listed above. After
1245 incubation, the cultures were gently mixed pipetted simultaneously from both sides by two
1246 people, ensuring that the rate of culture removal was identical for each side. Samples were fixed
1247 with Lugol's solution (final concentration 2%) and microscopically enumerated using a
1248 Sedgewick rafter counting chamber.
1249
1250 *2.7. Natural algal population experiments*
1251 Two experiments were conducted with *C. polykrikoides* bloom water or non-bloom water to
1252 observe the allelopathic effects of *C. polykrikoides* cultures and blooms within a natural
1253 phytoplankton community. For the experiment with bloom water, a surface bloom (with a *C.*
1254 *polykrikoides* cell density of 2,700 cells mL^{-1}) was sampled from Peconic Bay, Long Island, NY,
1255 USA on September 2, 2009. In a 200-mL sterile flask, 10 mL of *A. sanguinea* culture (final
1256 concentration 10 cells mL^{-1}) was mixed with 40 mL of bloom water as treatment. Two more

1257 aliquots of *A. sanguinea* culture were mixed with either GSe medium or 0.2 µm-filtered
1258 (Steriflip; Millipore) bloom water as controls. After gentle through-mixing, triplicate 10 mL
1259 aliquots were transferred into a 12-mL wells of 6-well plates (Corning, Corning, NY, USA) and
1260 incubated under the standard condition for 24 h. Triplicate 10 mL aliquots were also preserved
1261 with Lugol's solution (final 2%) at the start of experiments and after 24 h incubation for
1262 enumeration of cell concentrations. The salinities of bloom water and *A. sanguinea* culture were
1263 29 and 32, respectively.

1264 For the experiment with non-bloom water, surface water was sampled on August 22,
1265 2009 from Old Fort Pond (OFP), a tidal tributary of Shinnecock Bay, Long Island. In triplicate
1266 200-mL sterile flasks, a 50 mL aliquot of a *C. polykrikoides* CP1 culture (final cell density 2,100
1267 cells mL^{-1}) was combined with 50 mL seawater from OFP as the treatment. *C. polykrikoides*
1268 culture and the natural community were both mixed with 50 mL of 0.2 um-mesh filtrated OFP
1269 seawater as two distinct controls. After gentle mixing, triplicate 10 mL aliquots of each treatment
1270 were transferred into 12-mL wells of 6-well plates which were then incubated under the standard
1271 condition for 24 h. Initial and 24 h samples were preserved with Lugol's solution for
1272 enumeration of the six most abundant microphytoplankton: the diatoms *Skeletonema* cf.
1273 *costatum*, *Chaetoceros* sp., *Thalasiossira* sp., the flagellate *Euglena* sp, and the dinoflagellates
1274 *Gyrodinium* sp. and *Scrippsiella* sp. The salinity of seawater and culture was 30 and 32,
1275 respectively, measured before experiment.
1276
1277 *2.8. Statistics*
1278 One-way ANOVAs and t-test were performed to assess the effects of different treatments of *C.*
1279 *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 with SigmaStat 3.1. In all cases, significance levels were set at $p < 0.05$.

3. Results

3.1. Cochlodinium polykrikoides exhibited allelopathic effects on all competing species tested

Cochlodinium polykrikoides CP1 exhibited strong allelopathic effects on all target species examined (Figs. 1 and 2). The specific effects include loss of flagella and thus motility (for flagellates *A. sanguinea*, *G. aureolum*, *G. instriatum*, *H. rotundata*, *S.* cf. *trochoidea*, and *C. marina*), change of cellular morphology into a round shape (*R. salina*, *A. sanguinea*, *G. aureolum*, *G. instriatum*, *H. rotundata*, *S.* cf. *trochoidea*, and *C. marina*), and eventual cell disintegration and lysis (Fig. 1). These effects were observed in 15 minutes in some *A. sanguinea* cells, but took hours in other species (Fig. 1). After 24 h, *C. polykrikoides* CP1 at densities of 2,450 cells mL^{-1} caused mortalities in *A. anophagefferens*, *R. salina*, *I. galbana*, *T. weissflogii*, *A. sanguinea*, *G. aureolum*, *G. instriatum*, *H. rotundata*, *S.* cf. *trochoidea*, and *C. marina* of 90%, 61%, 79%, 57%, 93%, 59%, 91%, 74%, 96%, 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 – 79%; Fig 2).

3.2. Allelopathic effects of C. polykrikoides is not strain-specific

In experiments using different strains of *C. polykrikoides* (CP1, CPPB-12, and CPPB17) and the target species *A. sanguinea* and *G. 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. polykrikoides* are not strain-specific among strains isolated from different years and locations in NY.

3.3. Allelopathic effects of C. polykrikoides depend on the absolute and relative cell densities of donor and target species and exposure time

In bi-algal cultures of *C. polykrikoides* (110 - 1000 cells mL^{-1}) and *A. 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 $\leq$ 290 cells mL^{-1} (Fig. 4B). Hence, these allelopathic effects depended on the absolute cell density of both *C. polykrikoides* and *A. sanguinea* (Fig. 4A and 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*

1326 exceeded 1.0 (ANOVA, $p<0.001$; Fig. 4C). A second experiment conducted with *C.*
1327 *polykrikoides* and the target species *G. instriatum* demonstrated a similar dependence of the
1328 allelopathic effects on the initial ratio of *C. polykrikoides* : *G. instriatum* (Fig. 4D). At *C.*
1329 *polykrikoides* : *A. sanguinea* ratios exceeding 2, there was >50% mortality in *G. instriatum*,
1330 whereas at ratios of 1.2 and 0.5, 33% and 0% of *G. instriatum* cells were lost, respectively,
1331 compared to the control (Fig 4C).

1332 The allelopathic effects of *C. polykrikoides* also depended on exposure time. With initial
1333 concentrations of *C. polykrikoides* and *A. sanguinea* at 800 and 500 cells mL^{-1}, respectively, cell
1334 mortality in *A. sanguinea*, which was calculated in comparison with control, significantly
1335 increased from 60 to 95% with an increase in exposure time from 1 to 7 days (ANOVA,
1336 $p<0.001$; Fig. 5). There was no significant change in *C. polykrikoides* cell densities over this
1337 time frame.

1338

1339 *3.4.* ***Allelopathic effects of C. polykrikoides do not dependent on direct cell contact***

1340 Cultures of *C. polykrikoides* CP1 separated from target algae with 5μm-mesh nylon membranes
1341 exhibited significant allelopathic effects on *A. sanguinea*, *G. instriatum*, and *C. marina* (Fig. 6;
1342 ANOVA, $p<0.001$). During a 72 h exposure of 640 cell mL^{-1} of *A. sanguinea* to 1,740 cells mL^{-1}
1343 of CP1, cell densities of *A. sanguinea* declined to 130 cells mL^{-1}, while an indirect exposure
1344 (5μm-mesh nylon; Fig.6) led to a decrease to 540 cells mL^{-1}, which is significantly higher than
1345 with the direct exposure (ANOVA Holm-Sidak post hoc pairwise comparison, $p <0.001$), but
1346 significantly lower than the negative control (710 cells mL^{-1}; ANOVA Holm-Sidak post hoc
1347 pairwise comparison, $p < 0.001$). Direct exposure of *C. marina* (475 cells mL^{-1}) to CP1 (1,780
1348 cells mL^{-1}) led to a decrease of *C. marina* to 206 cells mL^{-1}, while the indirect exposure led to

1349 growth of *C. marina* to 2,400 cells mL^{-1}, which was significantly less than that of the negative
1350 control of *C. marina*, 3,360 cells mL^{-1} (ANOVA Holm-Sidak post hoc pairwise comparison, $p <$
1351 0.001; Fig. 6). Similarly, direct exposure of *G. instriatum* (600 cells mL^{-1}) to CP1 (1,420 cells
1352 mL^{-1}) for 72h led to a slight increase of *G. instriatum* to 780 cells mL^{-1}, while the indirect
1353 exposure led to growth of *G. instriatum* to 1,280 cells mL^{-1}, which was significantly higher than
1354 that of the direct exposure but significantly less than that of the negative control of *G. instriatum*,
1355 1,570 cells mL^{-1} (ANOVA Holm-Sidak post hoc pairwise comparison, $p <0.001$; Fig. 6). All the
1356 above results indicated that a direct contact between *C. polykrikoides* cells and the cells of target
1357 species is not necessary for *C. polykrikoides* to have an allelopathic effect and that at least some
1358 of the allelochemicals responsible are released from cells of *C. polykrikoides*, although direct
1359 exposure of target species to *C. polykrikoides* caused a much stronger effect on target species.
1360
1361 *3.5. Effects of different treatments of C. polykrikoides culture on its allelopathy*
1362 *Cochlodinium polykrikoides* CP1 cultures at 920 cells mL^{-1} completely lost their toxicity or
1363 allelopathic effects on *A. sanguinea* (with an initial cell density of 450 cells mL^{-1}) after
1364 treatments of boiling, freezing-and-thawing, sonication, and filtration (cell-free culture medium).
1365 Final cell densities of *A. sanguinea* in each of these treatments were not different from the
1366 negative control (without *C. polykrikoides*) (Fig. 7; ANOVA post hoc comparison, $p>0.05$). In
1367 contrast, 98% *A. sanguinea* cells in the positive control culture lysed after 24 h exposure to
1368 untreated *C. polykrikoides* cells (Fig. 7). The addition of 2.5 μg mL^{-1} of peroxidase (final)
1369 mitigated this impact, as there was no significant difference in the final cell density of *A.*
1370 *sanguinea* between this treatment and negative control cultures (Fig. 7; ANOVA post hoc
1371 comparison, $p>0.05$). The addition of catalase (1.0 U mL^{-1}) yielded a final cell density of *A.*

sanguinea which was significantly higher than the positive control (Fig. 7; ANOVA post hoc comparison, $p<0.001$) but significantly lower than the negative control (Fig. 7; ANOVA post hoc comparison, $p<0.001$), 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, $p>0.05$).

3.6. Allelopathic effects of C. polykrikoides cultures and blooms on natural phytoplankton communities

Akashiwo sanguinea cells added to a natural sample collected during a bloom of *C. polykrikoides* from the Peconic Estuary experienced significant mortality (76%), while in contrast, *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 (2,100 cells mL^{-1}), five of the six 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).

4. Discussion

4.1. Possible mechanisms and chemical nature of the allelopathic effects exhibited in C. polykrikoides

This study demonstrates that clonal cultures and blooms of *C. polykrikoides* from the coast of Northeastern US 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 in minutes, and included loss of motility, change of cell morphology, cell lysis, and cell death 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 *A. 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*, *C. marina*, or *G. instriatum*) through a 5μm-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 a 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 allelopathic effect in the cell-free medium which 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 the allelopathic effects by addition of the ROS-scavenging enzymes, peroxidase and catalase, into *C. polykrikoides* culture implies that reactive oxygen species (ROS)-like chemicals (e.g. O_2^-, OH•, HO_2•, ROO^-) are responsible for the algae-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 which have been proven to have a common mechanism for their toxicity to animals and

1441 allelopathic effects on phytoplankton (*Karlodinium veneficum* and *P. parvum*; Granéli & Hansen
1442 2006, Tillmann et al. 2008b). The consistency between allelopathic experiments, fish bioassays,
1443 bivalve bioassays, and shellfish larvae bioassays and the non-specificity of allelopathic effects on
1444 all microalgae tested suggests that labile, (ROS)-like chemicals are the allelopathic agents in *C.*
1445 *polykrikoides*, although further studies are needed to confirm this hypothesis.

1446 It has been previously suggested that elevated pH values in mixed cultures of donor and
1447 target species might be responsible for the observed allelopathic-like effects in some HAB
1448 species (Schmidt & Hansen 2001, Lundholm et al. 2005). However, the lack of significant
1449 differences among pH levels in cultures of *C. polykrikoides*, target species (i.e. no difference),
1450 and mixed cultures in the present study demonstrated that pH shock or pH increase during co-
1451 culturing was not responsible for the allelopathic effects of *C. polykrikoides*. Moreover, the
1452 presences of nitrate at levels more than an order of magnitude above the saturation constant of
1453 growth *C. polykrikoides* (Gobler et al., unpublished data) suggests nutrient limitation would not
1454 account for the dramatic and prompt results presented herein (e.g. >90% cell lysis in target
1455 species in $\leq$ 24 h; Fig 2).

1456

1457 *4.2 Ecological implications of the allelopathic effects exhibited in C. polykrikoides*

1458 The production of allelopathic chemicals is a strategy used by harmful algae to outcompete
1459 other, co-existing phytoplankton (Smayda 1997, Granéli & Hansen 2006, Tillmann et al. 2008b)
1460 and our results demonstrate this strategy likely plays a role in the occurrence of *C. polykrikoides*
1461 blooms. In cultures, *C. polykrikoides* is a slow-growing alga (μ = 0.4 d^{-1}; personal observation)
1462 compared to other diatoms and dinoflagellates including *G. instriatum* and *A. sanguinea*
1463 (Smayda 1997) which bloom in NY estuaries prior (1-4 wks.) to *C. polykrikoides* blooms or

1464 coexist with *C. polykrikoides* at high densities during bloom initiation (Gobler et al. 2007, Tang
1465 & Gobler pers. observations). Given this disadvantage in growth, *C. polykrikoides* must rely on
1466 other mechanisms, such as allelopathy to form mono-specific blooms. Since *C. polykrikoides*
1467 causes dramatic mortality in planktonic grazers including bivalve larvae (Tang & Gobler 2009b),
1468 planktivorous fish (Tang & Gobler 2009a), and zooplankton (Jiang et al. 2009), grazer deterrence
1469 is another process which likely contributes to bloom formation. Allelopathic effects on
1470 competitors together with grazing deterrence could promote *C. polykrikoides* blooms through
1471 positive feedback (Sunda et al. 2006) whereby higher *C. polykrikoides* cell densities yield fewer
1472 competitors and predators which in turn facilitates higher cell densities. Since the allelopathic
1473 effects of *C. polykrikoides* on co-occurring algae depend on absolute and relative cell
1474 abundances, allelopathy is more likely to contribute toward bloom maintenance when cell
1475 densities are high, than bloom initiation when cell densities are low. The ability of *C.*
1476 *polykrikoides* to form localized areas of high cell densities by diurnal migration (Gobler et al.
1477 2008, Kudela et al. 2008), however, could facilitate a scenario whereby *C. polykrikoides* could
1478 have allelopathic effects on competing algae, even during bloom initiation. In light of the
1479 conclusions of Putnam & Tang (1986), Smayda (1997), and Tillmann et al. (2008b), further
1480 confirmative studies identifying *C. polykrikoides* allelochemicals or/and toxins and *in situ*
1481 measurements of allelopathic agents and their bioactivity are needed to clarify the role of
1482 allelopathy in *C. polykrikoides* blooms, particularly during bloom initiation.
1483

1484 **Acknowledgements**

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Figure legends

Fig. 1. Morphology of selected target microalgae during exposure to *C. polykrikoides* CP1. (A-D) *A. sanguinea*, (E-H) *C. marina*, and (I-L) *G. instriatum*. (A, E, I) Normal cells, (B, F, J) cells lost flagella and motility and changed shape in general, (C, G, K) substantially deformed cells, (D, H, L) lysed cells. Exposure time is as shown.

Fig. 2. Percent cell mortality of 10 target species co-cultured with *C. polykrikoides* CP1 (2,450±390 cells mL^{-1}) for 24 h, expressed as the percent mortalities of the target cells relative to their respective controls. The error bars indicate 1× standard deviation of n = 3.

Fig. 3. (A) Percent cell mortality of *A. sanguinea* during 24 h co-culture with *C. 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 *G. 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}. The asterisks indicate significant difference between treatments and control and the error bars indicate 1× standard deviation of n =3.

Fig. 4. Percent cell mortality of the target species (*A. sanguinea*, *G. instriatum*) compared to the respective controls as a function of the initial cell densities of *C. polykrikoides* (strain CP1), *A. sanguinea,* and the ratio of *C. polykrikoides* to the target species in bi-algal cultures. (A) *A. sanguinea* (fixed at 1000 cells mL^{-1}) and *C. polykrikoides* varied (110 - 1000 cells mL^{-1}) over 24 h; (B) *C. polykrikoides* (fixed at 1000 cells mL^{-1}) and *A. sanguinea* varied (130 - 1000 cells mL^{-1}) over 24 h; (C) *C. polykrikoides* and *A. sanguinea*, with the initial cell densities varied (1000 cells mL^{-1} *C .polykrikoides* vs. 130 - 1000 cells mL^{-1} *A. sanguinea* and 1000 cells mL^{-1} *A. sanguinea* vs. 110 - 570 cells mL^{-1} *C. polykrikoides*) and 24 h culturing; (D) *C. polykrikoides* and *G. instriatum*, with the initial cell densities 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 indicate 1× standard deviation of n = 3. Small letters indicate significant differences among treatments.

Fig. 5. Percent cell mortality of the target species *A. sanguinea* relative to the respective controls (%) as a function of the exposure (co-culturing) time of *A. sanguinea* to *C. 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 averages of treatments (n =3) and their corresponding controls (n =3). Error bars indicate 1× standard deviation of n = 3 and the different small letters indicate significant difference among treatments.

Fig. 6. Cell densities of *Akashiwo sanguinea*, *Chattonella marina*, and *Gymnodinium instriatum* following co-culturing with direct and indirect exposure to *C. polykrikoides* CP1 via a 5μm-nylon mesh for 72 h. Concentrations of each target species (*A. sanguinea*, *C. marina*, and *G. instriatum*) were constant in the treatment (membrane partitioned), direct exposure (mixed culture), and negative control (target species only). Concentrations of *C. polykrikoides* in the treatment chamber and in the mixed culture were the same for each test species. Error bars

indicate 1× standard deviation of n = 3 and symbol * indicates significant difference from the control and between the direct and indirect exposures.

Fig. 7. Cell densities of *A. sanguinea* and *C. polykrikoides* CP1 after heat (100˚C for 15 min), freezing (in -80˚C), sonication, filtration (through 5μm-mesh membrane), and additions of peroxidase (final 2.5 μg mL^{-1}) and catalase (final 1.0 U mL^{-1}). Error bars indicate 1× standard deviation of n = 3. Lowercase letters indicate significant differences.

Fig. 8. Percent change in cell densities of *A. sanguinea* co-cultured with *C. polykrikoides* bloom water from the Peconic Estuary for 24 h. Cultures of *A. sanguinea* cultured in either GSe medium or 0.2um-mesh filtrated bloom water were used as controls. The asterisks indicate significant difference between treatment and controls or between two controls in percent change in cell densities in 24 h incubation, and error bars indicate 1× standard deviation of n = 3.

Fig. 9. Cell densities of dominant species present in water from Old Fort Pond co-cultured with *C. polykrikoides* CP1 and with 0.2um-mesh filtered *C. polykrikoides* culture medium after 24 h incubation. The asterisks indicate significant difference between treatment and control in cell density for each species, and error bars indicate 1× standard deviation of n = 3.

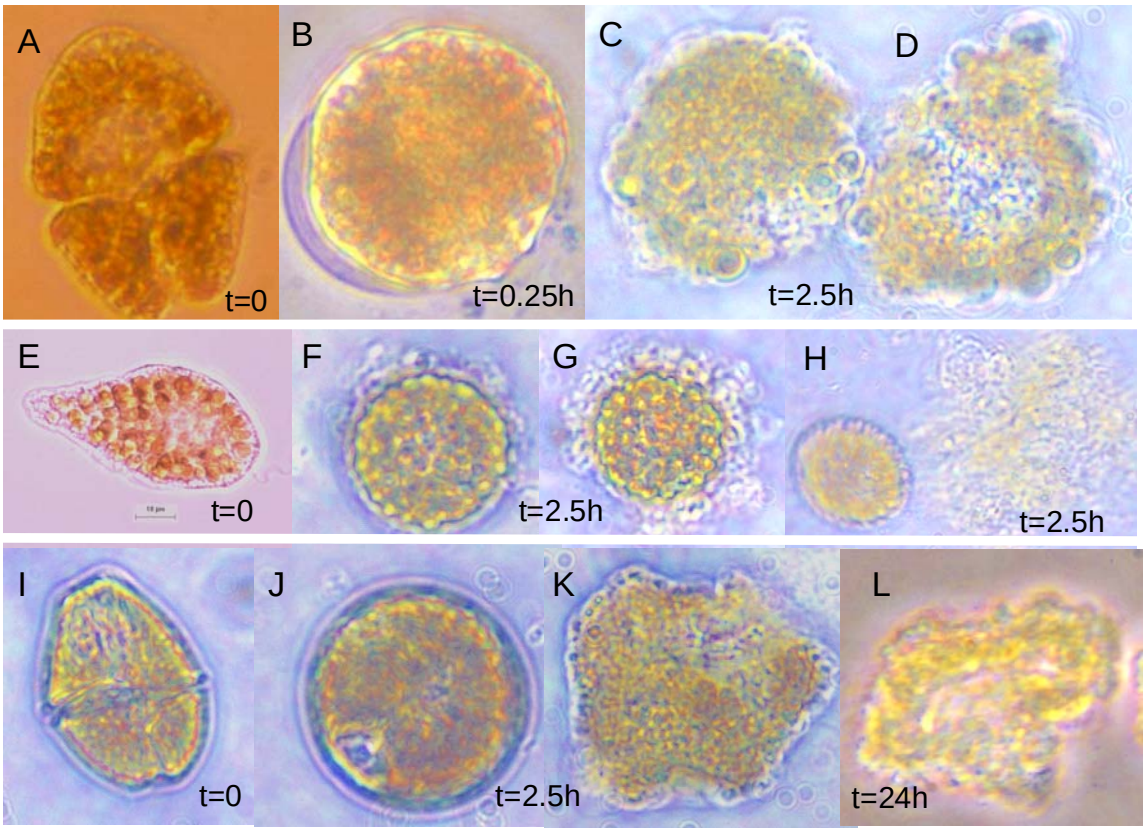

Fig. 1

1828

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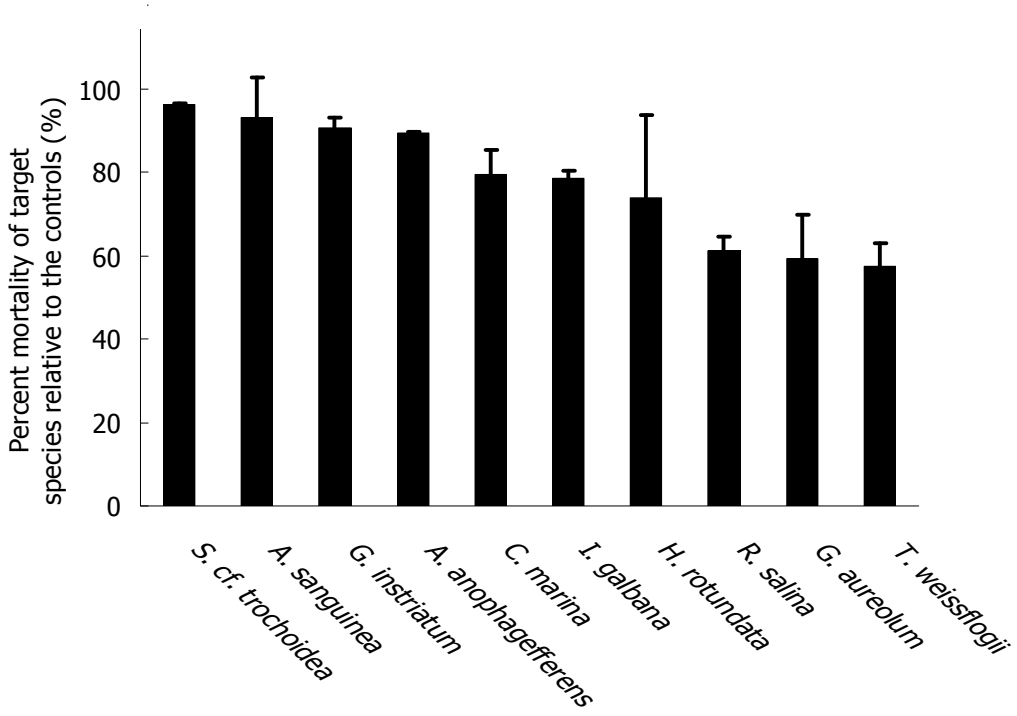

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1840 Fig. 2.

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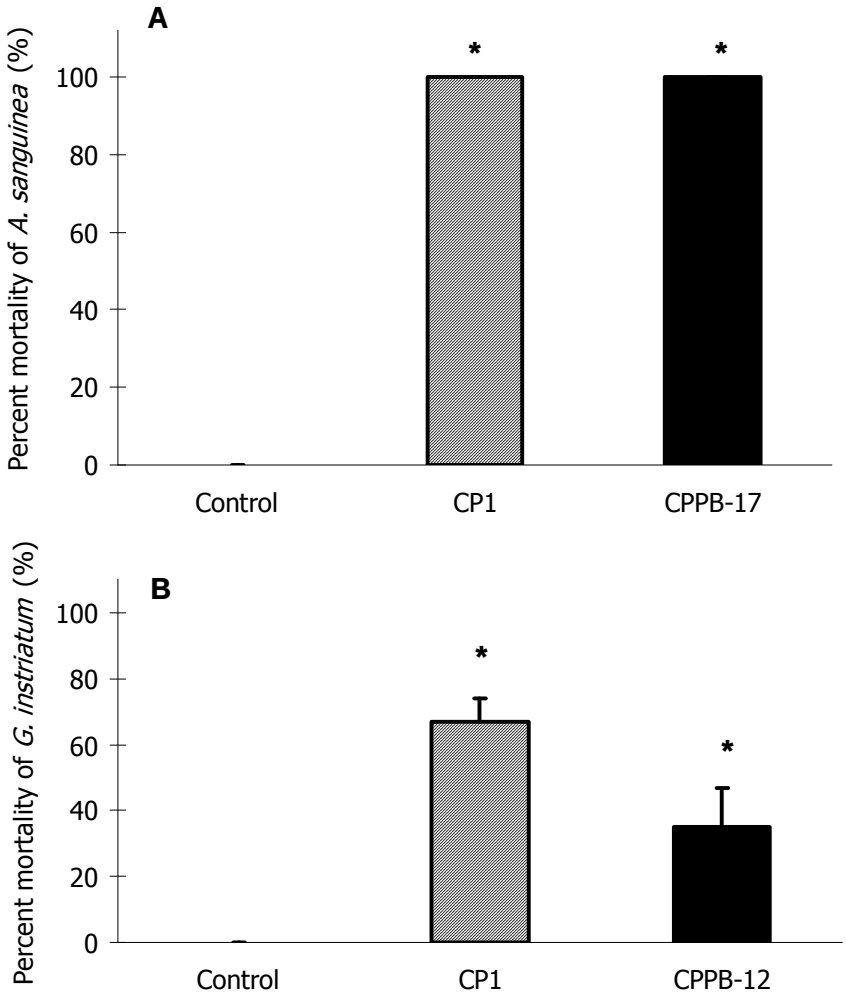

A
Percent mortality of A. sanguinea (%)
100
80
60
40
20
0
*
*
Control
CP1
CPPB-17
B
Percent mortality of G. instriatum (%)
100
80
60
40
20
0
*
*
Control
CP1
CPPB-12

Fig. 3.

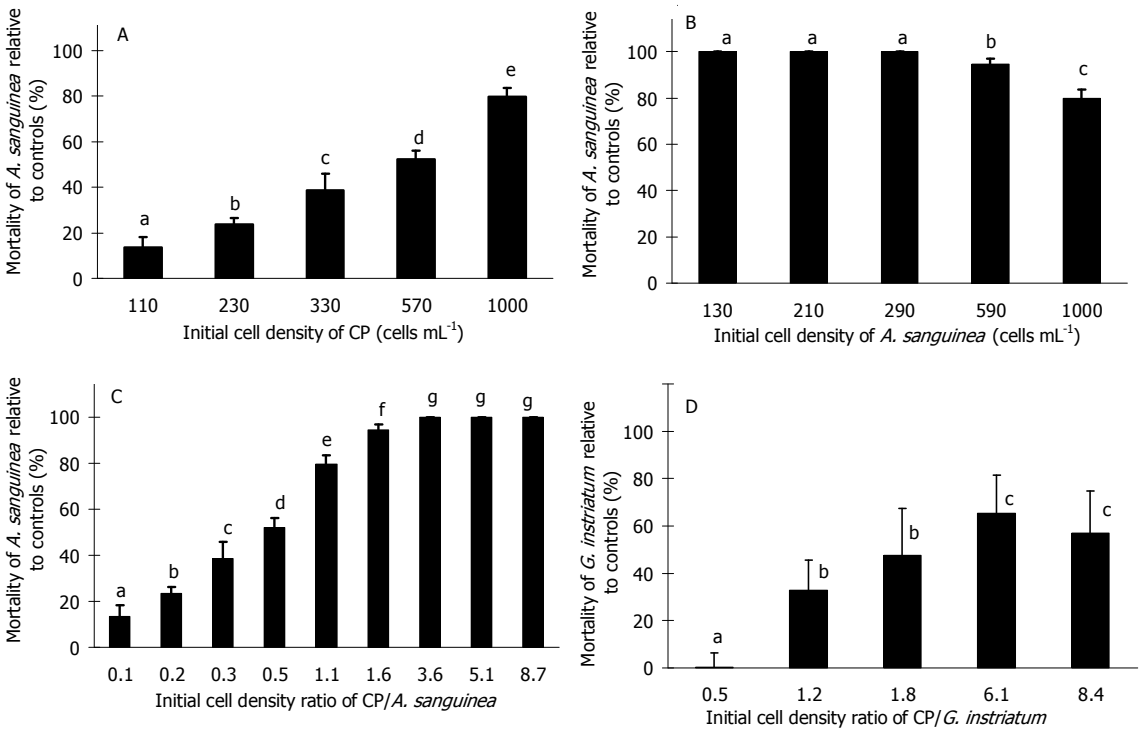

A
Mortality of A. sanguinea relative to controls (%)
100
80
60
40
20
0
a
b
c
d
e
110
230
330
570
1000
Initial cell density of CP (cells mL-1)
B
Mortality of A. sanguinea relative to controls (%)
a
a
a
b
c
130
210
290
590
1000
Initial cell density of A. sanguinea (cells mL-1)
C
Mortality of A. sanguinea relative to controls (%)
a
b
c
d
e
f
g
g
g
0.1
0.2
0.3
0.5
1.1
1.6
3.6
5.1
8.7
Initial cell density ratio of CP/A. sanguinea
D
Mortality of G. instriatum relative to controls (%)
a
b
b
c
c
0.5
1.2
1.8
6.1
8.4
Initial cell density ratio of CP/G. instriatum

Fig. 4

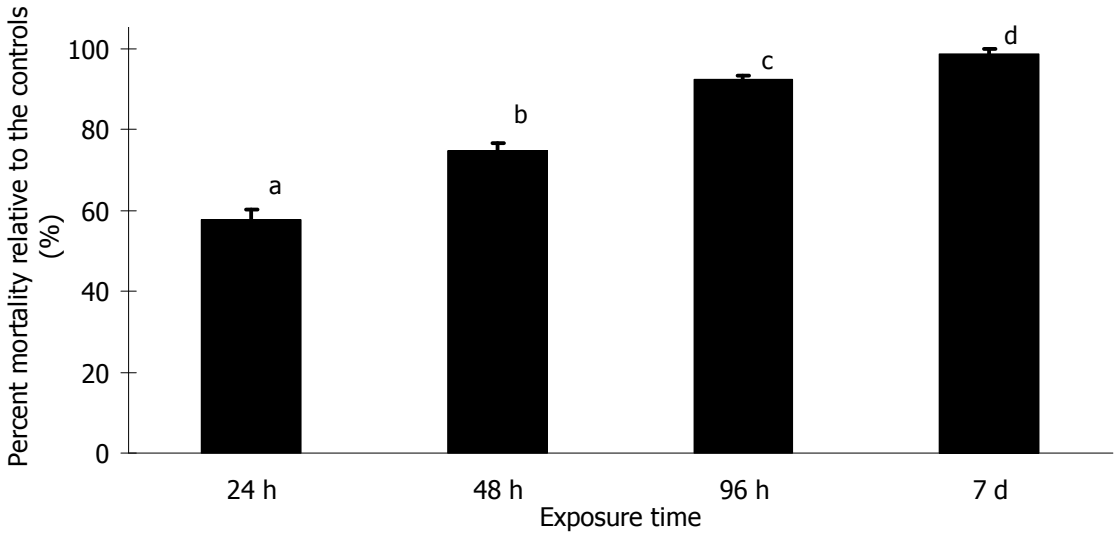

Percent mortality relative to the controls (%)
100
80
60
40
20
0
a
b
c
d
24 h
48 h
96 h
7 d
Exposure time

Fig. 5

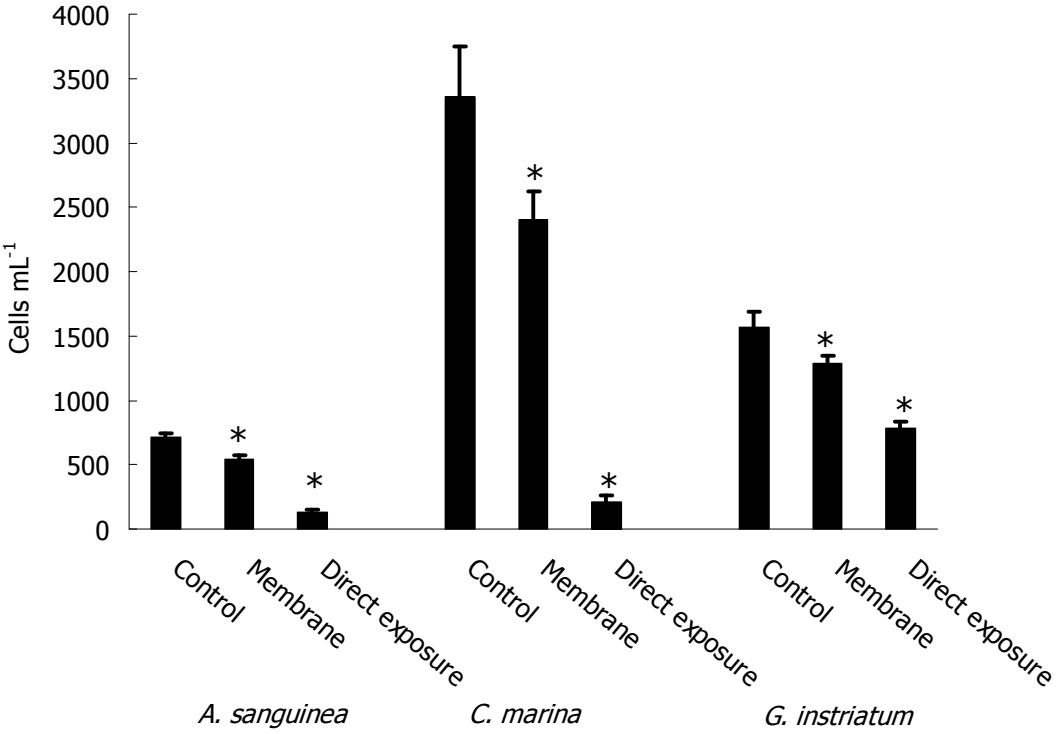
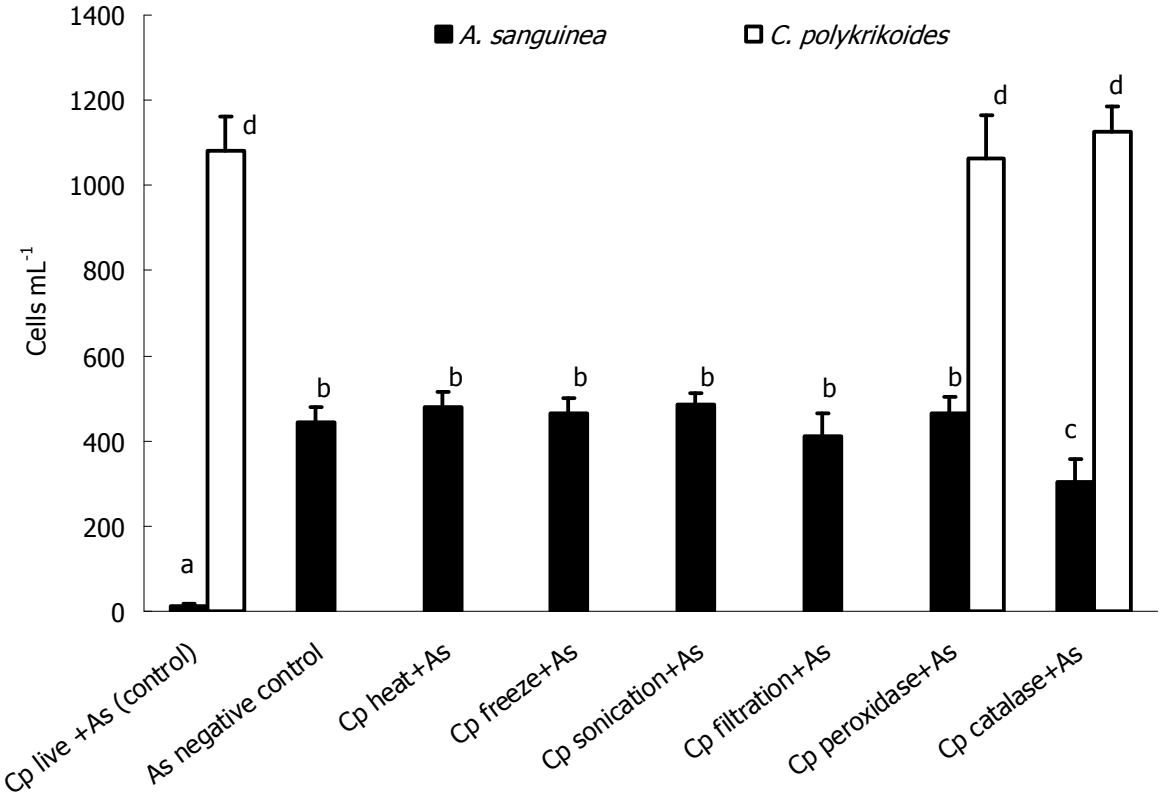

4000
3500
3000
2500
2000
1500
1000
500
0
Cells mL-1
*
Control
Membrane
Direct exposure
A. sanguinea
C. marina
G. instriatum

Fig. 6


1400
1200
1000
800
600
400
200
0
Cells mL-1
A. sanguinea
C. polykrikoides
a
d
b
b
b
b
b
b
d
c
d
Cp live +As (control)
As negative control
Cp heat+As
Cp freeze+As
Cp sonication+As
Cp filtration+As
Cp peroxidase+As
Cp catalase+As

1937 Fig. 7

1938

1939

1940

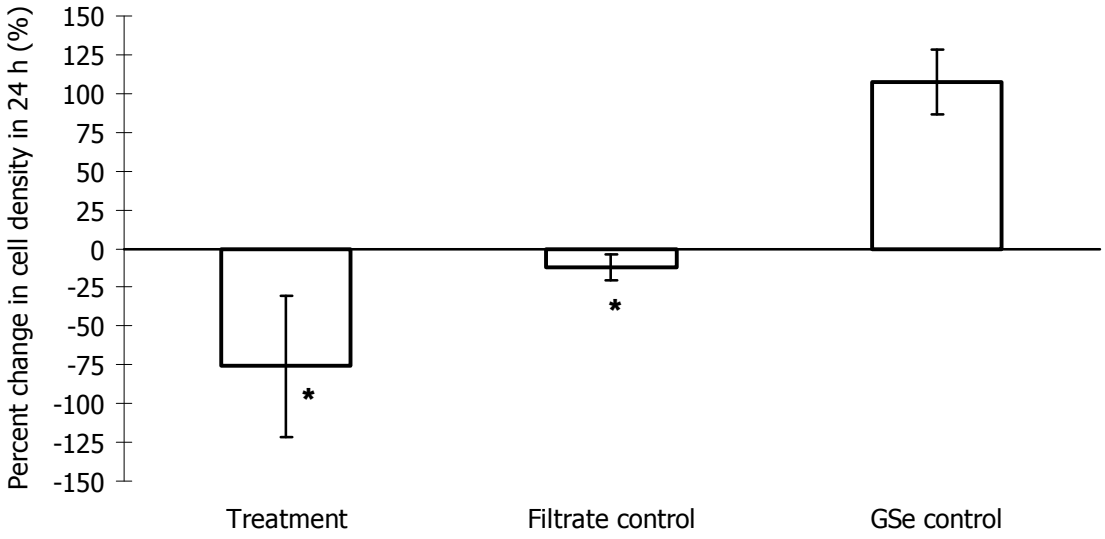
1941

1942

1943

1944

1945



1946

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1952 Fig. 8

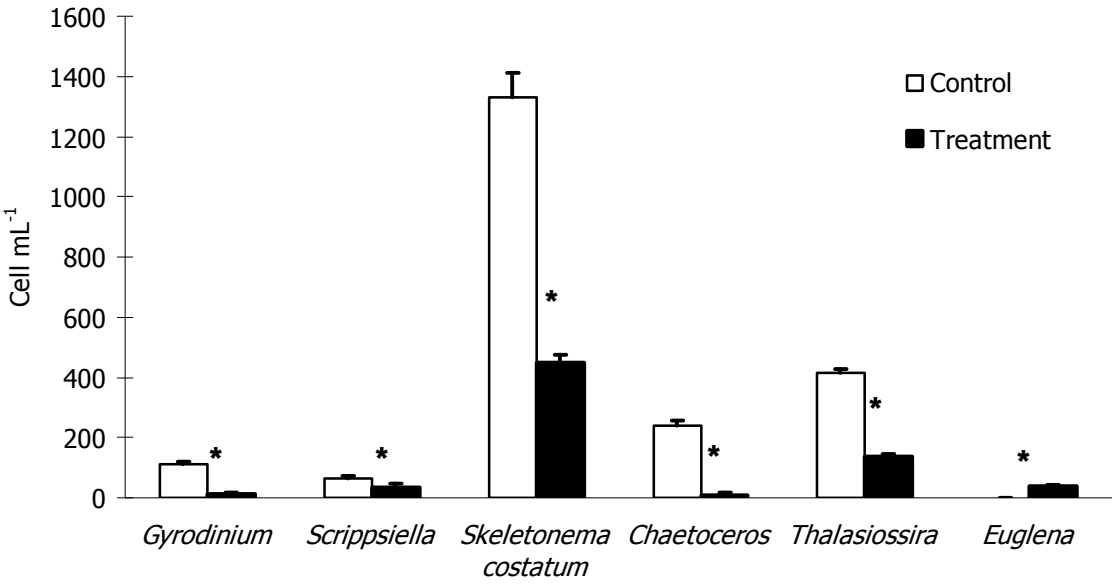
1600
1400
1200
1000
800
600
400
200
0
Cell mL-1
Control
Treatment
*
*
*
*
*
*
Gyrodinium
Scrippsiella
Skeletonema costatum
Chaetoceros
Thalasiossira
Euglena

Fig. 9

Chapter six: Deleterious consequences of a red tide dinoflagellate *Cochlodinium polykrikoides* Margalef for the calanoid copepod *Acartia tonsa* Dana

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Key words: harmful algae, copepods, survivorship, feeding, fecundity, *Cochlodinium polykrikoides*, *Acartia tonsa*

ABSTRACT

The dinoflagellate *Cochlodinium polykrikoides* Margalef has formed dense blooms and caused severe fish kills on a global scale in recent decades. Survivorship, feeding, and fecundity of the copepod *Acartia tonsa* Dana fed *C. polykrikoides* were investigated to assess potential impacts of these harmful events on herbivores. Survivorship of female *A. tonsa* was significantly reduced with increasing *C. polykrikoides* concentrations from 900 to 4700 μg C L^{-1} (500 – 2600 cells ml^{-1}). Copepods completely expired within 1.5 days at *C. polykrikoides* concentrations of 3300 and 4700 μg C L^{-1}(1800 and 2600 cells mL^{-1}), which are within the range of bloom densities of this alga. Stage-specific mortality of *A. tonsa* showed copepod susceptibility to *C. polykrikoides* decreased with development. Two bioassay experiments suggested that copepod mortality was due to multiple harmful compounds produced by *C. polykrikoides*. Ingestion rates of *A. tonsa* fed *C. polykrikoides* were 25 – 60% lower than ingestion rates on non-toxic *Rhodomonas lens* Pascher and Ruttner when the food concentrations ranged from 150 to 1500 μg C L^{-1}. *C. polykrikoides* supported higher egg production rates of *A. tonsa* than *R. lens* at the low algal concentrations (18 – 180 μg C L^{-1}), while egg production rates of *A. tonsa* fed *C. polykrikoides* were significantly less than those fed *R. lens* when the concentrations increased from 360 to 1080 μg C L^{-1}. Egg hatching success of *A. tonsa* fed *C. polykrikoides* ranging from 90 to 1080 μg C L^{-1} was very low (20 – 43%) compared to the higher values on *R. lens* (83 – 100%). Egg sizes of *A. tonsa* fed *C. polykrikoides* were significantly lower than those fed *R. lens*. All of these deleterious consequences may lead to *A. tonsa* population collapses during *C. polykrikoides* blooms.

INTRODUCTION

Harmful algal blooms (HABs) have increased in frequency, duration, and distribution in recent decades. Fish kills and accumulation of phycotoxins in shellfish with subsequent poisoning of humans have been well documented. However, studies of the interactions between harmful algae and their zooplankton grazers have been less common, and results are often controversial (Turner & Tester 1997, Turner 2006). These complex and inconsistent interactions are partly due to the wide variety of phycotoxins associated with more than 200 algal species from 20 genera (Landsberg 2002), substantial changes in toxicity levels of a single algal clone with culture age and nutrients (Granéli & Flynn 2006), and variations of grazers in terms of feeding patterns, binding sites of toxins, and structures of nervous systems (Turner & Tester 1997). Furthermore, phenotypic plasticity and rapid evolution of resistance to harmful algae can significantly shape the interactions between algae and herbivores (Hairston et al. 1999, Colin & Dam 2004).

Despite this complexity, zooplankton grazers are often considered as adversely affected by harmful algae. Effects include impaired feeding, avoidance behavior, physiological dysfunction, depressed growth and reproduction, and reduced population fitness (Turner & Tester 1997, Landsberg 2002, Prince et al. 2006, Barreiro et al. 2007, Cohen et al. 2007, Flynn & Irigoien 2009). Reduced feeding rates of zooplankton may be due to behavioral rejection of harmful algae prior to ingestion or physiological incapacitation (Ives 1987). Inability to continue feeding may result in low growth and reproduction, eventually causing a decline in population abundance. Zooplankton grazing may impact the development and termination of HABs. However, many studies suggest that the top-down controls are limited due to poisoning of grazers by phycotoxins and/or their relatively low growth rate (Turner & Tester 1997). Beyond directly feeding on

2026 harmful algae, zooplankton grazing may transport toxins along the food web and they may serve
2027 as vectors for higher trophic levels (Jester et al. 2009).

2028 The unarmored, chain-forming, gyrodinioid dinoflagellate *Cochlodinium polykrikoides*
2029 Margalef has formed dense blooms and caused severe economic damage in Southeast Asia
2030 during the past two decades (Lee 2008). Recently, *C. polykrikoides* blooms have been
2031 documented in many coastal waters ranging throughout temperate, sub-tropical, and tropical
2032 latitudes in both Asia and North America (Anton et al. 2008, Gobler et al. 2008, Curtiss et al.
2033 2008, Park et al. 2009). Cell densities during blooms usually range from 10^3 cell mL^{-1} to 10^4
2034 cells mL^{-1} (Anton et al. 2008, Gobler et al. 2008, Curtiss et al. 2008, Park et al. 2009). Bloom
2035 patches can achieve cell densities exceeding 10^5 cells mL^{-1} (Gobbler et al., 2008). Some studies
2036 have shown that *C. polykrikoides* isolated in East Asia can be mixotrophic, feeding on small
2037 phytoplankton species (<11μm) by engulfing the prey through the sulcus (Jeong et al. 2004).
2038 Strong diel vertical migration has been observed in field populations of *C. polykrikoides* (Park et
2039 al. 2001). *C. polykrikoides* has been reported to be resistant to attacks by six algicidal bacteria
2040 (Imai & Kimura 2008), and, in turn, *C. polykrikoides* inhibited growth of the dinoflagellate
2041 *Akashiwo sanguinea* and caused morphologically abnormal cells (Yamasaki et al. 2007). All of
2042 these attributes likely provide *C. polykrikoides* with competitive advantages over other occurring
2043 microalgae and, at least partly, explain the mechanisms of *Cochlodinium* bloom formation.

2044 Although the emergence of *Cochlodinium* blooms and subsequent severe fish kills have been
2045 well recorded, the precise toxic mechanisms of this alga are still poorly understood. *Cochldinium*
2046 blooms occurring along the coast of Japan were reported to release water-soluble ichthyotoxic
2047 substances with characteristics of paralytic shellfish toxins (Onoue et al. 1985) and three toxin
2048 fractions: neurotoxic, hemolytic and hemagglutinative (Onoue & Nozawa 1989). In Korean

2049 isolates, *C. polykrikoides* has been reported to generate the superoxide anion (O_2^-) and hydrogen
2050 peroxide (H_2O_2) (Kim et al. 1999), which resulted in the inactivation of transport-related enzyme
2051 activities in fish gills, a drop in blood pO_2, and abnormal secretion of gill mucus (Kim et al.
2052 2000). Interestingly, the production of reactive oxygen species (ROS) in two *C. polykrikoides*
2053 strains isolated in Japanese waters was very low compared to *Chattonella marina*, a species well-
2054 known for ROS production. Fish kills by these two strains were related to biologically active
2055 metabolites, such as cytotoxic agents and mucus substances (Kim et al. 2002). Further, the
2056 harmful effects of *C. polykrikoides* from the US east coast waters to fish was caused by a labile,
2057 extracellular toxic principle produced by actively growing cells (Tang & Gobler 2009).
2058 The interactions between *C. polykrikoides* and zooplankton have been poorly studied. *C.*
2059 *polykrikoides* retarded metamorphosis of the Pacific oyster (*Crassostrea gigas*) from the
2060 trochophore stage to the D-shaped larval stage (Matsuyama et al. 2001). The egg production
2061 rates and egg viability of the copepod *Acartia omorii* were low when fed *C. polykrikoides* (Shin
2062 et al. 2003). On the contrary, the planktonic ciliate *Strombidinopsis* sp. ingested *C. polykrikoides*
2063 and grew well (Jeong et al. 2008). In this study, the deleterious effects of *C. polykrikoides* on
2064 survival, feeding, and fecundity of the copepod *Acartia tonsa* Dana were investigated to assess
2065 potential impacts of harmful blooms on lower trophic grazers.
2066
2067 **MATERIALS AND METHODS**
2068 **Collection and culture of organisms.** The dinoflagellate *Cochlodinium polykrikoides* clone
2069 CP1 was isolated from Peconic Bay, Long Island, New York, USA in 2006 (Gobler et al. 2008).
2070 The flagellate *Rhodomonas lens* Pascher and Ruttner (CCMP 739) was obtained from The
2071 Provasoli-Guillard National Center for Culture of Marine Phytoplankton. The cultures were

2072 maintained in a temperature-controlled incubator at 20℃ with a 14:10 light-dark cycle
2073 (approximately 50 μmol photons m^{-2} s^{-1}). The cultures were maintained in exponential growth
2074 phase by biweekly dilution with f/2 medium. The length and width of more than 100 cells were
2075 measured under a compound microscope. The carbon contents of the two microalgae (Table 1)
2076 were estimated from their cell volumes (Stoecker et al. 1994).

2077 The copepod *Acartia tonsa* was collected from Stony Brook Harbor, Long Island Sound, New
2078 York, USA, with a 202-μm mesh plankton net. The population was continuously cultured in 20-
2079 L tanks at a density of 20 to 50 ind. L^{-1}. The copepods were offered *R. lens* at a carbon
2080 concentration of approximately 500 μg C L^{-1} and maintained at 20℃ with a 12:12 light-dark
2081 cycle (approximately 1 μmol photons m^{-2} s^{-1}). The low irradiance level minimized the potential
2082 effects of light on copepods and algal growth during experiments. Half of the copepod culture
2083 medium was refreshed twice a week with 0.2-μm filtered sea water (FSW).

2084 **Survival experiments.** A life table experiment was performed to compare survivorship of *A.*
2085 *tonsa* when fed *C. polykrikoides* at five concentrations ranging from 900 to 4700 μg C L^{-1} (500
2086 to 2600 cells mL^{-1}). Our experimental concentrations were within the range of *C. polykrikoides*
2087 densities observed in the field (Gobler et al. 2008). Copepod survivorship in FSW and two *R.*
2088 *lens* solutions (900 and 2200 μg C L^{-1}) were used as the controls. Approximately 400 female *A.*
2089 *tonsa* were transferred into a 5-L plastic container and acclimated in 0.2-μm filtered seawater for
2090 24 h. For each treatment, 20 – 48 healthy females were transferred individually into 6-well tissue
2091 culture plates. Each well was filled with 13 mL of the food medium and one *A. tonsa*. The
2092 copepods were checked every 12 h until they all died. Approximately 80% food medium was
2093 refreshed daily. All experiments in this study were conducted in a temperature-controlled
2094 incubator at 20^{o}C with a 12:12 light-dark cycle (approximately 1 μmol photons m^{-2} s^{-1}).

2095 An acute toxicity experiment was conducted to elucidate stage susceptibility of copepods to
2096 *C. polykrikoides*. The organisms from the first naupliar stage (N1), the fourth naupliar stage
2097 (N4), the first copepodite stage (C1), the fourth copepodite stage (C4), adult females, and eggs
2098 were exposed to a series of *C. polykrikoides* solutions ranging from 0 to 4700 μg C L^{-1} (0 to 2600
2099 cells mL^{-1}). Each treatment had four replicates. The organisms (n = 8 – 12) were individually
2100 held in tissue culture plates filled with *C. polykrikoides* solutions. After 24 h, the copepods were
2101 observed under a dissecting microscope.

2102 The mode of harmful effects of *C. polykrikoides* on copepods was explored using two 48-h
2103 bioassay experiments. Healthy female *A. tonsa* were exposed to either *C. polykrikoides* live
2104 culture, frozen and thawed culture, culture filtrate (0.2 μm), or 0.2-μm filtered seawater (the
2105 control). The culture density was 2200 μg C L^{-1} (1200 cells mL^{-1}). The procedures were the same
2106 as described above. Another experiment was designed to investigate whether the toxic reaction
2107 of copepods was dependent on physical contact with *C. polykrikoides* cells. The experiment was
2108 performed using cages made from polyethylene centrifuge tubes (50 mL) with sealed nylon-
2109 mesh bottoms. The mesh sizes were 100-μm, and 5-μm for treatment 1 and treatment 2,
2110 respectively. Cages with 100-μm mesh would permit the passage of *C. polykrikoides* cells while
2111 the 5-μm mesh did not, which was verified by using the microscope. Each treatment had four
2112 replicates. Healthy females (n = 8 – 12) were transferred into each cage. The cages in treatment 1
2113 and treatment 2 were immersed in a 4-L culture of *C. polykrikoides* at a concentration of 2200 μg
2114 C L^{-1} (1200 cells mL^{-1}). The cages with 5-μm mesh immersed in 4-L of 0.2-μm filtered seawater
2115 were used as the control. Copepod mortality was compared after 48 h.

2116 **Feeding experiments.** Active adult copepods with intact appendages were transferred into 2-
2117 L beakers with 0.2-μm filtered seawater for 24 h prior to the feeding experiments. Six food

2118 concentrations of *C. polykrikoides* and *R. lens* ranging from 150 to 1500 μg C L^{-1} were used to
2119 determine copepod ingestion rates. We used 3 or 4 replicates of 250-mL bottles for each
2120 experimental diet and concentration. The bottles without copepods were used as the controls.
2121 Ten active adult females were transferred into each bottle. The bottles were placed on a plankton
2122 wheel and rotated at 1 rpm for 24 h at 20°C with a 12:12 light-dark cycle (approximately 1μmol
2123 photons m^{-2} s^{-1}). At the beginning and end of the experiment, samples for cell densities were
2124 taken. Algal densities were approximated by measuring *in vivo* chlorophyll fluorescence with a
2125 Turner AU-10 fluorometer. Actual cell densities were quantified on Lugol's iodine preserved
2126 samples. *In vivo* fluorescence of *C. polykrikoides* and *R. lens* was significantly linearly related to
2127 algal concentration (the regression coefficients: r = 0.997 and 0.999, respectively, for both, P <
2128 0.001, unpublished data). The significant relationships between fluorescence and algal
2129 concentration provided a rapid and simple measurement to monitor algal concentration during
2130 this experiment. The ingestion rates (I) were calculated according to the equation described by
2131 Båmstedt et al. (2000):

2132 $$I = \frac{V \times \ln \frac{C_0'}{C_t'}}{t \times n} \times \frac{C_0 + C_t}{2}$$

2133 where C_0 and C_t are the food concentrations at the beginning and end of the experiment; C_t' is the
2134 final food concentration in the controls; V is the volume of the bottles; t is the duration of the
2135 experiment; n is the number of copepods.

2136 **Egg production and egg hatching experiments.** Egg production rates and hatching success
2137 were measured at algal concentrations of 18, 90, 180, 360, 540, and 1080 μg C L^{-1}.
2138 Approximately 300 healthy adult *A. tonsa* were transferred to each of 6 containers filled with 5-L
2139 of the appropriate diet suspension and acclimated for 24 h. Approximately 80% diet medium was

2140 refreshed daily. Two healthy female *A. tonsa* were then transferred from the container into a dish
2141 filled with 50-ml food solution. A 200-μm mesh was fixed above the bottom to minimize egg
2142 cannibalism. All eggs and nauplii were enumerated after a 24-h incubation. There were seven
2143 replicates for each treatment. Eggs were placed individually in 1-mL wells of a multi-depression
2144 dish contained within a closed plastic box. Distilled water was added to the bottom of the box to
2145 reduce evaporation from the wells. Fresh FSW was added to the wells. Eggs were observed once
2146 a day for 2-3 days. The measurements in the *C. polykrikoides* treatments ran for 10 days or until
2147 all copepods in the containers were dead. The measurements in *R. lens* treatments only ran for 1
2148 day.

2149 Copepod egg sizes were measured when exposed to *C. polykrikoides* and *R. lens* at
2150 concentrations of 90, 180, 360, 540, and 720 μg C L^{-1} during the 10-day period. Approximately
2151 600 healthy adult *A. tonsa* were transferred to each of 5 containers filled with 10-L of the
2152 appropriate diet suspension. Copepod eggs were collected by 60-μm mesh and 80% food
2153 solutions were refreshed every day. At least 15 eggs from a sample were measured under a
2154 compound microscope using the 100× magnification to determine the mean egg diameter.

2155 **Statistical analyses.** Survivorship curves were compared using the Gehan-Wilcoxon test
2156 (Pyke & Thompson 1986). Lethal median concentration (LC_{50}) was determined by applying a
2157 probit analysis. One-way ANOVAs followed by Tukey multiple comparison tests were used to
2158 compare means of different treatments in bioassay experiments. A two-level nested ANOVA
2159 was used to test the effects of algal species and concentration on ingestion rates. A two-way
2160 ANOVA was used to analyze the effects of algal species and concentration on egg production
2161 rates and hatching success. A three-way ANOVA was used to analyze the effects of algal

2162 species, concentration, and exposure time on egg sizes (Sokal & Rohlf 1995). All statistical
2163 analyses were conducted using SPSS 16.0 statistical package.
2164
2165 **RESULTS**
2166 **Survival experiments**
2167 Life table experiments revealed that survivorship of female *A. tonsa* was significantly reduced
2168 when fed *C. polykrikoides* compared to those starved or fed non-toxic *R. lens* (Fig. 1 and Table
2169 2). Survivorship of female *A. tonsa* significantly decreased with increasing *C. polykrikoides*
2170 concentrations (Fig. 1 and Table 2). Female *A. tonsa* experienced rapid mortality at high (3300
2171 and 4700 μg C L^{-1}, or ~ 1800 and 2600 cells mL^{-1}) and intermediate (1500 and 2200 μg C L^{-1}, or
2172 ~ 800 and 1200 cells mL^{-1}) concentrations of *C. polykrikoides* with 100% of individuals expiring
2173 within 1.5 and 3.5 day, respectively (Fig. 1). Survivorship of female *A. tonsa* fed *C.*
2174 *polykrikoides* was moderately improved at the low concentration of 900 μg C L^{-1} (~ 500 cells
2175 mL^{-1}) with individuals surviving 7 days (Fig. 1). All of these survival times were significantly
2176 shorter than those in FSW and in the *R. lens* control treatments (Fig. 1 and Table 2).
2177 Mortality of *A. tonsa* from early nauplii to adult females significantly increased with
2178 increasing *C. polykrikoides* concentrations after a 24-h exposure ($P < 0.001$ for all, one-way
2179 ANOVA, Fig. 2). In contrast, egg hatching was not affected by *C. polykrikoides* ($P > 0.05$, one-
2180 way ANOVA, Fig. 2). LC_{50} values indicated that the susceptibility of *A. tonsa* to *C.*
2181 *polykrikoides* decreased with development, especially from early copepodite to adult stage (Fig.
2182 3). Early nauplii of *A. tonsa* were approximately four times more sensitive to *C. polykrikoides*
2183 than adult females after 24-h exposure, with LC_{50}s of 607 μg C L^{-1} (334 cells mL^{-1}, 95%

2184 confidence interval: 399 – 877 μg C L^{-1}, 220 – 483 cells mL^{-1}) and 2511 μg C L^{-1} (1383 cells
2185 mL^{-1}, 95% confidence interval: 1769 –3602 μg C L^{-1}, 974 – 1983 cells mL^{-1}), respectively.

2186 Mortality of *A. tonsa* exposed to the frozen and thawed *C. polykrikoides* culture was
2187 significantly reduced to half of that exposed to the live culture (Fig. 4), but was significantly
2188 higher than that in FSW (Table 3). Copepods in the 0.2-μm culture filtrate had significantly
2189 increased survivorship compared to those in the live culture and their mortality did not
2190 significantly differ from that in FSW (Fig. 4 and Table 3). Copepod mortality in the cages with
2191 5-μm nylon mesh and immersed in *C. polykrikoides* live culture was significantly lower than that
2192 in the cages with 100-μm nylon mesh; however, it was significantly higher than that in FSW
2193 (Fig. 4 and Table 3).

2194 **Feeding experiments**

2195 The ingestion rates of *A. tonsa* were significantly affected by algal species ($F_{[1,29]}$ = 10.2347,
2196 $P < 0.01$, two-level nested ANOVA) and algal concentration ($F_{[10,29]}$ = 2.9841, $P < 0.05$, two-
2197 level nested ANOVA). The ingestion rates of *A. tonsa* fed *C. polykrikoides* were 25 – 60% lower
2198 than ingestion rates on *R. lens* (Fig. 5). The ingestion rates on *C. polykrikoides* and *R. lens* by *A.*
2199 *tonsa* significantly increased with their increasing concentration ($F_{[10,29]}$ = 2.9841, $P < 0.05$,
2200 two-level nested ANOVA, Fig. 5). Their maximum daily ingestion rates were 3.15 and 6.18 μg
2201 C $ind.^{-1}$ d^{-1}, respectively (Fig. 5).

2202 **Egg production and egg hatching experiments**

2203 The two-way ANOVA showed that egg production rates of *A. tonsa* after a 1-day exposure
2204 were significantly affected by algal species ($F_{[1,60]}$ = 13.9295, $P < 0.001$), algal concentration
2205 ($F_{[5,60]}$ = 8.0195, $P < 0.001$), and their interactions ($F_{[5,60]}$ = 13.8806, $P < 0.001$). Egg production
2206 rates of *A. tonsa* increased progressively with increasing *R. lens* concentration (Fig. 6). In

2207 contrast, egg production rates of *A. tonsa* moderately increased with increasing *C. polykrikoides*
2208 concentrations from 18 to 180 μg C L^{-1}, then were greatly reduced by the high concentrations of
2209 *C. polykrikoides* (180 – 1080 μg C L^{-1}, Fig. 6). *C. polykrikoides* supported higher egg production
2210 rates of *A. tonsa* than *R. lens* at low algal concentrations (18 – 180 μg C L^{-1}), while egg
2211 production rates of *A. tonsa* fed *C. polykrikoides* were greatly lower than those fed *R. lens* at high
2212 concentrations (360 – 1080 μg C L^{-1}, Fig. 6). The two-way ANOVA showed that egg hatching
2213 success of *A. tonsa* was significantly affected by algal species ($F_{[1,48]}$ = 30.8405, $P < 0.001$), but
2214 not by algal concentration ($F_{[51,48]}$ = 2.2991, $P = 0.06$). Egg hatching rates of *A. tonsa* were very
2215 high (82 – 100%) when fed *R. lens*, except the values at the lowest concentration (18 μg C L^{-1},
2216 Fig. 6). Egg hatching success was very low ranging from 20% to 43% when fed *C. polykrikoides*
2217 (Fig. 6).

2218 The two-way ANOVA showed that exposure time did not significantly change egg production
2219 rates ($F_{[26,155]}$ = 1.5491, $P = 0.055$, Fig. 7) and hatching rates ($F_{[26,142]}$ = 1.3164, $P = 0.165$, Fig.
2220 8) of *A. tonsa* when fed *C. polykrikoides*. The moderate concentrations of *C. polykrikoides* (90 –
2221 360 μg C L^{-1}) supported higher egg production rates of *A. tonsa* than the lowest concentration
2222 (18 μg C L^{-1}) and the higher concentrations (540 and 1080 μg C L^{-1}, Fig. 7). Egg production of
2223 *A. tonsa* when fed *C. polykrikoides* at 90 μg C L^{-1} persisted during the entire experiment (10 d).
2224 In contrast, egg production of *A. tonsa* only persisted for several days at the lowest and two
2225 highest concentrations of *C. polykrikoides*. *C. polykrikoides* at 1080 μg C L^{-1} reduced *A. tonsa*
2226 egg production to zero within two days (Fig. 7). The hatching successes of *A. tonsa* eggs when
2227 fed *C. polykrikoides* at 18 and 90 μg C L^{-1} were higher than other concentrations. *C.*
2228 *polykrikoides* at 1080 μg C L^{-1} reduced *A. tonsa* egg hatching success to zero within 1 d (Fig. 8).

2229 The three-way ANOVA showed that *A. tonsa* egg sizes were significantly affected by algal
2230 species ($F_{[1,2370]}$ = 89.337, P < 0.001), algal concentration ($F_{[4, 2370]}$ = 7.273, P < 0.001), and
2231 exposure time ($F_{[9, 2370]}$ = 2.35, P < 0.001, Fig. 9). Egg sizes of *A. tonsa* when fed *C.*
2232 *polykrikoides* were lower than those fed *R. lens* at each experimental concentration. The average
2233 egg sizes of *A. tonsa* when fed *C. polykrikoides* and *R. lens* for all concentrations were 76.40 μm
2234 and 77.60 μm, respectively. Egg sizes of *A. tonsa* when fed *C. polykrikoides* decreased from
2235 77.30 μm to 75.96 μm with increasing concentrations from 90 μg C L^{-1} to 720 μg C L^{-1}. In
2236 contrast, egg sizes of *A. tonsa* remained constant (77.34 – 77.89 μm) when fed non-toxic *R. lens*
2237 from 90 μg C L^{-1} to 720 μg C L^{-1}. The trend of egg sizes over time when fed either *C.*
2238 *polykrikoides* or *R. lens* was not clear (Fig. 9).
2239
2240 **DISCUSSION**
2241 Our study showed that the dinoflagellate *C. polykrikoides* had comparable or more deleterious
2242 impacts on copepods compared to other well-known toxic dinoflagellates. Copepods completely
2243 expired within 1.5 and 3.5 day, respectively, at high (3300 and 4700 μg C L^{-1}, ~ 1800 and 2600
2244 cells mL^{-1}) and intermediate (1500 and 2200 μg C L^{-1}, ~ 800 and 1200 cells mL^{-1}) concentrations
2245 of *C. polykrikoides*. Approximately 50% of *A. clausi* were dead during a 7-day exposure to the
2246 toxic strain *Alexandrium minutum* at a density of 650 cells mL^{-1} (Barreiro et al. 2007). The
2247 dinoflagellate *Karenia brevis* cultured at densities ranging from 1.8×10^4 to 2.1×10^4 cells mL^{-1}
2248 led to approximate 80% mortality of *A. tonsa* over a period of 5 days (Prince et al. 2006). Cohen
2249 et al. (2007) reported that the *K. brevis* culture at a density of 1×10^4 cells mL^{-1} caused
2250 approximately 10% mortality in *Temora turbinata*, 3% mortality in *A. tonsa*, and 1% mortality in
2251 *Centropages typicus* after a 24-h exposure. *Karlodinium corsicum* (as *Gyrodinium corsicum*) at

2252 concentrations around 1500 μg C L^{-1} killed approximate 50% *A. grani* after 6 days and all
2253 copepods after 12 days (da Costa et al. 2005). During blooms lasting one to two months in US
2254 eastern coast waters, typical densities of *C. polykrikoides* were $>10^3$ cells mL^{-1}, frequently 10^4
2255 cells mL^{-1} (Gobler et al. 2008, Mulholland et al. 2009). Since lethal concentrations of *C.*
2256 *polykrikoides* for *A. tonsa* are lower than their densities during the blooms, exposure to high
2257 densities of toxic *C. polykrikoides* cells for such extended periods may cause substantial
2258 mortalities within *A. tonsa* populations. The population dynamics of copepods are sensitive to
2259 variation in mortality, as a relatively small increase in female mortality can considerably change
2260 population growth by reducing recruitment. Even before blooms occur, the moderate densities of
2261 *C. polykrikoides* (~ 10^2 cells mL^{-1}) may lead to adverse effects on zooplankton, such as reduced
2262 feeding and fecundity. Thus, toxic blooms may reduce secondary production and further lead to
2263 food restriction for consumers at higher trophic levels.

2264 Extrapolating laboratory experiments to the natural environment can be complex. Rapid
2265 evolution of resistance may shape the interactions between zooplankton and toxic algae. Some
2266 studies have shown that grazer populations that have experienced recurrent HABs can evolve
2267 local adaptations to toxic algae (Hairston et al. 1999, Colin & Dam 2004). An artificial selection
2268 experiment showed that copepods evolved resistance to toxic algae over only 2-5 generations
2269 (Colin & Dam 2004). The rapid evolution of resistance may be an important feedback
2270 mechanism to minimize the potential deleterious effects of toxic algae on zooplankton. In New
2271 York, *C. polykrikoides* blooms only occur in eastern Long Island waters (Gobler et al. 2008).
2272 The copepod population used in this research was collected from Stony Brook Harbor, Long
2273 Island Sound, where no *C. polykrikoides* blooms have been observed. Thus, the adverse
2274 consequences may be maximized assuming there is no zooplankton gene flow between bloom

and non-bloom areas. Another potential factor is the complexity of plankton. Toxic algae rarely bloom in nature in the absence of other phytoplankters. Ingestion of *C. polykrikoides* with other concurrent phytoplankters or heterotrophic prey may dilute potential adverse effects on copepods. Some zooplankton has the ability to actively select a non-toxic diet (Turner & Tester 1997). The adverse effects of *C. polykrikoides* may be reduced by the presence of other occurring microalgae (Tang & Gobler 2009).

Stage-specific effects of HABs on zooplankton have rarely been considered in prior studies of the interactions between harmful algae and zooplankton. The present results showed the resistance of *A. tonsa* to *C. polykrikoides* increased with development. Early nauplii of *A. tonsa* were four times more sensitive to *C. polykrikoides* than adult females. These results are similar to the previous studies on stage-specific variations in sensitivity of copepods to toxic chemicals. The nauplii of *Tigriopus brevicornis* were two to four times more sensitive to three insecticides and two metals than the adults (Forget et al. 1998). The nauplii of *A. tonsa* were 28 times more sensitive to an organic pesticide (cypermethrin) than adults after 96 h of exposure (Medina et al. 2002). The greater sensitivity of copepod early life stages to toxic algae may be related to their relatively larger surface per unit volume, which may promote a greater diffusive flux of phycotoxins into the copepod body. Another possible explanation is that the later stages may have a greater ability to detoxify. Copepods may transfer toxins into fecal pellets and/or eggs, or eliminate them through excretion in dissolved form (Guisande et al. 2002). More developed metabolic systems in adults (Mauchline 1998) may improve detoxification abilities of copepods. Regardless, the studies on stage-specific effects of HABs on zooplankton may be necessary to understand their true impact on planktonic ecosystems. The investigation of all life stages also provides a more appropriate tool for predicting potential toxicity of harmful algae to copepod

2298 populations. Interestingly, live *C. polykrikoides* cells did not inhibit *A. tonsa* egg hatching. Tang
2299 and Dam (2001) reported a similar result that marine diatom exudates did not have negative
2300 effects on *A. tonsa* egg hatching.

2301 Mortality of *A. tonsa* exposed to the frozen and thawed *C. polykrikoides* culture was
2302 significantly lower than that in the live culture. The freezing and thawing treatment destroyed *C.*
2303 *polykrikoides* cells (personal observation). This result indicated that harmful effects were mainly
2304 dependent on the viability of *C. polykrikoides* cells. Similar results were observed on the lethal
2305 effects on fish by *C. polykrikoides* natural bloom waters (Gobler et al. 2008, Mulholland et al.
2306 2009) and pure cultures (Tang & Gobler, 2009). Copepod mortality exposed to the frozen and
2307 thawed *C. polykrikoides* culture was still significantly higher than that in FSW, which suggested
2308 that some harmful compound(s) remained after this treatment. The extracellular secretion and
2309 continuous accumulation of polysaccharides in *C. polykrikoides* medium were considered as one
2310 of the causes of fish kills (Kim et al. 2002). An extensive exocellular organic fibrillar matrix and
2311 a closely enclosing organic envelope surround the *C. polykrikoides* cells of our strain (Gobler et
2312 al. 2008). The freezing and thawing treatment may not completely eliminate the harmful effect of
2313 such polysaccharides. Direct contact with those polysaccharides or other harmful compounds
2314 located on the *C. polykrikoides* cell surface may be responsible for the death of some copepods
2315 in this treatment. Another possibility is that some harmful compounds in *C. polykrikoides* may be
2316 released when cells are broken. The result from our second bioassay supported the above
2317 explanations. The mortality of copepods in the cages with 5-μm nylon mesh and immersed in *C.*
2318 *polykrikoides* live culture was significantly lower than that in the cages with 100-μm nylon mesh.
2319 The separation from harmful compounds in *C. polykrikoides* cells or on cell surfaces by the 5-μm
2320 nylon mesh may account for the improved survival of copepods. Yamasaki et al. (2007) observed

2321 that cell contact with *C. polykrikoides* inhibited the growth of another dinoflagellate *Akaskiwo*
2322 *sanguinea* and caused morphologically abnormal cells. This result indicated some harmful
2323 compounds located on *C. polykrikoides* cell surface, but we still do not have evidence to exclude
2324 the possibility of the presence of harmful compounds in cells. Interestingly, the freezing of *C.*
2325 *polykrikoides* culture did not show toxic to juvenile fish (*Cypinodon variegates*) (Gobler et al.
2326 2008, Tang & Gobler 2009). This dissimilarity is probably due to the differences in the
2327 physiology of these organisms, such as different binding sites and tolerance to harmful
2328 compounds. This harmful fraction may impact on lower trophic copepods, but not vertebrate
2329 fish.

2330 Another harmful principle may be the dissolved, highly reactive, labile compounds released
2331 by live *C. polykrikoides* cells. The complete lack of the harmful effects of the 0.2-μm culture
2332 filtrate suggested that *C. polykrikoides* cells did not release water-soluble harmful compounds or
2333 that released compounds were very unstable. The second bioassay experiment supported the
2334 latter explanation. The mortality of copepods in the cages with the 5-μm nylon mesh and
2335 immersed in *C. polykrikoides* live culture was higher than that in the FSW. This result suggested
2336 that some water soluble harmful compounds released by *C. polykrikoides* cells may pass through
2337 the 5-μm nylon mesh and affect copepods. Kim et al. (1999) reported that reactive oxygen
2338 species (ROS) generated from *C. polykrikoides* was responsible for oxidative damage leading to
2339 fish kills. Tang and Gobler (2009) also reported that the ichthyotoxicity of *C. polykrikoides* could
2340 be caused by non-hydrogen peroxide, highly reactive, labile compounds such as ROS-like
2341 chemicals. Thus, we propose that multiple harmful compounds produced by *C. polykrikoides* are
2342 responsible to their deleterious effects on copepods.

C. polykrikoides significantly reduced ingestion rates of *A. tonsa* when compared to non-toxic *R. lens*. Two possible mechanisms, behavioral rejection and physiological incapacitation, have been postulated to explain such reduced feeding due to harmful algae (Ives, 1987). We did not directly test which mechanism was responsible for the reduced feeding by *C. polykrikoides*. Higher mortality of *A. tonsa* when exposed to *C. polykrikoides* than in FSW suggested that poisoning rather than starvation was the main mechanism for copepod death. Therefore, the physiological incapacitation may, at least partially, explain the reduced feeding of *A. tonsa* by *C. polykrikoides*. Our present experiments, however, did not directly rule out the possibility of feeding deterrents. Copepod feeding is shaped by prey size, motility, and quality (Berggreen et al., 1988; Hansen et al., 1994; Mauchline, 1998). The equivalent spherical diameters (ESD) for *C. polykrikoides* and *R. lens* were 28.2 μm and 7.97 μm, respectively. The optimal particle size for feeding by *A. tonsa* females was 14.8 μm (Berggreen et al., 1988). Clearance rates of *A. tonsa* females were nearly equal when fed the flagellate *R. baltica* (ESD: 6.91 μm) and the dinoflagellate *Scripsiella faröense* (ESD: 19.0 μm, Berggreen et al., 1988). Thus, it is reasonable to assume that the effect of size difference in this study was limited because the two algae used in this study were very similar to *R. baltica* and *S. faröense* in size. Egg sizes of *A. tonsa* when fed *C. polykrikoides* were smaller than *R. lens*. To our knowledge, this is the first report that toxic algae reduced copepod egg size. Cooney and Gehrs (1980) reported that there was a direct positive relationship between egg size and naupliar size in the calanoid copepod *Diaptomus clavipes*. Thus, copepod population fitness may be reduced by toxic algae since larger nauplii usually have lower mortality rates or matured more rapidly than smaller nauplii (Mauchline 1998). We do not know the mechanism by which a *C. polykrikoides* diet yielded smaller eggs of *A. tonsa*. The ingestion rates of *A. tonsa* on *C. polykrikoides* were 25 – 60% lower than values on

2366 *R. lens*. The lack of adequate nutrition and (or) physiological incapacitation would lead to
2367 impaired gametogenesis and spawning failure in copepods.

2368 The present results clearly showed that the red tide dinoflagellate *C. polykrikoides* at our
2369 experimental concentrations caused deleterious consequences for the copepod *A. tonsa*. Is *C.*
2370 *polykrikoides* really a toxic alga? Harmful effects of algae on zooplankton can be explained by
2371 the absence of essential nutrients or the presence of toxins (Turner & Tester 1997; Colin & Dam
2372 2002; Prince et al. 2006). One of major challenges in algae-grazer interactions is to separate
2373 potential toxic effects from nutritional inadequacy or deficiency. *C. polykrikoides* at high
2374 concentrations ($\geq$ 900 μg C L^{-1} or 500 cells mL^{-1}) significantly reduced survivorship of female *A.*
2375 *tonsa* compared to those starved in FSW. The lethal effects suggested that *C. polykrikoides* was a
2376 toxic prey for *A. tonsa* at high concentrations. Recently, the mixed diet approach has been
2377 developed to discern whether the suspect prey is beneficial, nutritionally inadequate, or toxic to
2378 grazers (Colin & Dam 2002). In another study, we conducted mixed diet experiments at four
2379 concentrations (100 μg C L^{-1}, 200 μg C L^{-1}, 600 μg C L^{-1}, and 1000 μg C L^{-1}) and three durations
2380 (1 d, 3 d, and 5 d). The results showed that harmful effects on *A. tonsa* at 1000 μg C L^{-1} were
2381 caused by *C. polykrikoides* toxicity. However, the nutritional value of *C. polykrikoides* was
2382 greater than or equal to the standard diet of *R. lens* at 100 μg C L^{-1} and 200 μg C L^{-1}. These
2383 results showed that the nutritional value of *C. polykrikoides* to *A. tonsa* ranged from beneficial to
2384 toxic with increasing cell density. The density-dependent nutritional value of this alga suggests
2385 that *C. polykrikoides* can be nutritious or toxic for *A. tonsa* depending on ambient concentrations.
2386

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2392

2393 LITERATURE CITED

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Table 1. Characters of two algae used in the experiments

Alga	Length (μm)	Width (μm)	Equivalent spherical diameter (μm)	Carbon content (pg cell^{-1})
Cochlodinium polykrikoides	34±4.7	27±4.1	28.2	1816
Rhodomonas lens	11±1.2	7.0±1.0	7.97	39.5

Table 2. Gehan-Wilcoxon test results of survivorship curves in the life table experiment. Arrow indicates whether the survivorship curve indicated by the column header is greater (up arrow) or less (down arrow) than that indicated in the row header. Significant differences are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) and ns (not significant). CP = *Cochlodinium polykrikoides*; RL = *Rhodomonas lens*; FSW = 0.2-μm filtered seawater; The numbers indicate algal carbon concentrations (μg C L^{-1}).

	CP 900	CP 1500	CP 2200	CP 3300	CP 4700	FSW	RL 900	RL 2200
CP 900	----							
CP 1500	***↑	----						
CP 2200	***↑	**↑	----					
CP 3300	***↑	***↑	***↑	----				
CP 4700	***↑	***↑	***↑	ns	----			
FSW	*↓	***↓	***↓	***↓	***↓	----		
RL 900	*↓	***↓	***↓	***↓	***↓	ns	----	
RL 2200	***↓	***↓	***↓	***↓	***↓	**↓	ns	----

Table 3. Results of Tukey multiple comparison tests for mortality in two bioassay experiments. Significant differences are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) and ns (not significant). CP = *Cochlodinium polykrikoides*; RL = *Rhodomonas lens*; FSW = 0.2-µm filtered seawater;

	(A)					(B)			
	Live CP	Frozen and thawed CP	0.2-µm filtered CP	FSW		100-µm cage in CP	5-µm cage in CP	5-µm cage in FSW	
Live CP	----				100-µm cage in CP	----			
Frozen and thawed CP	***	----			5-µm cage in CP	***	----		
0.2-µm filtered CP	***	***	----		5-µm cage in FSW	***	*	----	
FSW	***	***	ns	----					

Figure Legends

Fig. 1. *Acartia tonsa*. Survivorship when exposed to five *Cochlodinium polykrikoides* solutions (CP 900 μg C L^{-1}, CP 1500 μg C L^{-1}, CP 2200 μg C L^{-1}, CP 3300 μg C L^{-1}, and CP 4700 μg C L^{-1}), two *Rhodomonas lens* solutions (RL 900 μg C $L^{-1}$and RL 2200 μg C L^{-1}), and 0.2 μm-filtered seawater (FSW).

Fig. 2. *Acartia tonsa*. Stage-specific mortality (mean ± SD) when exposed to *Cochlodinium polykrikoides* for 24 h. N1: the first naupliar stage, N4: the fourth naupliar stage, C1: the first copepodite stage, C4: the fourth copepodite stage.

Fig. 3. *Acartia tonsa*. 24-h LC_{50} and 95% confidence intervals for five development stages when exposed to *Cochlodinium polykrikoides*. N1: the first naupliar stage, N4: the fourth naupliar stage, C1: the first copepodite stage, C4: the fourth copepodite stage.

Fig. 4. *Acartia tonsa*. Bioassay experiments conducted for 48 h. (A) Percent mortality (mean ± SD) when exposed to either *Cochlodinium polykrikoides* live culture, frozen and thawed culture, culture filtrate (0.2-μm), or 0.2-μm filtered seawater. (B) Percent mortality (mean ± SD) in the cages that were covered by 100- μm or 5-μm nylon mesh and immersed in *C. polykrikoides* live culture or 0.2-μm filtered seawater.

Fig. 5. *Acartia tonsa*. Ingestion rates (mean ± SD) when fed either *Cochlodinium polykrikoides* or *Rhodomonas lens*.

Fig. 6. *Acartia tonsa*. Egg production rates (mean ± SD) and hatching success (mean ± SD) when fed either *Cochlodinium polykrikoides* or *Rhodomonas lens* for 1 day as a function of algal concentration.

Fig. 7. *Acartia tonsa*. Egg production rates (mean ± SD) when fed *Cochlodinium polykrikoides* as a function of exposure time.

Fig. 8. *Acartia tonsa*. Egg hatching success (mean ± SD) when fed *Cochlodinium polykrikoides* as a function of exposure time.

Fig. 9. *Acartia tonsa*. Egg sizes (mean ± SD) when fed either *Cochlodinium polykrikoides* or *Rhodomonas lens* as a function of exposure time.

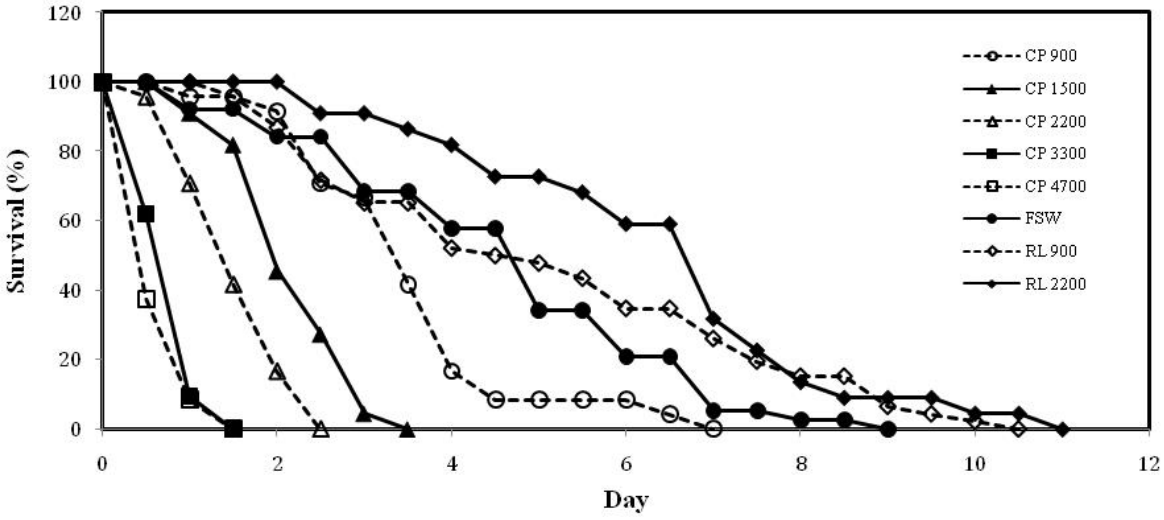
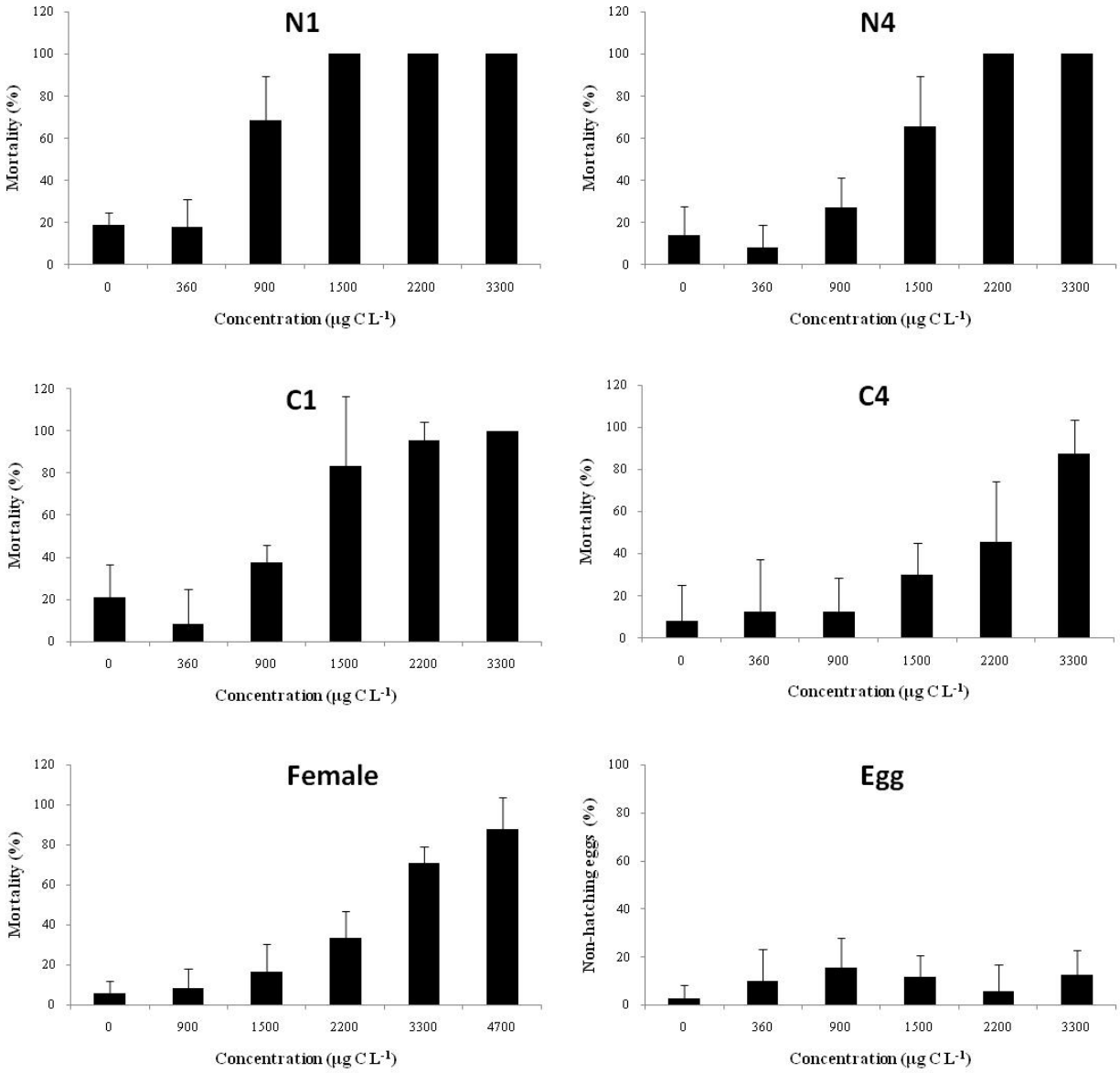


Figure 1 (final size)

2577



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2580 Figure 2 (final size)

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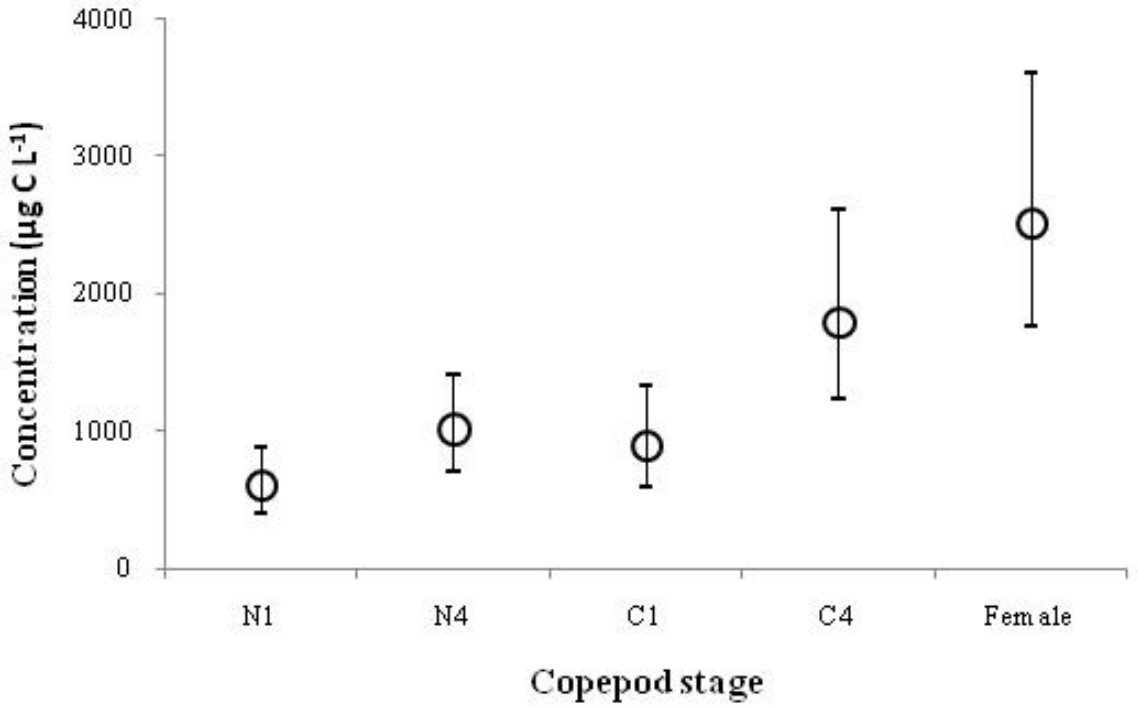
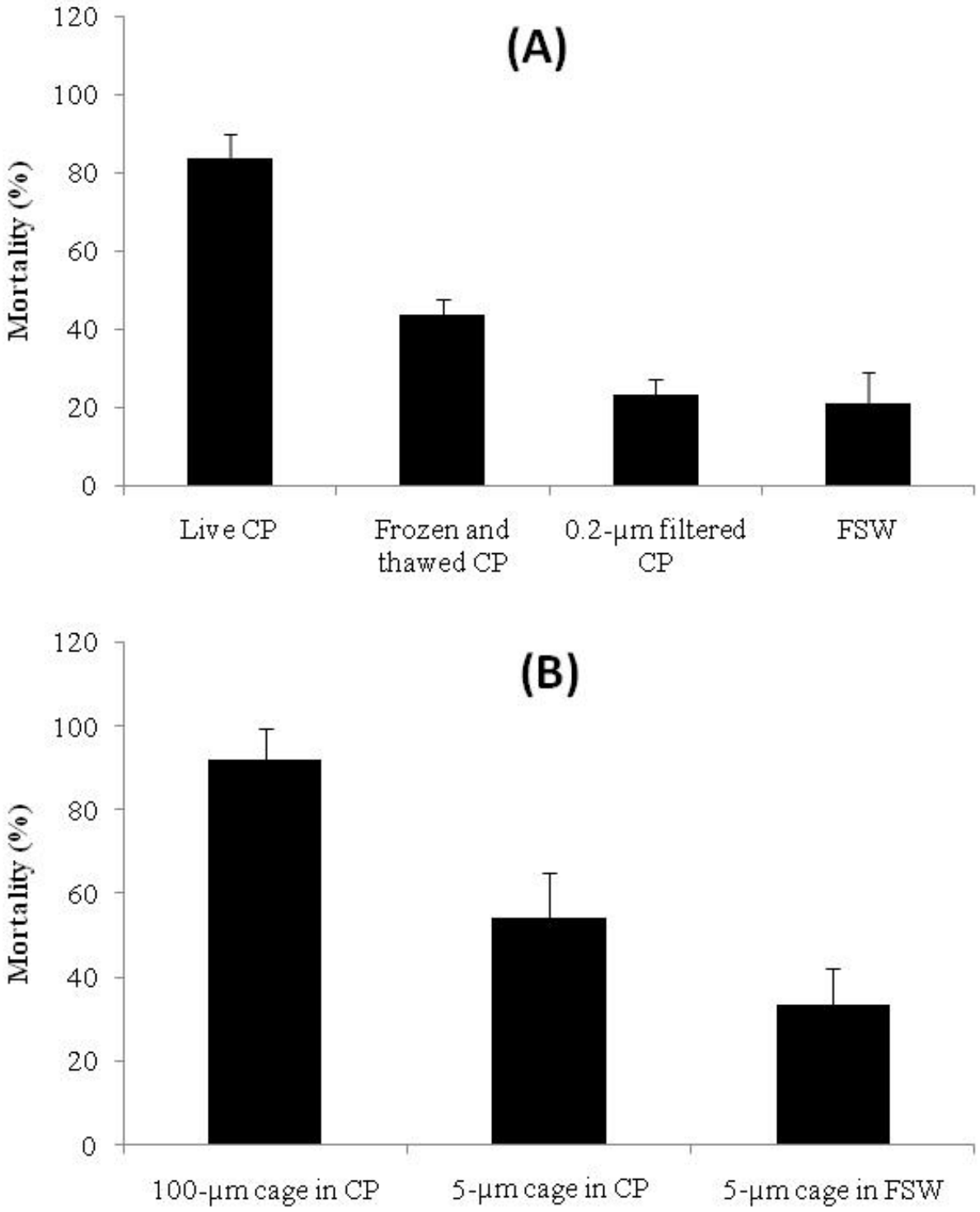


Figure 3 (double final size)



2587

2588 Figure 4 (double final size)

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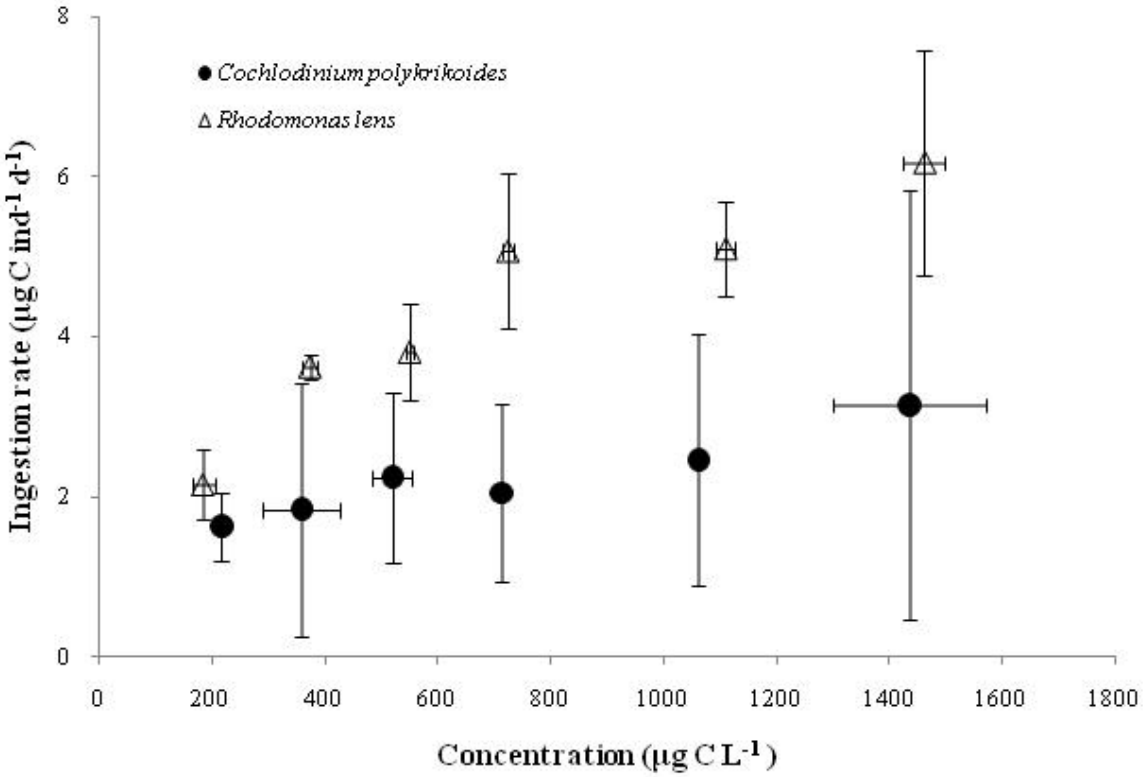

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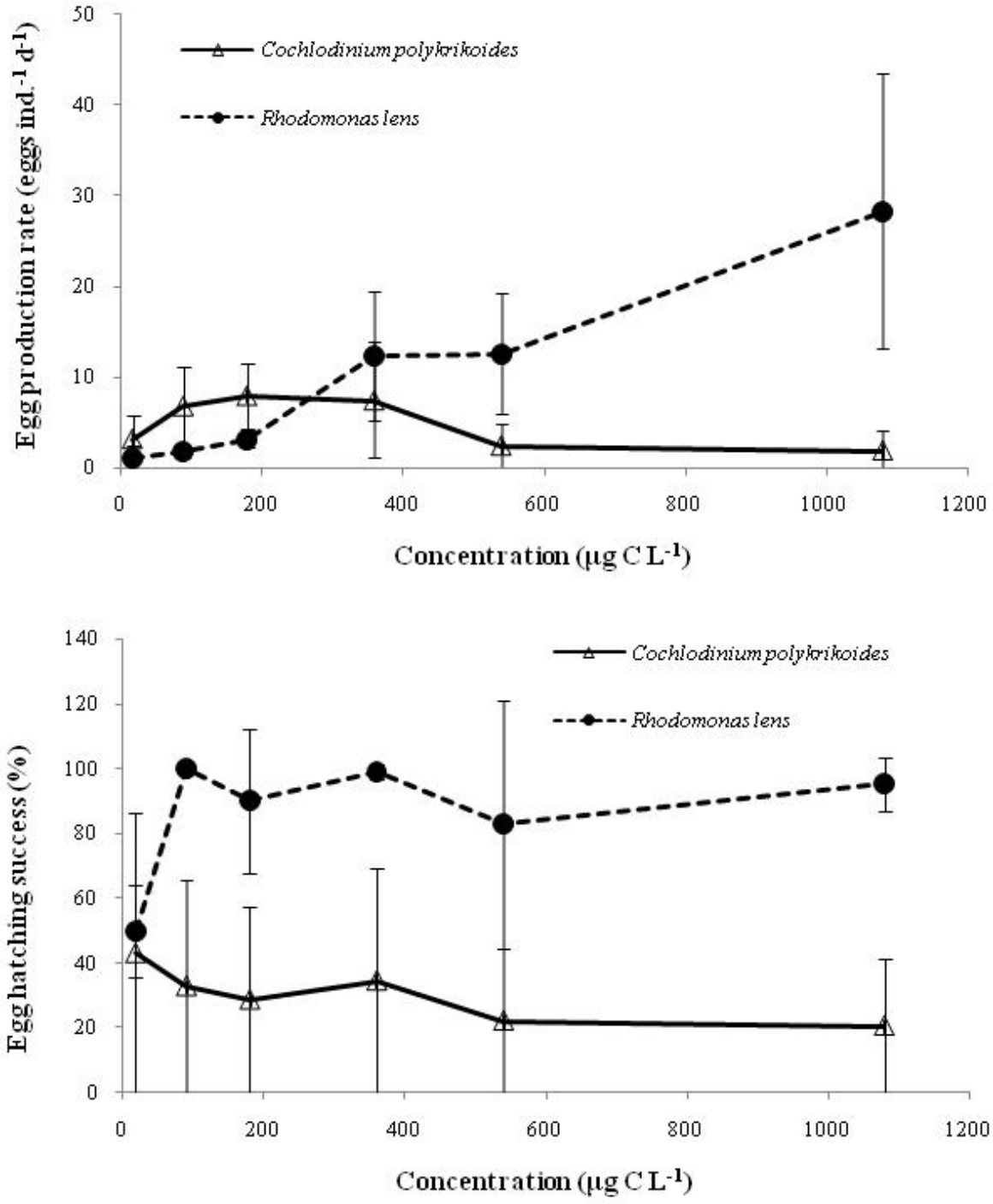

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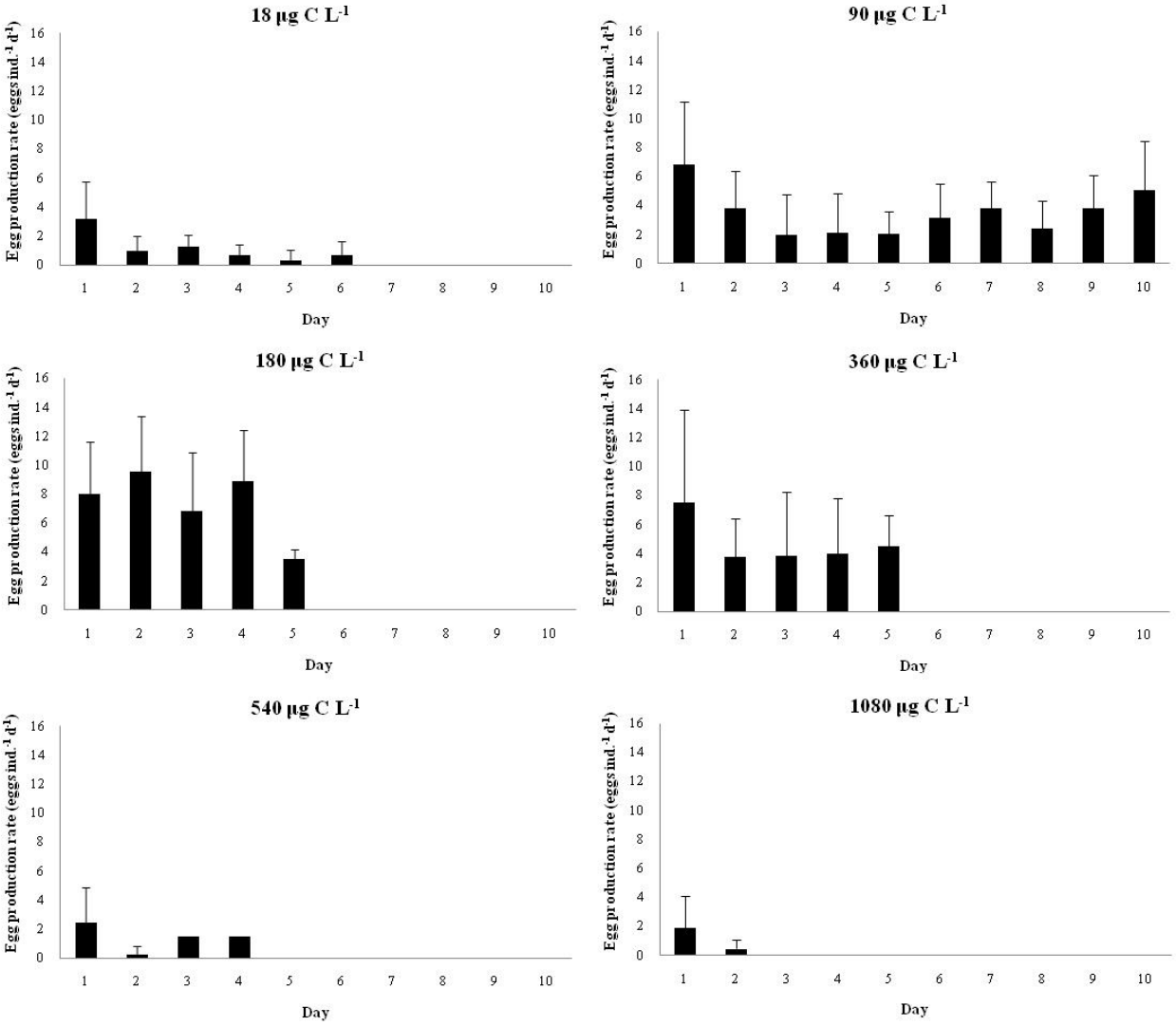

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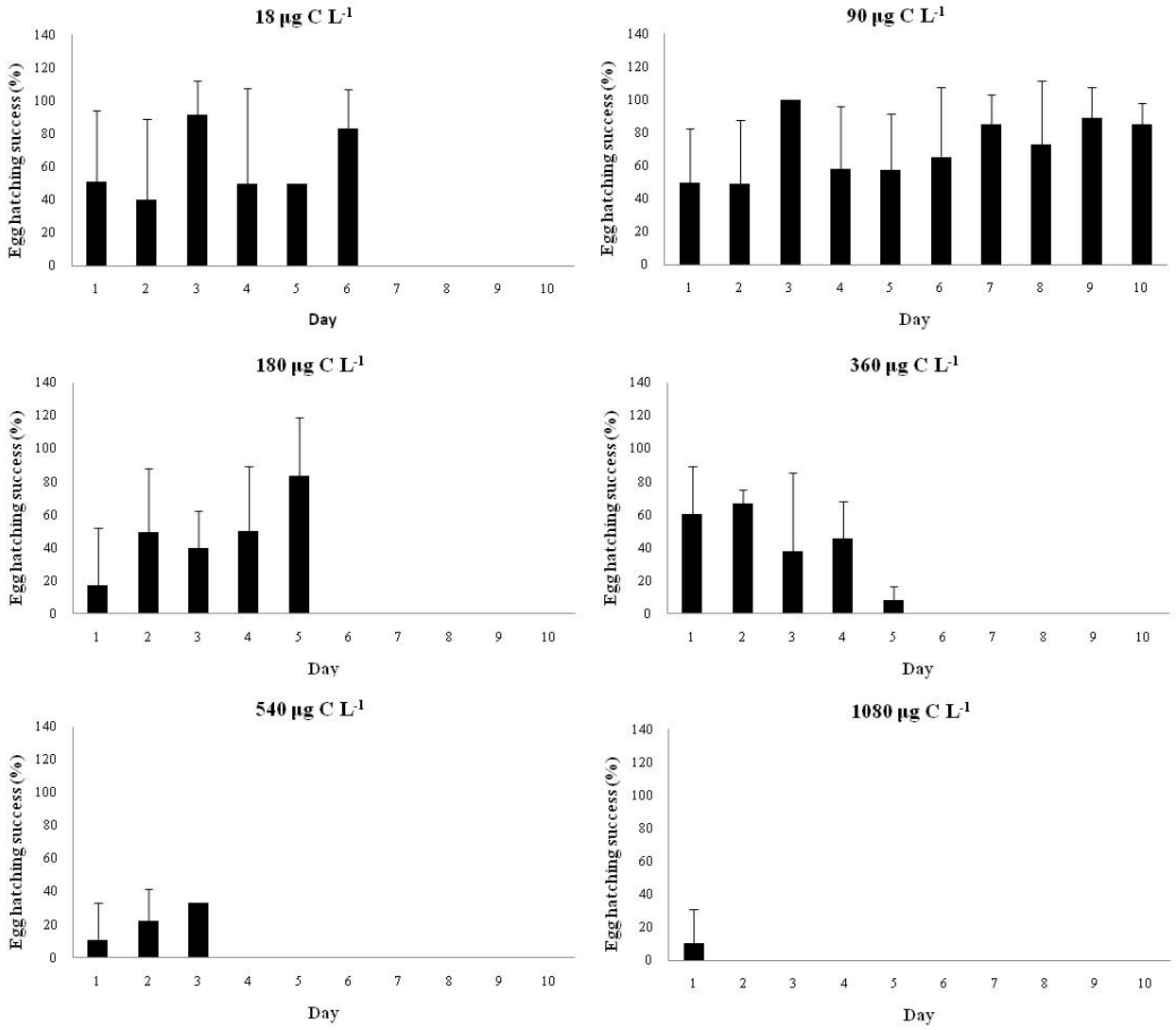

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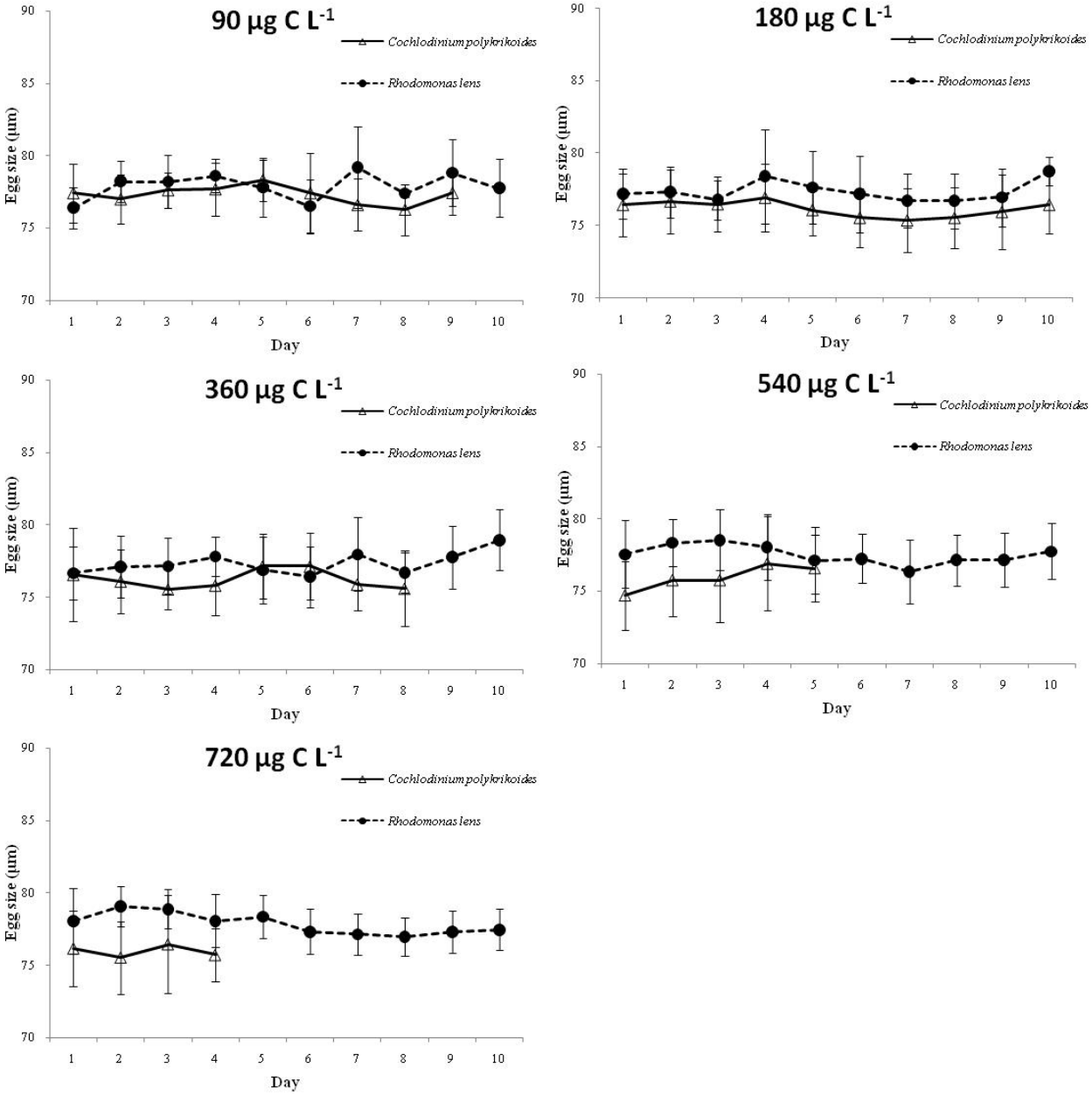

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Chapter seven: Density-dependent nutritional value of the dinoflagellate *Cochlodinium polykrikoides* to the copepod *Acartia tonsa*

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Running head:

C. polykrikoides nutritional value

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Abstract

The nutritional value of the dinoflagellate *Cochlodinium polykrikoides* to the copepod *Acartia tonsa* was evaluated over a range of ecologically-relevant cell densities. Based on egg production rate, egg hatching success, and naupliar recruitment rate of *A. tonsa*, mixed-diet experiments indicated *C. polykrikoides* was nutritionally insufficient or had no nutritional value to *A. tonsa* at 600 μg C L^{-1} (330 cells mL^{-1}), and was toxic at 1000 μg C L^{-1} (550 cells mL^{-1}) when compared with non-toxic flagellate *Rhodomonas lens*. However, the nutritional value of *C. polykrikoides* to *A. tonsa* at 100 and 200 μg C L^{-1} (55 and 110 cells mL^{-1}) was greater than or equal to *R. lens*. The density-dependent nutritional value of *C. polykrikoides* to *A. tonsa* was also demonstrated in the long-term survival experiments. Survivorship of *A. tonsa* fed *C. polykrikoides* was lower than those fed *R. lens* at 900 and 1800 μg C L^{-1}. In contrast, *C. polykrikoides* supported higher survivorship of *A. tonsa* than *R. lens* at 180 and 540 μg C L^{-1}. The nutritional value of *C. polykrikoides* to *A. tonsa* decreased from beneficial to deleterious with increasing cell density. A putatively 'harmful' alga is not always deleterious to grazers and its ecological effects may be distinctly different during bloom and non-bloom periods.

Introduction

One long-standing ecological question in aquatic sciences is why a major fraction of dense phytoplankton blooms in aquatic environments, generally dominated by diatoms or dinoflagellates, is ungrazed and sinks out of the euphotic zone (Litchman and Klausmeier 2008). The low grazing pressure on these blooms has been attributed to the inability of herbivore populations, mainly copepods, to take advantage of the blooms due to the latter's long development time, ranging from weeks to months (Mauchline 1998), relative to fast algal reproductive rates. An alternative explanation is that predation, especially cannibalism, constrains the cohort size of copepods (Ohman and Hirche 2001). On the other hand, the nutrition hypothesis argues that not all algae are good food sources due to nutritional inadequacies, morphological defenses, and/or chemical defenses. These traits are known to depress herbivore feeding and negatively affect herbivore fitness (Miralto et al. 1999; Prince et al. 2006). The nutritional value of algae is usually considered species-specific and varies greatly in terms of digestion resistance, biochemical composition, and toxin production (Sterner and Schulz 1998). Some genera of phytoplankton such as *Rhodomonas*, *Chlamydomonas*, and *Scenedesmus* are typically considered as high-quality food sources for zooplankton (Sterner and Schulz 1998; Koski et al. 2008). Many biochemical components in algae including certain vitamins, amino acids, and fatty acids are nutritionally important for zooplankton success (Jónasdóttir 1994). Element imbalances can reduce phytoplankton quality and limit zooplankton growth (Litchman and Klausmeier 2008). Incomplete digestion, possibly due to thickened cell walls or increased extracellular mucilage, can also contribute to the low quality of some algae (Sterner and Schulz 1998). When algae produce toxins, grazers are often deleteriously affected due to impaired feeding, physiological dysfunction, depressed growth and reproduction, and

reduced population fitness (Landsberg 2002; Prince et al. 2006). Therefore, the negative effects of algae on zooplankton may be explained by both the absence of essential nutrients and the presence of toxins. A major challenge in understanding the nutritional ecology of zooplankton is separating potential toxic effects of prey from their nutritional inadequacy (Colin and Dam 2002).

The mixed-diet technique has been developed to discern whether a given phytoplankton species is beneficial, nutritionally inadequate, or toxic to grazers (Jónasdóttir et al. 1998). This approach is based on the premise that grazer responses, such as clearance rate, egg production rate, and egg hatching success, are linearly related to the proportion of good and poor prey in a mixed diet. Grazers are offered sole diets of the suspected prey (the treatment), a well-known good prey (the control), and mixed diets. A reference line is drawn connecting the responses of the grazer feeding on the 100% suspect and 100% control prey. If the responses of the grazer with the suspected prey are higher than or similar to values with the control prey, the suspect prey is likely to be a nutritionally beneficial food. If the responses of the grazer fed the suspected prey are lower than values with the control prey, deleterious effects due to either toxicity or nutritional insufficiency are suggested. If the responses of the grazers with mixed diets fall along the reference line, the suspect prey has no nutritional value since the responses of the grazers are entirely determined by the dilution of the control prey. If the grazer responses fed mixed diets fall above the reference line, the suspect prey has some nutritional value. And, if the values of the grazer with mixed diets fall below the reference line, the suspect prey is toxic because it detracts from the beneficial effects of the control prey.

Using mixed-diet experiments, Colin and Dam (2002) investigated whether several algae that had been previously reported to have harmful effects on grazers were in fact toxic to the copepod

2700 *Acartia tonsa*. The experiments performed at a concentration of 250 μg C L^{-1} indicated only a
2701 highly toxic *Alexandrium* sp. strain was toxic to female *A. tonsa* and other algae (low toxicity
2702 *Alexandrium* sp. strain, *Heterosigma carterae*, *Thalassiosira rotula*, and *Phaeodactylum*
2703 *tricornutum*) could not be considered toxic (Colin and Dam 2002). The red tide dinoflagellate
2704 *Karenia brevis* is usually considered to be toxic, but mixed-diet experiments at a single food
2705 concentration showed it was only nutritionally inadequate for *A. tonsa* (Prince et al. 2006;
2706 Speekmann et al. 2006). Although the diatoms *P. tricornutum* and *T. rotula* produce
2707 polyunsaturated aldehydes (PUA), mixed-diet experiments at 240 μg C L^{-1} showed that *P.*
2708 *tricornutum* did not have any effects on the copepod *Temora longicornis* and *T. rotula* had a
2709 beneficial effect (Koski et al. 2008). Variability between results of mixed-diet experiments and
2710 previous reports may not only reflect differences among copepod species, but also imply that
2711 using a single food concentration in experiments does not adequately reflect the nutritional value
2712 of an alga to zooplankton.
2713 The dinoflagellate *Cochlodinium polykrikoides* Margalef has formed dense blooms and
2714 caused severe fish kills in Southeast Asia and North America during the past two decades
2715 (Gobler et al. 2008). *C. polykrikoides* negatively affects marine algae (Tang and Gobler 2010),
2716 copepods (Jiang et al. 2009), shellfish, and fish (Tang and Gobler 2009). However, the precise
2717 mode of toxicity in this species has not been completely resolved. The production of reactive
2718 oxygen species (ROS) is likely the main mode of toxicity to marine organisms (Kim et al. 1999;
2719 Tang and Gobler 2009). Cytotoxic agents and mucus substances may also contribute to the
2720 deleterious effects of *C. polykrikoides* (Kim et al. 2002).
2721 In this study we used the mixed-diet approach to determine whether *Cochlodinium*
2722 *polykrikoides* was beneficial, nutritionally insufficient, or toxic to the calanoid copepod *Acartia*

tonsa. *A. tonsa* is an abundant species in many neritic and estuarine environments. The copepod is common in US estuaries where *C. polykrikoides* blooms occur, and is capable of consuming *C. polykrikoides* (Jiang et al. 2009). Based on egg production rate, egg hatching success, and naupliar recruitment rate of *A. tonsa*, mixed-diet experiments showed that the nutritional value of *C. polykrikoides* ranged from beneficial to deleterious with increasing cell density. Long-term survival experiments also supported this conclusion. This density-dependent nutritional quality provides new insights into the ecological effects of putatively harmful algae and their bloom dynamics, and may explain some aspects of plant-herbivore interactions, in general.

Methods

Collection and culture of organisms----The dinoflagellate *Cochlodinium polykrikoides* clone CP1 was isolated from Peconic Bay, Long Island, New York, United States of America in 2006. The flagellate *Rhodomonas lens* Pascher and Ruttner (CCMP 739) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton. The cultures were maintained in a temperature-controlled incubator at 20°C with a 14 h light:10 h dark cycle (approximately 50 μmol photons m^{-2} s^{-1}). The cultures were maintained in exponential growth phase by biweekly dilution with autoclaved f/2 medium prepared with 0.2-μm filtered seawater (FSW, salinity 30). The carbon contents of *C. polykrikoides* and *R. lens* were 1816 and 39.5 pg $cell^{-1}$, respectively (Jiang et al. 2009).

The copepod *Acartia tonsa* was collected from Stony Brook Harbor, New York, with a 202-μm mesh plankton net. The population was continuously maintained in 20-L tanks at 20℃ with a 12 h light:12 dark regime. Copepods were offered *Rhodomonas lens* at a near saturating

concentration of 500 μg C L^{-1} (Mauchline 1998) every day. Half of the seawater in the copepod culture was replaced with fresh FSW twice a week.

Mixed-diet experiments----The experiments were performed at 4 concentrations of total algal carbon: 100, 200, 600, and 1000 μg C L^{-1}. The corresponding densities of *Cochlodinium polykrikoides* were 55, 110, 330, and 550 cells mL^{-1}, which represented their densities from initiation to the development of blooms in the natural environment (Gobler et al. 2008). For each concentration, the carbon fractions of *C. polykrikoides* in diets were nominally 100%, 75%, 50%, 25%, and 0%. Each experimental algal suspension was prepared by diluting algal cultures at the concentration of approximately 1800 μg C L^{-1} (1000 cells mL^{-1} for *C. polykrikoides* and 45600 cells mL^{-1} for *Rhodomonas lens*) with FSW. On day 0, 150 *Acartia tonsa* adults were isolated from culture and kept in a 2-L beaker containing sole diets of *C. polykrikoides* or *R. lens*, or mixed diets. Approximately 80% of the algal suspension was changed daily. Although *C. polykrikoides* has been reported as a mixotrophic alga when fed picoplankton (Jeong et al. 2004), our initial study with 50% *C. polykrikoides* and 50% *R. lens* at 600 μg C L^{-1} showed that *C. polykrikoides* did not feed on *R. lens* since the ratio of two species did not significantly change after 24 h (paired two sample *t*-test, $t = 1.4887$, df = 3, $p = 0.2333$, authors' unpubl. data). Another feeding experiment of *A. tonsa* with 50% *C. polykrikoides* and 50% *R. lens* at 600 μg C L^{-1} showed that *A. tonsa* did not selectively feed on *R. lens* (paired two sample *t*-test, $t = 1.4910$, df = 3, $p = 0.2327$, authors' unpubl. data).

Copepod performances were assessed by 3 functional responses: egg production rate, egg hatching success, and naupliar recruitment rate. Egg production rate (eggs $female^{-1}$ d^{-1}) and egg hatching success (%) of *Acartia tonsa* for each treatment were measured on days 1, 3, and 5. The experiments at 1000 μg C L^{-1} did not persist beyond 3 d due to the massive mortality of *A. tonsa*

in the 100% *Cochlodinium polykrikoides* treatment. Two healthy females were transferred into 5 to 7 replicated glass dishes filled with 50 mL of algal suspension. A 202-μm mesh was fixed above the bottom to minimize egg cannibalism. All eggs and nauplii were quantified after 24 h. Eggs were then incubated in 1-mL wells of a multi-depression dish filled with FSW. The dishes were contained within a closed plastic box with distilled water added to the bottom of the box to minimize evaporation from the wells. Eggs were examined daily for 2 to 3 d. All experiments in this study were performed at 20°C with a 12 h light:12 h dark cycle. The irradiance level was approximately 1 μmol photons m^{-2} s^{-1} to minimize the potential effects of light on copepods and algal growth during experiments. Naupliar recruitment rate (nauplii $female^{-1}$ d^{-1}) was calculated by multiplication of egg production rate and the proportion of hatched eggs.

Survival experiment----A 10-d experiment was carried out to compare survivorship of *Acartia tonsa* when fed *Cochlodinium polykrikoides* and *Rhodomonas lens* at 4 carbon concentrations ranging from 180 to 1800 μg C L^{-1} (100 to 1000 cells mL^{-1} for *C. polykrikoides*). Approximately 300 *A. tonsa* females were transferred into a 2-L beaker and acclimated in FSW for 24 h. For each treatment 16 to 44 healthy females were transferred individually into 15-mL wells of 6-well tissue culture plates. Each well was filled with 1 female and 13 mL of the experimental algal suspension. The copepods were examined and 80% of the algal suspension was refreshed daily.

Statistical analyses----Egg production rate, egg hatching success, and naupliar recruitment rate on 5 diet treatments were compared by one-way ANOVA for each carbon concentration and exposure time, respectively. Multiple comparisons among the fractions were made using the Tukey post hoc test for equal sample sizes or the Gabriel post hoc test for slightly unequal sample sizes. The original data was transformed to meet the assumptions of ANOVA when

2791 necessary. A linear regression line and 95% confidence limits were set for the responses of
2792 copepods fed 100% *Cochlodinium polykrikoides* and 100% *Rhodomonas lens* when their means
2793 were significantly different. The linear regression line was treated as the reference line in mixed-
2794 diet experiments. *T*-tests were used to compare differences between the predicted means from the
2795 reference line and the observed means on each mixed diet. The overall difference between the
2796 observed data on 3 mixed diets and the reference line was compared using Fisher's procedure of
2797 combining probabilities from 3 independent *t*-tests. Survivorship curves of copepods when fed
2798 two algae were compared using the Gehan-Wilcoxon test. Statistical analyses were conducted
2799 using SPSS 16.0 statistical package.
2800
2801 **Results**
2802 When *Acartia tonsa* females were fed 100% *Cochlodinium polykrikoides* at 100 μg C L^{-1},
2803 egg production rates on days 1, 3, 5 and naupliar recruitment rates on days 1, 3 were significantly
2804 higher than the control *Rhodomonas lens* (Fig. 1, one-way ANOVA with post hoc tests, $p < 0.05$
2805 for all). Similarly, copepod responses when fed 100% *C. polykrikoides* at 200 μg C L^{-1} were
2806 significantly improved (e.g., egg production rates on days 3, 5 and naupliar recruitment rate on
2807 day 3, Fig. 2, one-way ANOVA with post hoc tests, $p < 0.05$ for all). In contrast, all responses of
2808 *A. tonsa* when fed 100% *C. polykrikoides* at 600 μg C L^{-1} were significantly reduced compared
2809 with the controls (Fig. 3, one-way ANOVA with post hoc tests, $p < 0.05$ for all) except egg
2810 production rate and naupliar recruitment rate on day 1. The overall egg production rates of *A.*
2811 *tonsa* when fed mixed diets on day 3, egg hatching success on day 5, and naupliar recruitments
2812 rates on days 3, 5 were significantly above the reference lines connecting the two monoculture
2813 diets at 600 μg C L^{-1} (Fig. 3, Fisher's procedure of combining probabilities, df = 6, $p < 0.05$ for

all), while the overall egg production rate on day 5 and egg hatching success on days 1, 3 were not (Fisher's procedure of combining probabilities, df = 6, $p > 0.05$). Furthermore, all egg production and naupliar recruitment rates with mixed diets at 1000 μg C L^{-1} were significantly below the references lines (Fig. 4, Fisher's procedure of combining probabilities, df = 6, $p < 0.01$ for all), except for the egg hatching success on day 1 which was significantly above the reference line (Fisher's procedure of combining probabilities, df = 6, $p < 0.01$).

The nutritional value of the dinoflagellate *Cochlodinium polykrikoides* to the copepod *Acartia tonsa* decreased from beneficial to deleterious with increasing *C. polykrikoides* concentration (Table 1). The nutritional value of *C. polykrikoides* was more beneficial than or equal to *Rhodomonas lens* at 100 and 200 μg C L^{-1}. In contrast, *C. polykrikoides* was nutritionally inadequate or had no nutritional value to *A. tonsa* relative to *R. lens* at 600 μg C L^{-1}. The nutritional value of *C. polykrikoides* to *A. tonsa* became toxic at 1000 μg C L^{-1}.

Survivorship of *Acartia tonsa* females when fed *Cochlodinium polykrikoides* was significantly higher than *Rhodomonas lens* at 180 μg C L^{-1} (Fig. 5; Gehan-Wilcoxon test, df = 1, $p < 0.05$) and 540 μg C L^{-1} (Fig. 5; Gehan-Wilcoxon test, df = 1, $p < 0.01$). In contrast, survivorship of *A. tonsa* when fed *C. polykrikoides* was significantly lower than *R. lens* at 900 μg C L^{-1} (Fig. 5; Gehan-Wilcoxon test, df = 1, $p < 0.05$) and 1800 μg C L^{-1} (Fig. 5; Gehan-Wilcoxon test, df = 1, $p < 0.001$).

Discussion

Ecological significance of density-dependent nutritional value---- Our results showed *Cochlodinium polykrikoides* had variable nutritional effects on the copepod *Acartia tonsa* over concentrations ranging from 100 to 1000 μg C L^{-1}. Contrary to expectation, *C. polykrikoides*,

2837 which has been reported as a harmful red-tide alga (Gobler et al. 2008; Tang and Gobler 2009,
2838 2010), was more beneficial to *A. tonsa* than the flagellate *Rhodomonas lens* at low
2839 concentrations. Harmful algae are typically considered universally deleterious to target
2840 organisms (Landsberg 2002), even though harmful effects often vary with growth stage,
2841 inorganic nutrients, organic matter, temperature, salinity, light, and grazers (Granéli and Flynn
2842 2006). Our results, however, clearly showed that *C. polykrikoides* was a nutritious alga for
2843 grazers at low densities, which challenges the traditional view on harmful algae. On the other
2844 hand, *C. polykrikoides* was toxic to *A. tonsa* at the highest concentration of 1000 μg C L^{-1} (550
2845 cells mL^{-1}). Jiang et al. (2009) found that survivorship of *A. tonsa* females was significantly
2846 reduced when fed *C. polykrikoides* monocultures at high concentrations ($\geq$ 900 μg C L^{-1}, 500
2847 cells mL^{-1}) compared to copepods starved in filtered seawater. These results along with our
2848 current findings using mixed diets indicate the deleterious mode of this alga is related to cellular
2849 toxicity rather than nutritional insufficiency. There is no doubt that *C. polykrikoides* is highly
2850 deleterious to a variety of marine organisms at high densities, but its effects on ecosystems at
2851 low densities are likely different than those observed at high densities.

2852 This study demonstrates that the ecological effects of putatively harmful algae in natural
2853 systems can be density-denpendent. Typical densities of *C. polykrikoides* in US eastern coast
2854 waters during blooms have been > 10^3 cells mL^{-1}, frequently exceeding 10^4 cells mL^{-1}, with
2855 bloom events persisting for approximately one month during late summer (Gobler et al. 2008).
2856 Harmful effects of *C. polykrikoides* on copepods may only occur at high densities during blooms.
2857 In contrast, the alga may serve as a good nutritional resource and support copepod production
2858 when its cell densities are low. Such density-dependent nutritional value may shed light on the
2859 controversy regarding the interaction between diatoms and copepods (Miralto et al. 1999).

Diatoms, which were traditionally considered an ideal food sources for copepods, have been reported to cause impaired recruitment of copepods, especially when fed high concentrations of diatoms (Miralto et al. 1999). The nutritional inadequacy hypothesis argues that reduced egg production or hatching of copepods fed diatoms was due to the deficiency in some mineral or lipid (Jones and Flynn 2005). The toxicity hypothesis states that the negative effects on copepods were specifically related to the production of polyunsaturated aldehydes (PUA) by diatoms (Miralto et al. 1999). One important but often overlooked factor, cell density, may contribute to the diatom-copepod controversy. Most laboratory experiments and some field observations (Miralto et al. 1999) showing harmful effects on zooplankton were conducted at high diatom concentrations. In the context of our results, we hypothesize that a density-dependent nutritional value of diatoms may account for the observed discrepancies of diatom-copepod interactions.

Density-dependent nutritional quality of algae may provide some insights into the formation of monospecific *Cochlodinium polykrikoides* blooms. The maximum growth rate of *C. polykrikoides* is approximately 0.4 d^{-1} (Kim et al. 2004), which is comparable to some dinoflagellates but slower than most diatoms and flagellates (Smayda 1997). Hence, killing zooplankton during early stages of bloom development would be a dangerous strategy for *C. polykrikoides* since this would facilitate the dominance of fast growing competitors within algal community (Flynn 2008). Although supporting grazers at low densities of *C. polykrikoides* would depress its populations, grazers also control the population size of fast-growing algae. Several attributes of *C. polykrikoides*, such as mixotrophy (Jeong et al. 2004), allelopathy (Tang and Gobler 2010), and resistance to algicidal bacteria (Imai and Kimura 2008), may elevate its population density and facilitate bloom formation. Once a bloom population with high cell densities is established, *C. polykrikoides* gains advantages with competing algae and subsequent

killing grazers would benefit *C. polykrikoides*, particularly since its allopathic effects on competing algae are also maximal under elevated cell densities (Tang and Gobler 2010). Thus, the harmful effects of *C. polykrikoides* to grazers do not contribute to the bloom initiation, but become increasing important as blooms develop and likely contribute towards bloom maintenance as its nutritional value switches from beneficial to deleterious with increasing cell density. In addition to the support from our empirical study, the competition-predation hypothesis is also consistent with model simulations of algal blooms, which indicate that the ability of an alga to kill a generalist zooplankton predator can only be considered advantageous when the alga has strong competitive advantages with regard to substrate affinity and/or maximum growth rates (Flynn 2008). Testing the competition-predation hypothesis, in combination with some important factors in trophic interactions (e.g., grazing deterrence, nutrient regeneration by zooplankton) and other traditional hypotheses (e.g., nutrient-uptake adaptations, allelopathy, and turbulence effects, Smayda 1997; Flynn 2008), will enable us better understand bloom formation of slow-growing dinoflagellates, such as *C. polykrikoides*.

Proposing the competition-predation hypothesis puts forward a new question regarding how *Cochlodinium polykrikoides* cells at low densities avoid being completely decimated by grazers before they gain a window of opportunity for bloom formation. Our preliminary observations suggest that *C. polykrikoides* cells can detect the presence of grazers and increase cell chain length (authors' unpubl. data). Chain formation in *C. polykrikoides* could be an effective defense by creating predator-prey size mismatch. Additionally, since the swimming speeds of the dinoflagellates *Gymnodinium catenatum* and *Alexandrium affine* increased by 1.5 times from single cells to chains of 4 cells (Fraga et al. 1989), chain formation in *C. polykrikoides* could increase motility and subsequent escape ability. Thus, induced chain formation by grazers may

help *C. polykrikoides* to avoid grazing even when their nutritional value is beneficial to zooplankton at low densities.

Possible mechanisms of density-dependent nutritional value----The mechanism(s) of density-dependent nutritional quality of *Cochlodinium polykrikoides* is not clear. The nutritional value of phytoplankton indicated by zooplankton performances is an overall balance between positive factors (e.g., nutritional compounds such as fatty acids), and negative factors (e.g., toxins). Production of fatty acids and toxins (harmful compounds) by microalgae is greatly variable, even on a daily or hourly scale (Sterner and Schulz 1998; Granéli and Flynn 2006). Toxin production in some dinoflagellates is positively related to cell density (Granéli and Flynn 2006). *C. polykrikoides* cells should have had equally nutritional value at the beginning of the experiments since they were diluted from the same culture at approximately 1000 cells mL^{-1}. After the dilutions, *C. polykrikoides* cells may respond to density changes and thus production of fatty acids and harmful compounds may change with cell densities and/or growth rate, although this likelihood is small given the slow growth of this species and the short duration between transfers (24 h). A more plausible explanation is that zooplankton responses to harmful compounds are dose dependent. *C. polykrikoides* would not have deleterious effects on *Acartia tonsa* when cell concentrations are below a threshold value. We hypothesize that the amount of nutritional components in *C. polykrikoides* probably exceeds *Rhodomonas lens* and thus zooplankton perform better when fed *C. polykrikoides* at low concentrations. Jónasdóttir (1994) reported that egg production of the copepods *A. tonsa* and *A. hudsonica* was positively correlated with some specific fatty acids [20:5(n-3), 22:6(n-3), and 18:0]. The relative concentrations of fatty acids 20:5(n-3) and 18:0 to total fatty acids were 17.5% and 2.1% in *C. polykrikoides* (Dorantes-Aranda et al. 2009), which were higher than those in *R. lens* (Jónasdóttir 1994). These two fatty

2929 acids may contribute to the high nutritional value of *C. polykrikoides* for *A. tonsa* at lower cell
2930 densities. On the other hand, deleterious effects on zooplankton occur when *C. polykrikoides*
2931 concentration exceeds a critical level and further increase with increasing cell concentrations.
2932 The potential modes of toxicity in *C. polykrikoides* include the production of reactive oxygen
2933 species (ROS, Kim et al. 1999; Tang and Gobler 2009) and the production of mucus
2934 polysaccharides (Kim et al. 2002), both of which are extracellular and would increase in total
2935 toxicity with increasing cell densities. Hence, the nutritional value of some algae, such as *C.*
2936 *polykrikoides*, which is inherently nutritious but also produces harmful compounds, may
2937 frequently range from beneficial to deleterious with increasing cell density. Given the known
2938 variations in production of fatty acids, toxins, or harmful compounds among algal clones and
2939 species (Granéli and Flynn 2006), one must take care in extrapolating prior chemical
2940 composition data of algae to the present study. Investigations of production of these compounds
2941 by both *C. polykrikoides* and other algae at different cell densities with respect to the
2942 physiological responses of copepods are expected to provide more insight regarding the
2943 mechanisms influencing the density-dependent nutritional value of phytoplankton.
2944 Some zooplankton exposed to recurrent HABs can rapidly evolve and adapt to toxic algae
2945 (Colin and Dam 2004). The copepods used in this study came from Stony Brook Harbor, Long
2946 Island Sound, where no *Cochlodinium polykrikoides* blooms have been observed. Thus, it is
2947 unlikely that these copepods have evolved resistance to any putative *C. polykrikoides* toxins.
2948 Some copepods compensate for low food quality by increasing the quantity of food consumed
2949 (Mauchline 1998). Although ingestion rates (μg C copepod^{-1} d^{-1}) of *Acartia tonsa* on *C.*
2950 *polykrikoides* were reduced 40 – 60% relative to *Rhodomonas lens* when food concentrations
2951 increased from 350 to 1500 μg C L^{-1}, there was no significant difference in ingestion rates

between the two diets at approximately 200 μg C L^{-1} (Jiang et al. 2009). Thus, the high quality of *C. polykrikoides* to *A. tonsa* relative to *R. lens* at low concentrations was not due to higher ingestion rates.

Interpreting mixed-diet experiments----Our findings regarding the density-dependent nutritional value of phytoplankton suggests that algal food quality should be assessed using multiple concentrations. Previous studies using mixed-diet experiments (Colin and Dam 2002; Prince et al. 2006; Speekmann et al. 2006) were performed only at a single food concentration. The results showed that several toxic algae were nutritionally insufficient for copepods (Colin and Dam 2002; Prince et al. 2006; Speekmann et al. 2006). However, similar experiments performed under a wide range of environmentally-realistic prey densities may alter this conclusion. Usually, a food-limiting concentration is used in mixed-diet experiments (Jónasdóttir et al. 1998; Colin and Dam 2002), which is appropriate when toxins are intracellular and grazer performances are influenced by how much of the toxin they ingest. Within this context, the experimental concentrations for algae with intracellular toxins must be below the feeding saturation point. However, the modes of toxicity for many harmful algae depend on extracellular toxins, exudates, or cell surface contact (Landsberg 2002). In these cases, the toxic reactions of grazers are influenced by the concentration of the toxic algae in the environment and not by how many toxic algae they ingest. Thus, the experimental abundances of these algae would not be limited by the feeding saturation point. As discussed above, the toxic modes of *C. polykrikoides* are extracellular reactions (Kim et al. 1999; Kim et al. 2002; Tang and Gobler 2009). Even in the case of intracellular toxins, if toxin production is density-dependent (Granéli and Flynn 2006), the concentrations above the feeding saturation point should also be examined. Toxin amounts ingested by grazers could differ, even if ingestion rates were constant.

2975 Our results indicated that egg hatching success was a less sensitive indicator of the nutritional
2976 condition of copepods than egg production rates in mixed-diet experiments. Egg production rates
2977 of *Acartia tonsa* fed *Cochlodinium polykrikoides* were higher than when fed *Rhodomonas lens* at
2978 the concentration of 100 μg C L^{-1}, while the differences in hatching success were not significant.
2979 Also, the toxic effects of *C. polykrikoides* at 1000 μg C L^{-1} were more evident for egg production
2980 rates than egg hatching success. Furthermore, egg hatching success of *A. tonsa* was more
2981 variable than egg production rates in our experiments. Some extremely low values of egg
2982 hatching were observed when copepods were fed mixed diets. In some cases, egg hatching
2983 success was not statistically reliable because low egg production did not provide adequate
2984 sample numbers for hatching experiments. Alternatively, events, such as unfertilized eggs, may
2985 influence hatching results. Copepod behavioral changes including prey switching when feeding
2986 on mixed diets (Mauchline 1998) may also contribute to higher variation in the responses of *A.*
2987 *tonsa* compared to mono-specific diets.
2988 The size, motility, and quality of prey all influence copepod feeding (Berggreen et al. 1988;
2989 Mauchline 1998). The equivalent spherical diameters (ESD) of *Cochlodinium polykrikoides* and
2990 *Rhodomonas lens* are 28.2 and 7.97 μm (Jiang et al. 2009). The optimal particle size for feeding
2991 by *Acartia tonsa* females is about 15 μm (Berggreen et al. 1988). Clearance rates of *A. tonsa*
2992 females were nearly equal when fed on the flagellate *R. baltica* (ESD: 6.91 μm) and the
2993 dinoflagellate *Scripsiella faröense* (ESD: 19.0 μm, Berggreen et al., 1988). Thus, the effects of
2994 prey size on feeding of *A. tonsa* should have been minimal since the two algae used in the
2995 present study were very similar in size to *R. baltica* and *S. faröense* (Berggreen et al. 1988).
2996 Although copepods may actively select for particular prey (Mauchline 1998), we did not observe
2997 significant prey selection by *A. tonsa* when fed 50% *C. polykrikoides* and 50% *R. lens* at 600 μg

2998 C L^{-1} (authors' unpubl. data). Given that prey selection was only examined within this treatment,
2999 care should be taken when extrapolating this result to all treatments.
3000 Our results may challenge the traditional view that harmful algae are chronically deleterious
3001 to ecosystems. The nutritional value of the red tide dinoflagellate *Cochlodinium polykrikoides* to
3002 the copepod *Acartia tonsa* ranged from beneficial to deleterious with increasing cell densities.
3003 Therefore, the ecological roles of *C. polykrikoides* during bloom and non-bloom periods may be
3004 distinctly different. Density-dependent nutritional quality also suggests that supporting grazers
3005 may benefit slow-growing *C. polykrikoides* at low densities since grazers may keep fast-growing
3006 algae in check. Once *C. polykrikoides* gains a competitive advantage at high concentrations, its
3007 effect on grazers may then switch to deleterious, which leads to monospecific blooms. Testing
3008 our results under field conditions is expected to bring more insights into the complexity of such
3009 planktonic interactions.
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Tang, Y., and C. J. Gobler. 2010. Allelopathic effects of clonal isolates and blooms of the harmful dinoflagellate *Cochlodinium polykrikoides* on competing phytoplankton. Mar. Ecol. Prog. Ser. (in press)

3091 Table 1. Nutritional value of *Cochlodinium polykrikoides* to *Acartia tonsa* inferred from the
3092 mixed-diet experiments with *Rhodomonas lens*. EPR: egg production rate; EHS: egg hatching
3093 success; NRR: naupliar recruitment rate; + +: more beneficial; +: equal beneficial; -: nutritional
3094 insufficient; --: no nutrition; ---: toxic

Concentrations (μg C L^{-1})	Time (day)	EPR	EHS	NRR
100	1	+ +	+	+ +
	3	+ +	+	+ +
	5	+ +	+	+
200	1	+	+	+
	3	+ +	+	+ +
	5	+ +	+	+
600	1	+	--	+
	3	-	--	-
	5	--	-	-
1000	1	---	-	---
	3	---		---

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Figure Legends

Fig. 1. Performances of *Acartia tonsa* vs. the percent carbon of *Rhodomonas lens* at the total carbon concentration of 100 μg C L^{-1}. The linear regression line (solid line) and 95% confidence limits (dotted line) are set for the performance with 0% and 100% *R. lens* when they are significantly different.

Fig. 2. Performances of *Acartia tonsa* vs. the percent carbon of *Rhodomonas lens* at the total carbon concentration of 200 μg C L^{-1}. Regression line and 95% confidence limits as Fig. 1.

Fig. 3. Performances of *Acartia tonsa* vs. the percent carbon of *Rhodomonas lens* at the total carbon concentration of 600 μg C L^{-1}. Regression line and 95% confidence limits as Fig. 1; Significant differences between the observed means on mixed diets and the predicted means from the regression line are indicated by asterisks (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) and ns (not significant).

Fig. 4. Performances of *Acartia tonsa* vs. the percent carbon of *Rhodomonas lens* at the total carbon concentration of 1000 μg C L^{-1}. Regression line and 95% confidence limits as Fig. 1; Statistical symbols as Fig. 3.

Fig. 5. Survivorship of *Acartia tonsa* when exposed to either *Cochlodinium polykrikoides* or *Rhodomonas lens* at 4 concentrations.

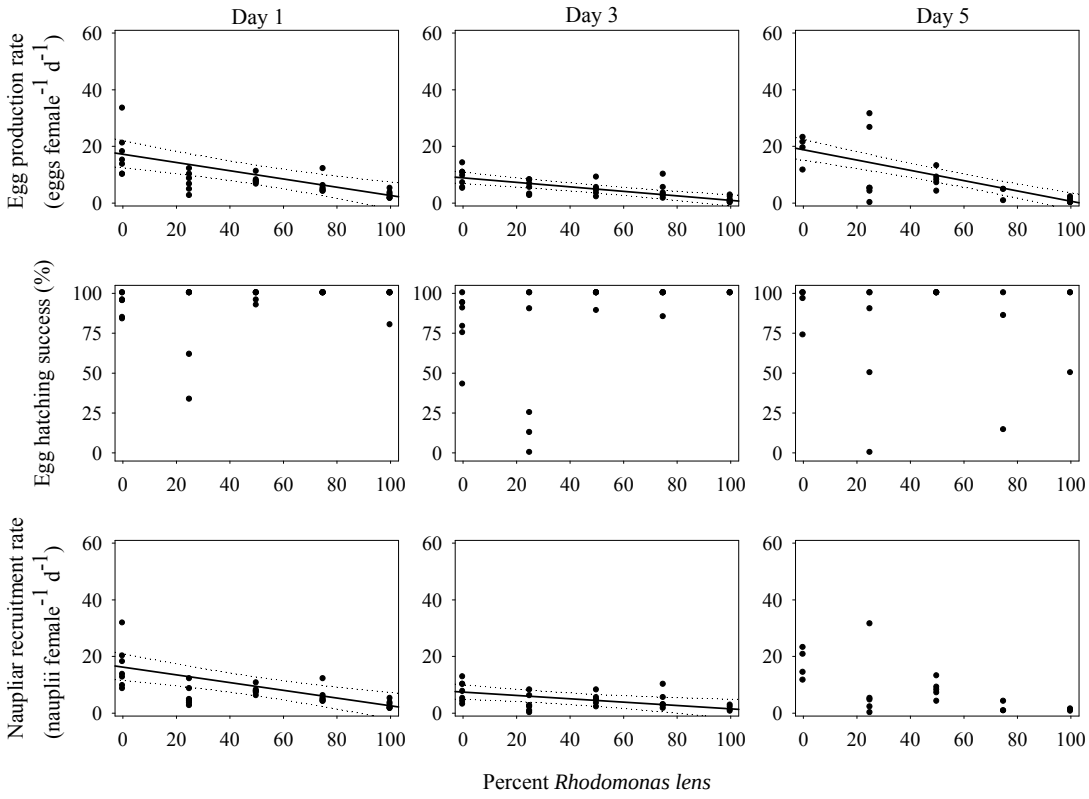

Figure 1 Jiang et al.

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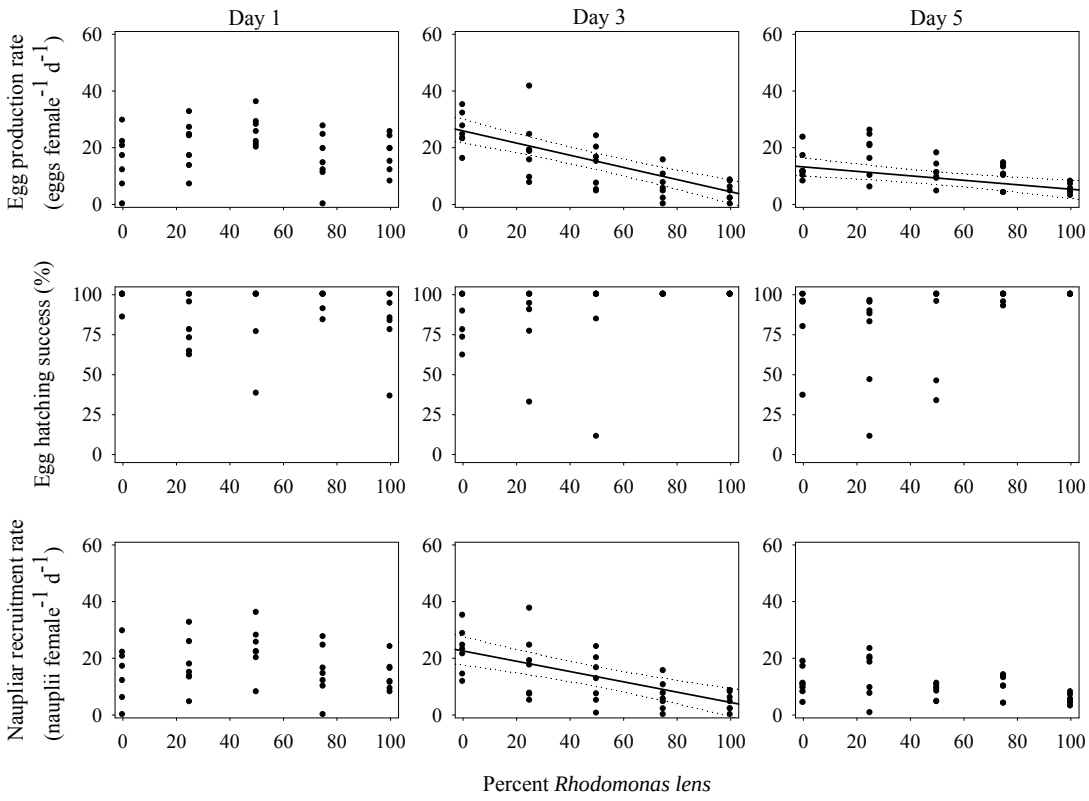

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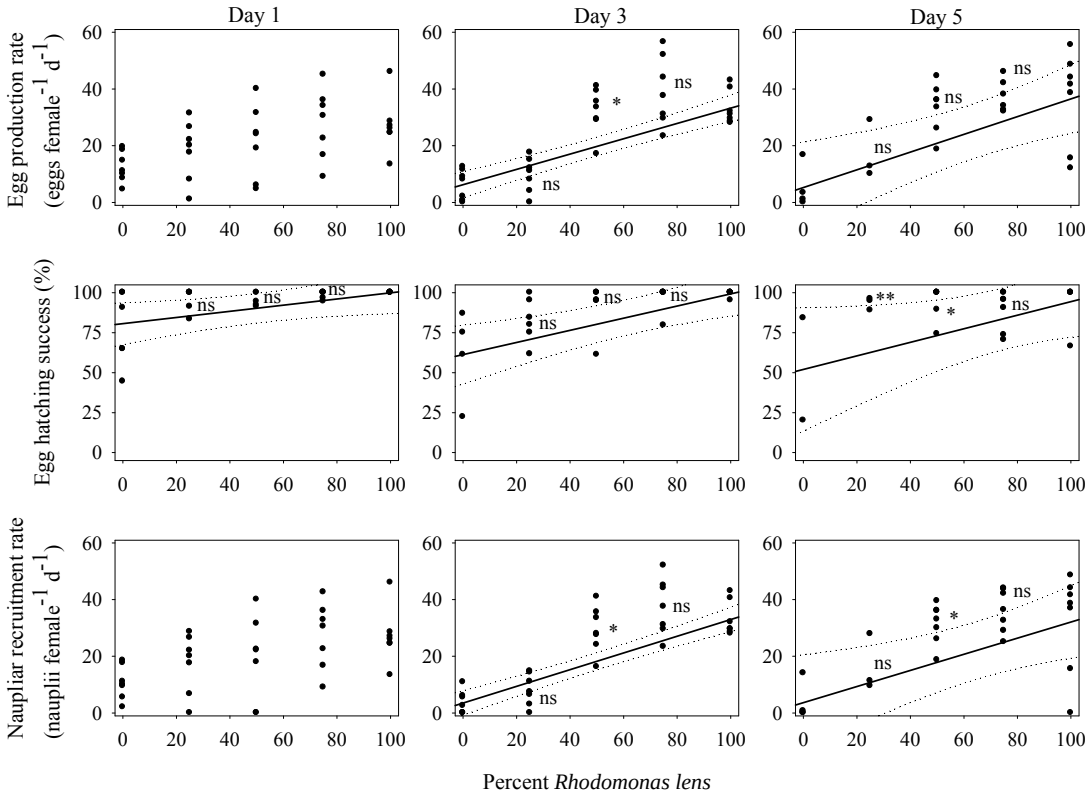

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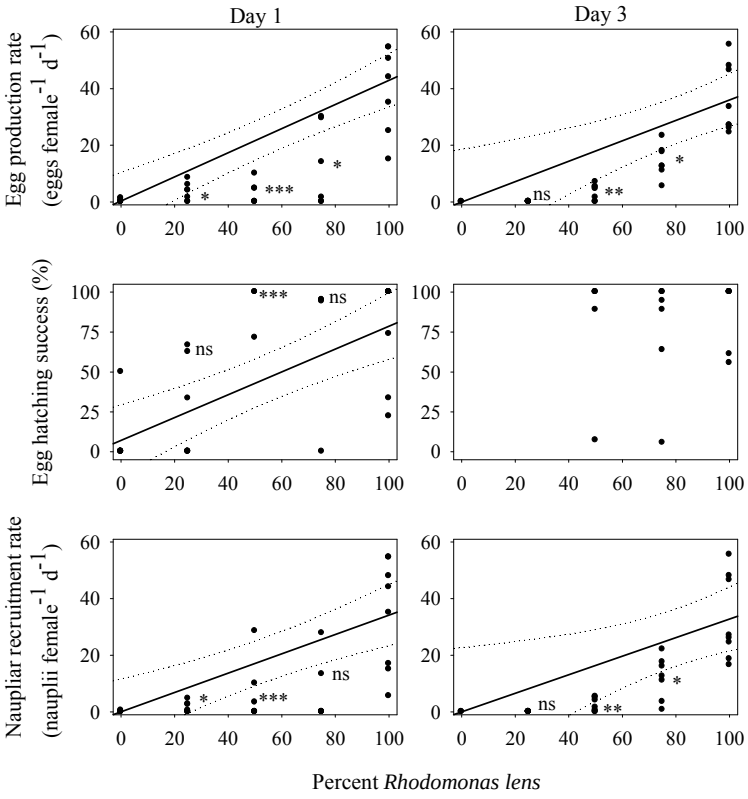

Figure 4 Jiang et al.

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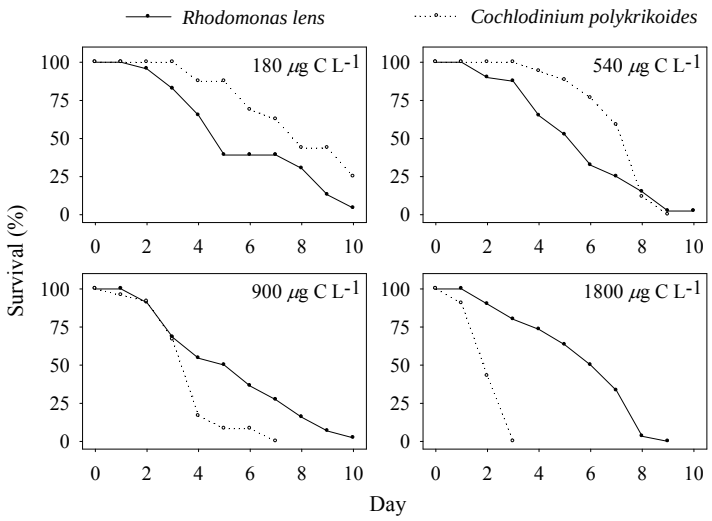

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Chapter eight: Grazers and vitamins shape chain formation in a bloom-forming dinoflagellate, *Cochlodinium polykrikoides*

In preparation for Harmful Algae

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Key words: chain formation, dinoflagellate, grazer, vitamin

ABSTRACT

Predators influence the phenotype of prey through both natural selection and induction. We investigated the roles of grazers and nutrients on chain formation in a dinoflagellate *Cochlodinium polykrikoides*, which has formed dense blooms and caused deleterious effects on marine ecosystems around the world. Field populations of *C. polykrikoides* displayed a significantly larger variation in chain length compared to laboratory cultures. Chain formation in a culture of *C. polykrikoides* was significantly enhanced when exposed for 48 h to adults or fresh (<24 h post-isolation) exudates of the copepod *Acartia tonsa*. In the field, chain length of *C. polykrikoides* was positively correlated to *A. tonsa* abundance. These results suggest that dissolved chemical cues released by *A. tonsa* can induce chain formation in *C. polykrikoides*. In addition, nutrient amendment experiments demonstrated that 3 vitamins (B_1, B_7, and B_{12}) enhanced the chain length of *C. polykrikoides* both singly and collectively, while trace metals and inorganic nutrients did not. Chain formation may be an effective anti-grazing defense by creating predator-prey size mismatch and increasing prey motility, which is especially vital for population persistence of *C. polykrikoides* during bloom initiation.

INTRODUCTION

Almost all species have evolved adaptations for interactions with other species. While prey develop numerous defenses to avoid predation, predators evolve means to breach these defenses resulting in an evolutionary "arms race" of adaptation and counteradaptation (Vermeij 1994, Brodie & Brodie 1999, Agrawal 2001, Smetacek 2001). Selection on prey is often stronger than on predators due to the "life-dinner principle" which argues that it is worse to lose life than to miss a dinner (Brodie & Brodie 1999). Reciprocal adaptations between species regulate population dynamics, shape community structure, affect biogeochemical cycles, and drive genetic diversification (Agrawal 2001, Pohnert et al. 2007, Hay 2009). In aquatic environments, phytoplankton have developed morphological and chemical defenses against predation. Many phytoplankton have protective external structures, such as siliceous or calciferous shells, spines, and horns (Litchman & Klausmeier 2008). Thickened cell walls, increased extracellular mucilage, or fast gut passage of some phytoplankton can lead to incomplete digestion and subsequent imbalances in lipids or unknown compounds important for the reproductive success of herbivores (Dutz et al. 2008). A wide variety of harmful compounds are produced by more than 200 algal species from 20 genera to deter grazing or directly kill herbivores (Landsberg 2002). Compared to constitutive defenses, induced defenses by the presence or action of predators may be an effective way to minimize the cost of defense (Agrawal 2001). Since zooplankton grazing usually varies both on temporal and spatial scales, the evolution of inducible defenses should be favored compared to constitutive defenses. Zooplankton grazing induces more toxin production in some phytoplankton (Jang et al. 2003, Selander et al. 2006). More interestingly, some phytoplankton are induced to release some volatile chemicals once

attacked by zooplankton, which serve as directional cues by predators of zooplankton, such as seabirds, reef fishes, harbor seals, and whale sharks (Hay 2009). The presence of grazers also promotes colony formation in some phytoplankton which reduces grazing pressure due to size mismatch (Lürling 2003, Long et al. 2007).

Colony formation occurs in some species of cyanobacteria, Bacillariophyceae, Charophyceae, Chlorophyceae, Pavlovophyceae and Prymnesiophyceae (Beardall et al. 2009). These colonies are formed by assemblages of fully differentiated and morphologically identical cells of the same genotype. The colony structures are diverse including one-dimensional filaments (chains), two-dimensional mats (plates), or three-dimensional cylinders, spheres, or amorphous structures (Beardall et al. 2009). Hessen & Van Donk (1993) discovered that the cladoceran *Daphnia magna* released dissolved chemicals to stimulate colony formation in *Desmodesmus subspicatus* (formerly known as *Scenedesmus subspicatus*). Such induced colony formations have been mainly documented in freshwater green algae *Scenedesmus* and *Desmodesmus* (Hessen & Van Donk 1993, Lürling & Van Donk 1997, Lürling 2003), and the marine prymnesiophyte *Phaeocystis* (Tang 2003, Long et al. 2007). Signal of colony induction may be non-predator specific. Colony formation in *S. acutus* was evoked by cladocerans, rotifers, and copepods (Lürling & Van Donk 1997). Two protozooplankton species and one copepod grazer all stimulated colony enlargement in *P. globosa*, although the extent of enlargement varied (Tang 2003). A recent study, however, showed that size-specific feeding induced consumer-specific, but opposing, morphological transformations in *P. globosa* (Long et al. 2007). Ciliates that consumed single cells of *P. globosa* enhanced colony formation. In contrast, copepods that fed on colonies suppressed colony formation (Long et al. 2007).

Some species in the Dinophyceae are considered as pseudocolonial organisms relative to the colonial organisms discussed above (Beardall et al. 2009). Dinoflagellates usually possess 2 flagella in a single cell. The transverse flagellum wraps around the equator of the cell in the cingulum and serves to push and spin the cell in the water as it swims. The longitudinal flagellum trails behind in the sulcus and acts as a steering wheel (Hackett et al. 2004). Some species can form chains as a result of a series of incomplete cell divisions, resulting in the total number of flagella not matching the number of nuclei in chains (Beardall et al. 2009). Chains of some dinoflagellates swim faster than individual cells (Fraga et al. 1989), which may increase migration ability and escape success from predation. However, it has not been investigated whether the chain formation in these pseudocolonial organisms can be induced by the presence of grazers as in colonial organisms.

The unarmored gyrodinioid dinoflagellate *Cochlodinium polykrikoides* Margalef has formed dense blooms and caused severe fish kills in Southeast Asia and North American during the past 2 decades (Gobler et al. 2008, Kudela et al. 2008, Lee 2008). *C. polykrikoides* forms chains consisting of 2 or more cells. The cells in a chain are morphologically different. The leading cell possesses a conical epicone and a flattened hypocone. The last cell has a truncated epicone and a conical hypocone. The intermediate cells are more or less compressed longitudinally (Matsuoka et al. 2008). It is possible that chain formation in *C. polykrikoides* serves as an adaptive strategy of grazing defense by creating a predator-prey size mismatch. The calanoid copepod *Acartia tonsa* Dana is an abundant species in many estuarine environments where *C. polykrikoides* blooms occur. *C. polykrikoides* produces harmful compounds to reduce copepod feeding and even kill copepods at high cell densities (Jiang et al. 2009). Further study has showed that the nutritional value of *C. polykrikoides* to *A. tonsa* ranged from beneficial to deleterious with

increasing cell density (authors' unpubl. data). The aim of the present study was to test the hypothesis that the presence of zooplankton grazers would induce chain formation in *C. polykrikoides*. We compared chain structures in a field population (presence of grazers) and a cultured population (absence of grazers), investigated chain changes following directly adding grazers, and explored the relationship between chain length and grazer abundance. Given the variation of chain length in cultured cells through its natural growth cycle even without grazers, we further tested the hypothesis that some nutrients would influence chain formation in *C. polykrikoides* using nutrition amendment experiments. Resolving factors which influence chain formation provided further insights into *C. polykrikoides* bloom dynamics and improved our understanding on predator-prey interactions.

MATERIALS AND METHODS

Collection and culture of organisms. The dinoflagellate *Cochlodinium polykrikoides* clone CP1 was isolated from Peconic Bay, Long Island, New York, USA in 2006 (Gobler et al. 2008). The culture was grown in f/2 medium under a standard incubation condition (at 20℃ with a light/dark cycle of 14 h/10 h at approximately 50 μmol photons m^{-2} s^{-1}). The copepod *Acartia tonsa* was collected from Stony Brook Harbor, Long Island Sound, NY, with a 202-μm mesh plankton net. The copepod population was continuously cultured in 20-l tanks at a density of 20 to 50 ind. l^{-1}. Copepods were daily offered the flagellate *Rhodomonas lens* Pascher and Ruttner (CCMP 739) at a carbon concentration of approximately 500 μg C l^{-1}.

Field population. Field sampling was conducted every other day in Old Fort Pond, Shinnecock Bay, NY, from August 29 to October 6, 2008. The water depth was approximately 1.5 m and seawater was vertically well-mixed. Whole seawater samples (120 ml) were preserved

in 5% Lugol's iodine for enumeration of *Cochlodinium polykrikoides*. Twenty to 50 l of seawater was filtered onto a 64-µm mesh and preserved in 5% formalin buffered with hexamethylentetramin for determination of *Acartia tonsa* stage-specific abundance. At least 400 *C. polykrikoides* cells and their chain lengths were recorded using a Sedgewick Rafter counting chamber under a compound microscope. At least 100 *A. tonsa* adults and copepodites were counted under a dissecting microscope.

Cultured population. We also examined chain length of *Cochlodinium polykrikoides* in a cultured population. Approximately 50 ml of an exponentially growing *C. polykrikoides* was transferred to a 2-l flask containing 1.5 l of autoclaved f/2 medium. The initial cell density of *C. polykrikoides* was approximately 10 cells ml^{-1}, which represented a cell density prior to a field bloom. The culture was kept at 20℃ under a light/dark cycle of 14 h/10 h at approximately 50 µmol photons m^{-2} s^{-1}. A sample (20 ml) was preserved daily in 5% Lugol's iodine for enumeration of *C. polykrikoides* cells and their chain lengths. Growth rates of *C. polykrikoides* were calculated from daily increases in cell densities using the equation: $r = \ln (C_t/C_{t-1})$, where t is time (d).

Grazer addition experiments. An exponentially growing culture of *Cochlodinium polykrikoides* was diluted with sterile 0.2-µm filtered seawater (FSW) and randomly divided into 4 treatments with 6 replicates. Each replicate had 200 ml of *C. polykrikoides* culture in a 250-ml flask. The cell density of *C. polykrikoides* in the experiment was 100 cells ml^{-1}, which represented a cell density during bloom initiation (Gobler et al. 2008). The first treatment was the control without adding anything. To produce grazer exudates, *Acartia tonsa* females were maintained in sterile FSW at a density of 30 ind. l^{-1} for 24 h. Solution from the incubation was filtered through 0.2-µm GF/F filters and these filtered exudates were stored for 24 h under the

3309 standard incubation condition or administered immediately after filtration. One ml of fresh or
3310 stored exudates was added to flasks twice daily to create the second and third treatments,
3311 respectively. Healthy *A. tonsa* females were added to the fourth set of the treatment flasks at a
3312 density of 30 ind. L^{-1}. After a 48-h incubation of all treatments under the standard condition,
3313 samples (20 ml) from each flask were fixed in 5% Lugol's iodine for examination of chain length
3314 of *C. polykrikoides*.

3315 **Nutrient amendment experiments.** Nutrient amendment experiments were performed to
3316 explore the role of nutrients in chain formation of *Cochlodinium polykrikoides*. An exponentially
3317 growing culture of *C. polykrikoides* was diluted with sterile FSW and randomly divided into 6
3318 treatments with 6 replicates. Each replicate had 200 ml of *C. polykrikoides* culture in a 250-ml
3319 flask with an initial density of 100 cells ml^{-1}. The first treatment was the control without adding
3320 any nutrients. Trace metals, nitrate, phosphate, vitamins, and f/2 working solution were,
3321 respectively, added into other 5 treatments. The composition and concentration of each nutrient
3322 followed the f/2 medium recipe (Guillard 1975). After 48 h, chain length of *C. polykrikoides*
3323 were examined under a compound microscope.

3324 After an increased chain length of *Cochlodinium polykrikoides* was observed in a mixed-
3325 vitamin treatment, we further determined which vitamin(s) contributed to the increased chain
3326 length. An exponentially growing *C. polykrikoides* was diluted by f/2 medium without vitamins
3327 and randomly divided into 8 treatments with 8 replicates. In each replicate, 200 ml of *C.*
3328 *polykrikoides* culture was maintained in a 250-ml flask with a final density of 100 cells ml^{-1}. The
3329 first treatment was control without adding any vitamins. Vitamins B_1, B_7, and B_{12} were added to
3330 other treatments both individually and collectively. The final concentrations of vitamins B_1, B_7,

and B_{12} were 1.00×10^{-4}, 5.00×10^{-7}, and 5.00×10^{-7} g l^{-1}, respectively. Chain length of *C. polykrikoides* was investigated after 48 h.

Data analysis. Chain length data from each sampling were pooled for the field population and the cultured population, respectively, and their frequency distributions were compared using a *G*-test. The means of chain length among treatments in the grazer and nutrient addition experiments were compared by one-way ANOVA followed by a Tukey post hoc test, respectively. Linear regressions were used to explore the relationships of *Cochlodinium polykrikoides* chain length with *Acartia tonsa* abundance and *C. polykrikoides* growth rates, respectively.

RESULTS

Chain formation in the field and cultured populations

A bloom of *Cochlodinium polykrikoides* was observed from the late August to the early October in Old Fort Pond (Fig. 1A). *C. polykrikoides* achieved an initial peak in cell densities (564 to 1120 cells ml^{-1}) in early September. The cell density of *C. polykrikoides* was lower during the second peak (50 to 350 cells ml^{-1}) which occurred from September 20 to October 2. The chain structure of *C. polykrikoides* varied during the field bloom. The chain length of *C. polykrikoides* ranged from 1.15 to 2.30 cells $chain^{-1}$ in the field population (Fig. 1A). Single cells were most abundant, followed by 2 cells, 4 cells, and 3 cells. Chains with 5 – 8 cells were also observed in the field bloom, although their percentages were very small (Fig. 1B). The chain length of *C. polykrikoides* in the field bloom was positively related to the abundance of *Acartia tonsa* adults and copepodites ($r^2 = 0.3580$, $F_{1,17} = 9.479$, $p < 0.01$, Fig. 2).

After inoculation into fresh medium from a stock culture, the density of *Cochlodinium polykrikoides* exponentially increased from 12.5 to 418.8 cells ml^{-1} with a mean net population growth rate of 0.11 d^{-1} (SD ± 0.09, n = 32) during the first 33 d (Fig. 3A). The population entered into a relatively stationary phase on day 34 (Fig. 3A). The maximum density of *C. polykrikoides* was 452.3 cells ml^{-1} on day 45. Cell density varied during the stationary phase, although the mean growth rate was almost zero (0.002 ± 0.076 d^{-1}, n = 26). The chain length of *C. polykrikoides* ranged from 1.05 to 2.10 cells $chain^{-1}$ in the cultured population (Fig. 3A). The chain structure of *C. polykrikoides* switched from 2 cells to single cells as cell growth rates were reduced zero. The 4-cell type occurred during the first 35 d and diminished thereafter. The chains with 5 – 8 cells were not observed in the cultured population (Fig. 3B). The growth rate of *C. polykrikoides* was positively correlated to the chain length in the cultured population (r^2 = 0.2611, $F_{1,57}$ = 20.14, $p < 0.001$, Fig. 4).

The chain structure of *Cochlodinium polykrikoides* in the field bloom was significantly different from the culture bloom (*G*-test, G_7 = 7490, $p < 0.001$). The percentages of 1 and 2 cells of *C. polykrikoides* were higher in the cultured population than in the field population. In contrast, the percentages of other chain types (>2 cells) were lower in the cultured population than in the field population (Fig. 5). The mean chain length of *C. polykrikoides* in the cultured and field populations was 1.32 and 1.88 cells $chain^{-1}$, respectively.

Grazer and nutrient addition experiments

The exposure to adult females or fresh exudates (0-h storage) of *Acartia tonsa* significantly enhanced the chain length of *Cochlodinium polykrikoides* from 1.44 (SD ± 0.038) to 1.59 (SD ± 0.044) and 1.60 (SD ± 0.027) cells $chain^{-1}$, respectively (Tukey post hoc test, $p < 0.001$ for both, Fig. 6A). In contrast, the stale *A. tonsa* exudates after the 24-h storage did not significantly

increase the chain length of *C. polykrikoides* (Tukey post hoc test, $p = 0.163$). None of the additions of trace metals, nitrate, and phosphate significantly enhanced the chain length of *C. polykrikoides* (Tukey post hoc test, $p > 0.05$ for all, Fig. 6B). However, the additions of vitamins either solely or with other nutrients (f/2 treatment) significantly increased the chain length of *C. polykrikoides* from 1.46 (SD ± 0.058) to 1.60 (SD ± 0.050) and 1.61 (SD ± 0.028) cells chain^{-1}, respectively (Tukey post hoc test, $p < 0.001$ for both). The chain length of *C. polykrikoides* was significantly increased after adding vitamins B_1, B_7, and B_{12} both singly and collectively (Tukey post hoc test, $p < 0.05$ for all, Fig. 6C). The chain length of *C. polykrikoides* in the B_1+B_{12} treatment was significantly higher than these in the B_1+B_7 and B_7+B_{12} treatments (Tukey post hoc test, $p < 0.05$ for both), but not significantly differ from other vitamin treatments (Tukey post hoc test, $p > 0.05$ for all).

DISCUSSION

Benefits and costs of chain formation

Our results showed that the presence of grazers induced chain formation in *Cochlodinium polykrikoides*. Chain length of *C. polykrikoides* was significantly increased when exposed to females and fresh exudates of the copepod *Acartia tonsa* (Fig. 6A). In addition, the mean chain length of *C. polykrikoides* in the wild population facing chronic predation pressure was 42% higher than that in the cultured population without predators. Furthermore, the chain length of *C. polykrikoides* in the wild population was positively correlated to the grazer abundance (Fig. 2). Induced chain formation is likely an effective defense against grazing for *C. polykrikoides*. The mean length of *C. polykrikoides* chains was 44.9 μm for the cultured population and 63.9 μm for

3399 the field populations (Fig. 7). In the laboratory, exposure to *A. tonsa* females and fresh *A. tonsa*
3400 water for 2 d enhanced the mean chain length from 48.9 to 54.4 μm. The optimal particle size for
3401 feeding by *A. tonsa* females is 14.8 μm and the relationship between clearance rates and food
3402 size is a bell-shape, decreasing markedly from this optimal size (Fig. 7, Berggreen et al. 1988).
3403 Thus, copepod grazing on *C. polykrikoides* chains with 4 cells may almost completely be
3404 depressed (Fig. 7). Chain formation in *C. polykrikoides* would substantially reduce clearance rate
3405 of *A. tonsa*.

3406 In addition to creating a size mismatch, induced chain formation in *C. polykrikoides* could
3407 also lessen feeding pressure via behavioral changes. Predation risk is mainly determined by the
3408 rate of encounter between predator and prey and the probability of successful prey escape. Many
3409 motile microplankton can perceive fluid deformation generated by predators and may escape
3410 typical zooplankton feeding currents (Titelman 2001). Successful prey escape depends on their
3411 remote detection and motility (Titelman and Kiørboe 2001, Kiørboe et al. 2009). While the high
3412 motility behavior of *A. tonsa* nauplii enhanced encounter rates with a larger copepod predator
3413 (adult *Centropages typicus*) by increasing the velocity difference between predator and prey, its
3414 escape success was approximately 10-fold that of *Temora longicornis* nauplii with low motility.
3415 The combined consequence was the predation risk on *T. longicornis* nauplii was approximately
3416 3-fold that of *A. tonsa* nauplii (Titelman 2001). The swimming speeds of two dinoflagellates
3417 *Gymnodinium catenatum* and *Alexandrium affine* increased by 1.5 times from a single cell to a
3418 chain of 4 cells (Fraga et al. 1989). Therefore, enhanced swimming speeds of *C. polykrikoides*
3419 due to chain formation may also increase its escape ability and reduce predation risk, although it
3420 may also increase encounter rate with predator.

3421 The morphological plasticity induced by the presence of zooplankton predators in
3422 *Cochlodinium polykrikoides* was relatively low compared to *Scenedesmus* (Lürling & Van Donk
3423 1997), *Desmodesmus* (Hessen & Van Donk, 1993), and *Phaeocystis* (Tang 2003).
3424 Dinoflagellates are usually considered as pseudocolonial organisms due to their incomplete cell
3425 division (Beardall et al. 2009). More investigations on induced chain formation among
3426 dinoflagellates are needed to test whether the relatively low morphological plasticity is a
3427 characteristic for pseudocolonial organisms when compared to colonial organism.
3428 Since the resources which may be allocated to all traits are limited, defenses against
3429 predation may lead to a fitness cost in other traits (Litchman & Klausmeier 2008). Although a
3430 trade-off between colony formation and growth has often been proposed for phytoplankton
3431 (Agrawal 1998), this expected negative relationship has not been observed in many
3432 phytoplankton (Lürling & Van Donk 1997, 2000, Tang et al. 2008). In fact, colony formation has
3433 been related to higher growth rates in some species (Veldhuis et al. 2005, Takabayashi et al.
3434 2006). A positive relationship between chain length and growth rate was observed in
3435 *Cochlodinium polykrikoides* cultures indicating that, when nutrients are saturating, other factors
3436 may counteract any growth cost due to chain formation in *C. polykrikoides*. The swimming speed
3437 of *C. polykrikoides* may be improved when switching from single cells to chains (Fraga et al.
3438 1989). In the field, higher motility likely enables *C. polykrikoides* to actively migrate and seek
3439 resources, such as light and nutrients. It is possible, however, that chain formation in *C.*
3440 *polykrikoides* may lead to reduced growth in an ecosystem setting when nutrient concentrations
3441 are lower. One possible cost of chain formation in *C. polykrikoides* may be a lowered specific
3442 light absorption coefficient for pigment molecules due to the "package effect". Theory and
3443 observation have showed that photon absorption per unit pigment can be higher in single cells

than in colonies (Beardall et al. 2009). Another possibility is the increased diffusive limitation of nutrients. The thickness of a diffusion boundary layer around all objects in a fluid medium is positively related to the size of objects. When the concentrations of nutrients are low, diffusive limitation is more likely in colonies than in single cells (Beardall et al. 2009). Finally, chain formation in *C. polykrikoides* may increase the risk of infection with pathogens, which may easily spread from 1 cell to others in a chain. These possible costs of chain formation may impose an evolutionary constraint on responses to natural selection favoring chain formation in *C. polykrikoides*. This constraint may partially explain the dominance of 1 and 2-cell chains and the low degree of the morphological plasticity of *C. polykrikoides*.

The principle of economy of design implies that unused structures of organisms may be reduced and lost since it is costly to develop and maintain them (Agrawal 2001). The strain CP1 of *Cochlodinium polykrikoides* has been maintained in the laboratory without grazing pressure for 3 years after the isolation. Thus, the costs of the chain formation may outweigh the benefits in *C. polykrikoides* cultures. Our observation supported the theoretical predication. The mean chain length of *C. polykrikoides* in the cultured population was 42% lower than in the field population. The natural population had more long chains (>2 cells) than the culture of *C. polykrikoides*. Similarly, the maximal chain lengths of *Skeletonema costatum* were lower in the batch culture than in the natural population (Takabayashi et al. 2006).

Effects of grazers and nutrients on chain formation

The presence of grazers can induce chain formation in *Cochlodinium polykrikoides*. Physical contact with grazers was not necessary for the induction since the fresh *Acartia tonsa* exudates induced chain formation as well as live *A. tonsa* females. Hence, it seems that *C. polykrikoides* detects the chemical cues of potential grazers and initiates chain formation, a putative defense

system. Success of induced plasticity is dependent on the predictability of a changing environment (Agrawal 2001). Zooplankton grazing varies greatly with time and space due to their heterogeneous distribution and composition. Degradable chemical cues are more likely to reflect the real-time risk of grazing. The stale *A. tonsa* exudates after the 24-h storage did not induce chain formation in *C. polykrikoides*. A similar phenomenon was observed in a *Desmodesmus – Daphnia* interaction (Lürling & Van Donk 1997). These results suggest that the chemical cues released by aquatic grazers are not persistent. The rapid degradation ensures the reliability of the chemical cues since they reflect the actual not the past risk of grazing.

Even without the grazer effect, the chain structure of *Cochlodinium polykrikoides* varied in the cultured population as a function of growth stages, with chains being almost completely absence in the stationary growth stage. This variation implied that nutrients might influence chain formation. Nutrient addition experiments indicated that chain formation of *C. polykrikoides* was stimulated by vitamins and not by inorganic nutrients or trace metals. Further experiments demonstrated that vitamins B_1, B_7, B_{12} were each capable of increasing the chain length of *C. polykrikoides*. B vitamins are involved in multiple biochemical pathways and serve as enzyme cofactors and antioxidants in algal metabolism (Croft et al. 2006). A compilation of 306 species reveals that >50% algae require B_{12}, while 22% required B_1 and 5% required B_7 to grow (Croft et al. 2006). We have found *C. polykrikoide*s had an absolute requirement for B_{12} and B_1 for growth (C. J. Gobler unpubl. data). Trace amounts of these vitamins in natural waters can influence phytoplankton productivity, succession, and their interactions with other organisms (Sañudo-Wilhelmy et al. 2006, Gobler et al. 2007). To our knowledge, the present study is the first report on the role of vitamins in algal chain formation. While the mechanism by which B vitamins facilitate chain formation in *C. polykrikoides* is unknown, B vitamins may function as

cofactors for enzymes involved in this process. Given the universal response to all vitamins and the well known heterotrophic nature of dinoflagellates (Hackett et al. 2004), B vitamins may also serve as an organic carbon source to *C. polykrikoides* and thus enhancing its cellular carbon supply, irrespective of photosynthesis. Regardless of the mechanism, these results demonstrate that while grazers can induce chain formation, an ample supply of B vitamins also stimulated chain formation in *C. polykrikoides*.

Ecological significance of chain formation

Induced chain formation by grazers may influence the population dynamics of *Cochlodinium polykrikoides*. The nutritional value of *C. polykrikoides* to *Acartia tonsa* ranged from beneficial to deleterious with increasing concentration from 100 to 1,000 μg C l^{-1} (authors' unpubl. data). In view of this density-dependent nutritional value, we have proposed that slow-growing *C. polykrikoides* might be palatable to predators when their densities are low, but kill predators when they obtain advantages over competitors at high densities. An unresolved question is how *C. polykrikoides* can avoid being completely grazed down at low densities prior to bloom formation. Induced chain formation by grazers provides *C. polykrikoides* with a morphological defense against grazing, irrespective of cell densities. Although *C. polykrikoides* cells are nutritionally beneficial to copepods at low densities, copepods cannot over-grazed them due to their ability to form chains. Thus, the induced chain formation is especially important for the persistence of *C. polykrikoides* prior to bloom formation.

CONCLUSIONS

Understanding the adaptations of species to interactions with other species is an important goal of ecology and the study of evolution. Although the dinoflagellate *Cochlodinium polykrikoides* forms chains with multiple cells in both the field and cultured populations, the

field population displayed a greater morphological plasticity. Grazer addition experiments and the positive relationship between the chain length and grazer abundance in the field suggest that the presence of grazers could induce the chain formation in *C. polykrikoides*. The chemical cues of *Acartia tonsa* were water soluble and degradable, which may ensure the reliability of the chemical cues since they reflect the actual not the past risk of grazing. In addition, chain formation in *C. polykrikoides* was also enhanced by B vitamins. Chain formation in *C. polykrikoides* may serve as a morphological defense by creating a predator-prey size mismatch and enhancing cellular motility. This ecological strategy assists *C. polykrikoides* cells to avoid being completely grazed during non-bloom periods.

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Figure Legends

Fig. 1 *Cochlodinium polykrikoides*. Chain length, cell density, and chain structure in Old Fort Pond, Shinnecock Bay, NY, in 2008.

Fig. 2 *Cochlodinium polykrikoides*. Relationship between chain length and abundance of *Acartia tonsa* adults and copepodites in Old Fort Pond, Shinnecock Bay, NY, in 2008. The linear regression (solid line) and 95% confidence limits (dotted lines)

Fig. 3 *Cochlodinium polykrikoides*. Chain length, cell density, and chain structure during a 60-d incubation of an isolated culture.

Fig. 4 *Cochlodinium polykrikoides*. Relationship between chain length and growth rate in the cultured population. Linear regression and 95% confidence limits as Fig. 2

Fig. 5 *Cochlodinium polykrikoides*. Frequency distribution of chain structure in the field and cultured populations.

Fig. 6 *Cochlodinium polykrikoides*. Effects of grazers and nutrients on chain length after a 48-h incubation. (A) Grazer addition experiment: adding the fresh (0 h storage) and stale (24 h storage) exudates of the copepod *Acartia tonsa*, and adult females; (B) Nutrient amendment experiment: adding trace metals, nitrate, phosphate, vitamins, and f/2 medium; (C) Vitamin amendment experiment: adding vitamin B_1, vitamin B_7, and vitamin B_{12} both singly and

collectively. Error bars represent the standard deviation of 6 or 8 replicates in each treatment; letters indicate significant differences among treatments.

Fig. 7 *Acartia tonsa*. Clearance rate of adult females relative to equivalent spherical diameter of algae (filled circle; redrawn from Berggreen et al. 1988). The size of 3 dominant cell types (1, 2, and 4 cells) of *Cochlodinium polykrikoides* (open square) and the mean chain length in the field and cultured populations (open circle) was indicated to show reduced grazing due to chain formation.

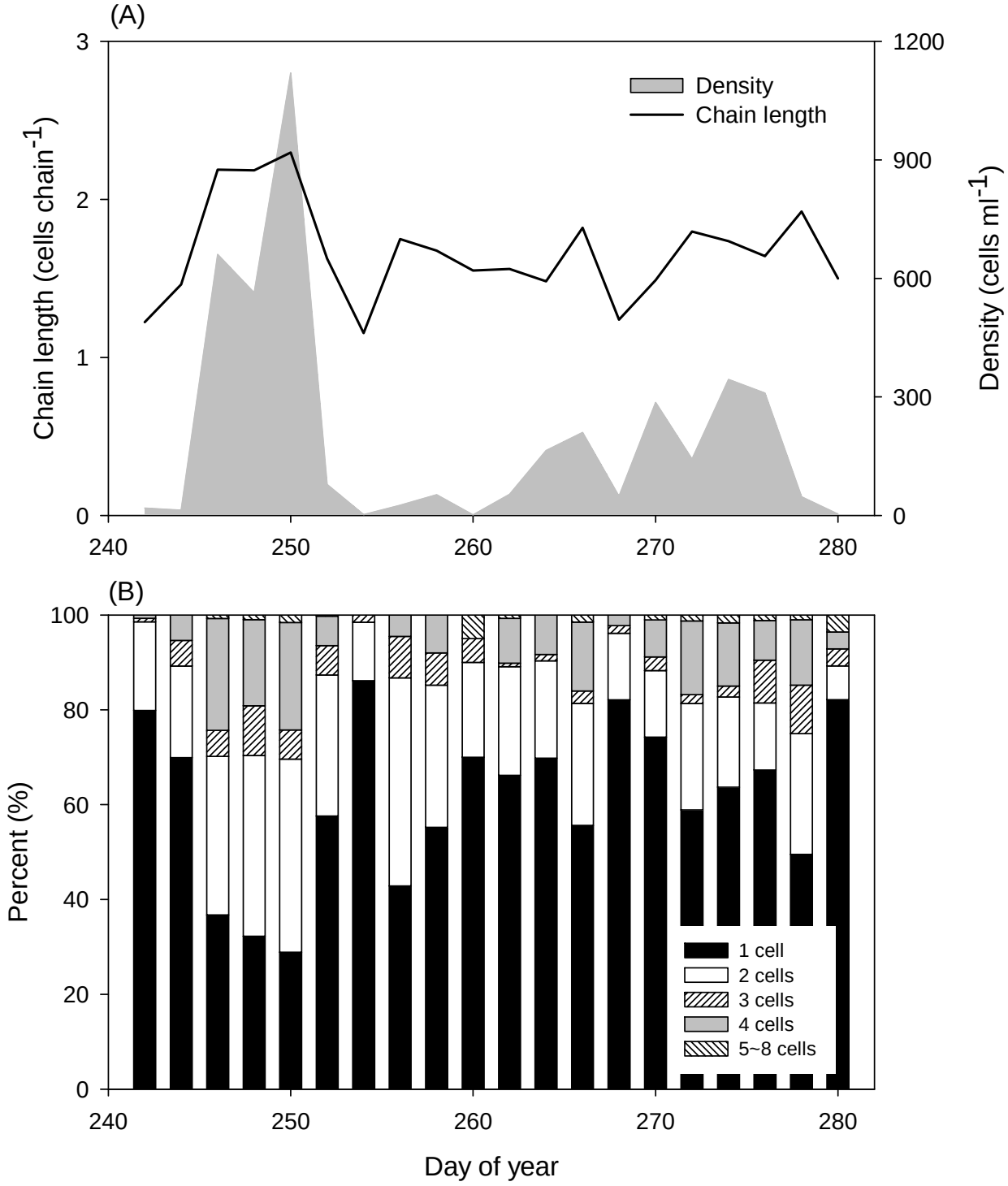
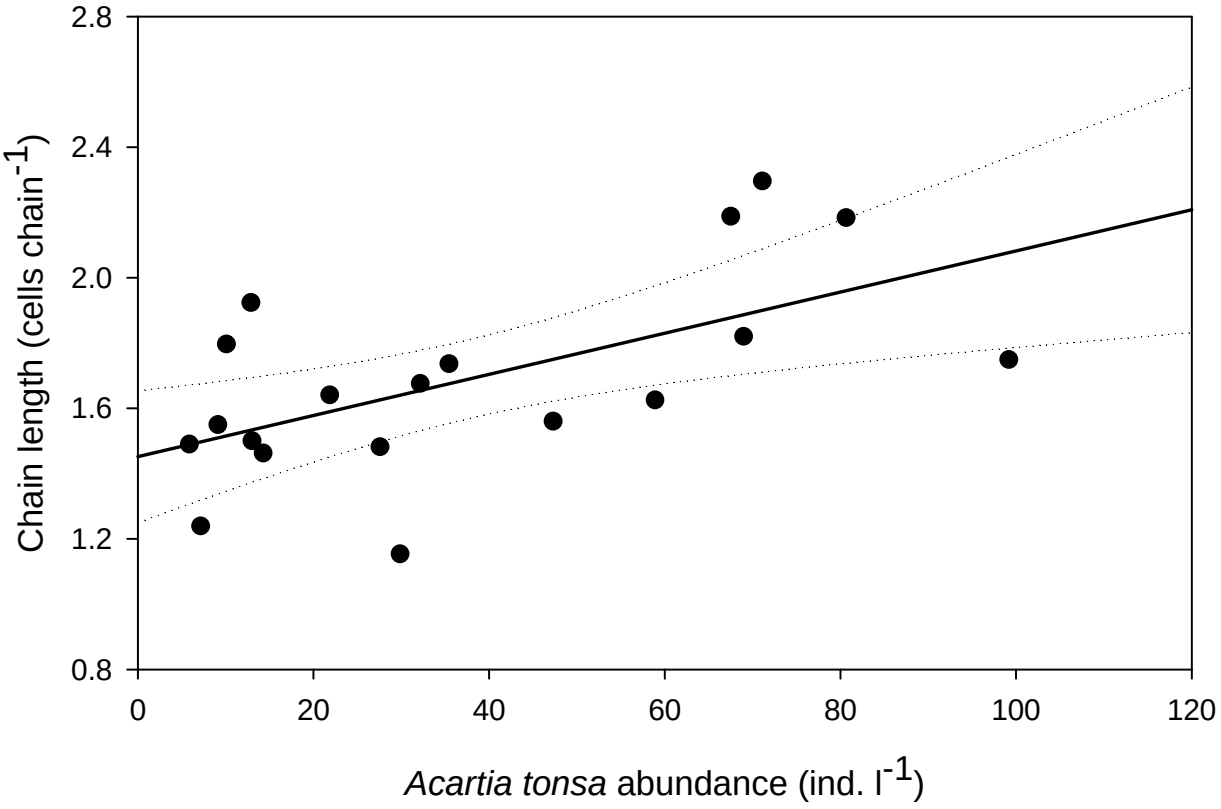


Fig. 1 Jiang et al.



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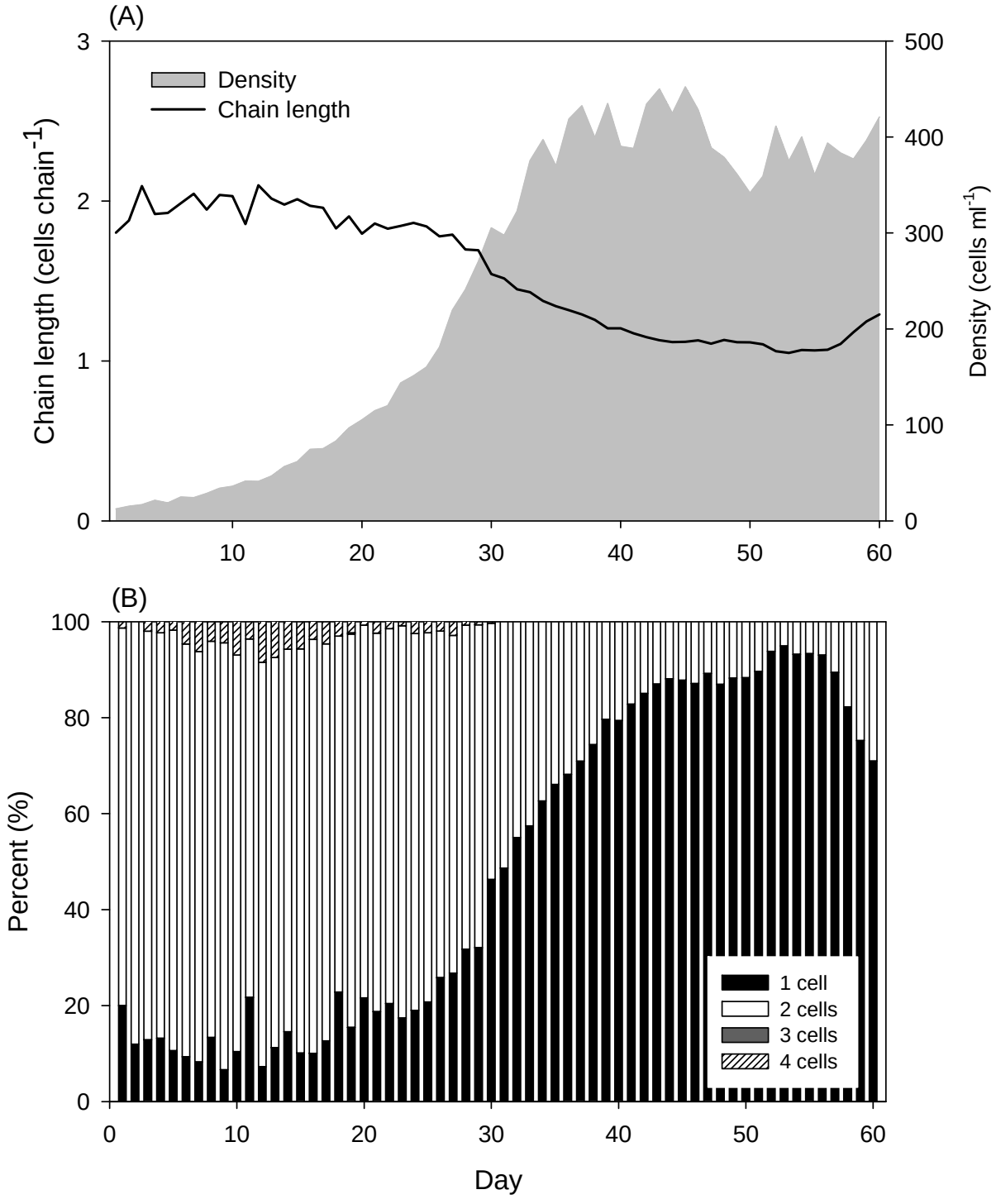

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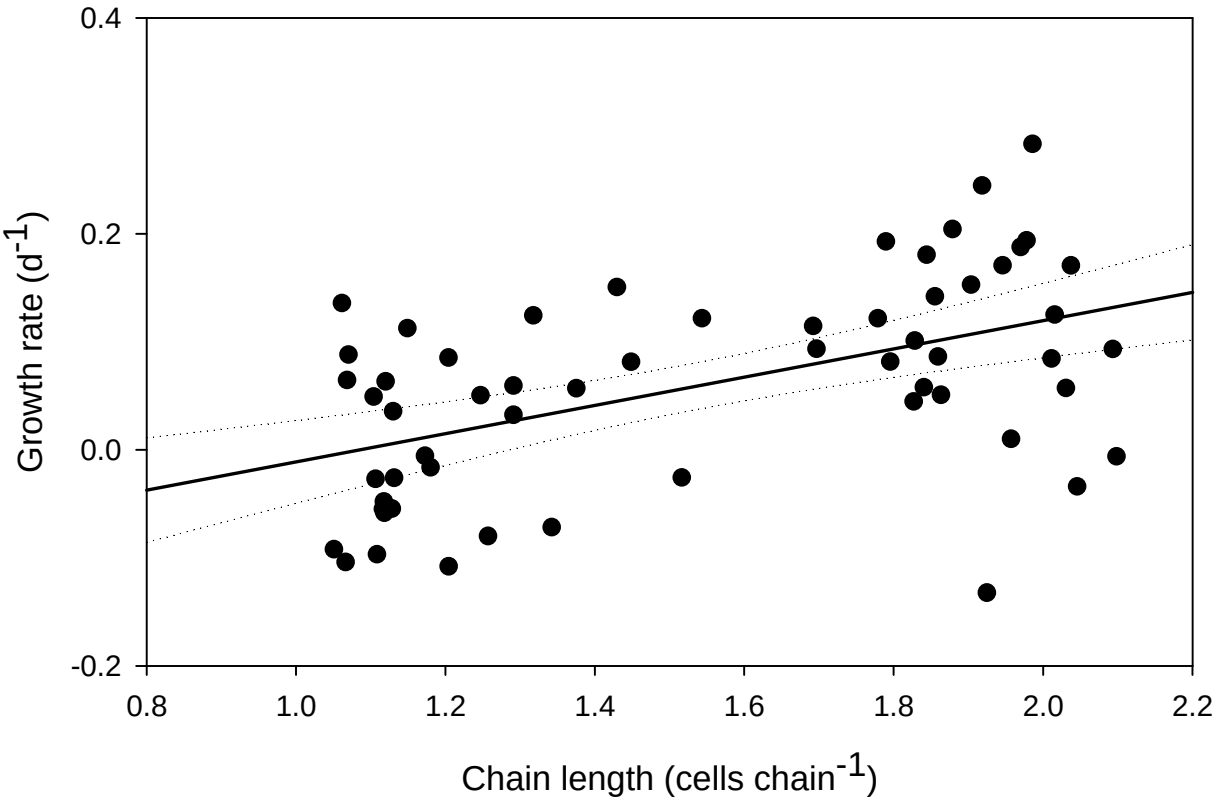

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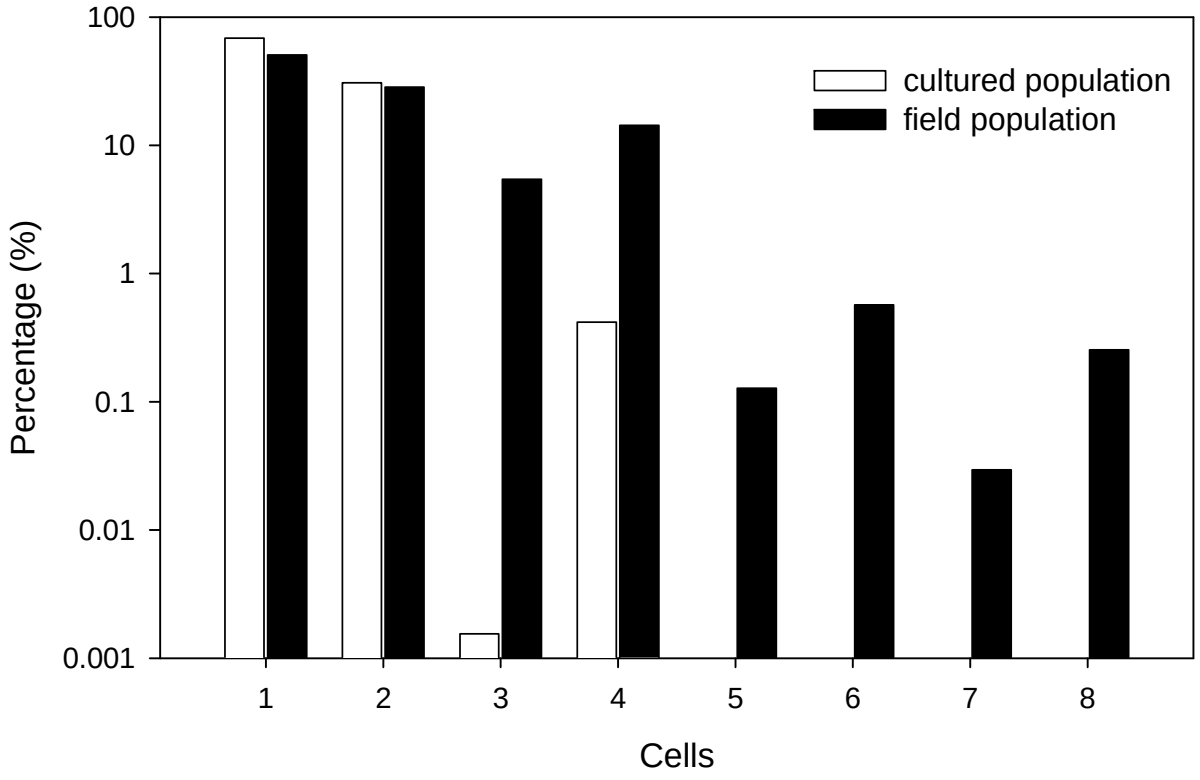

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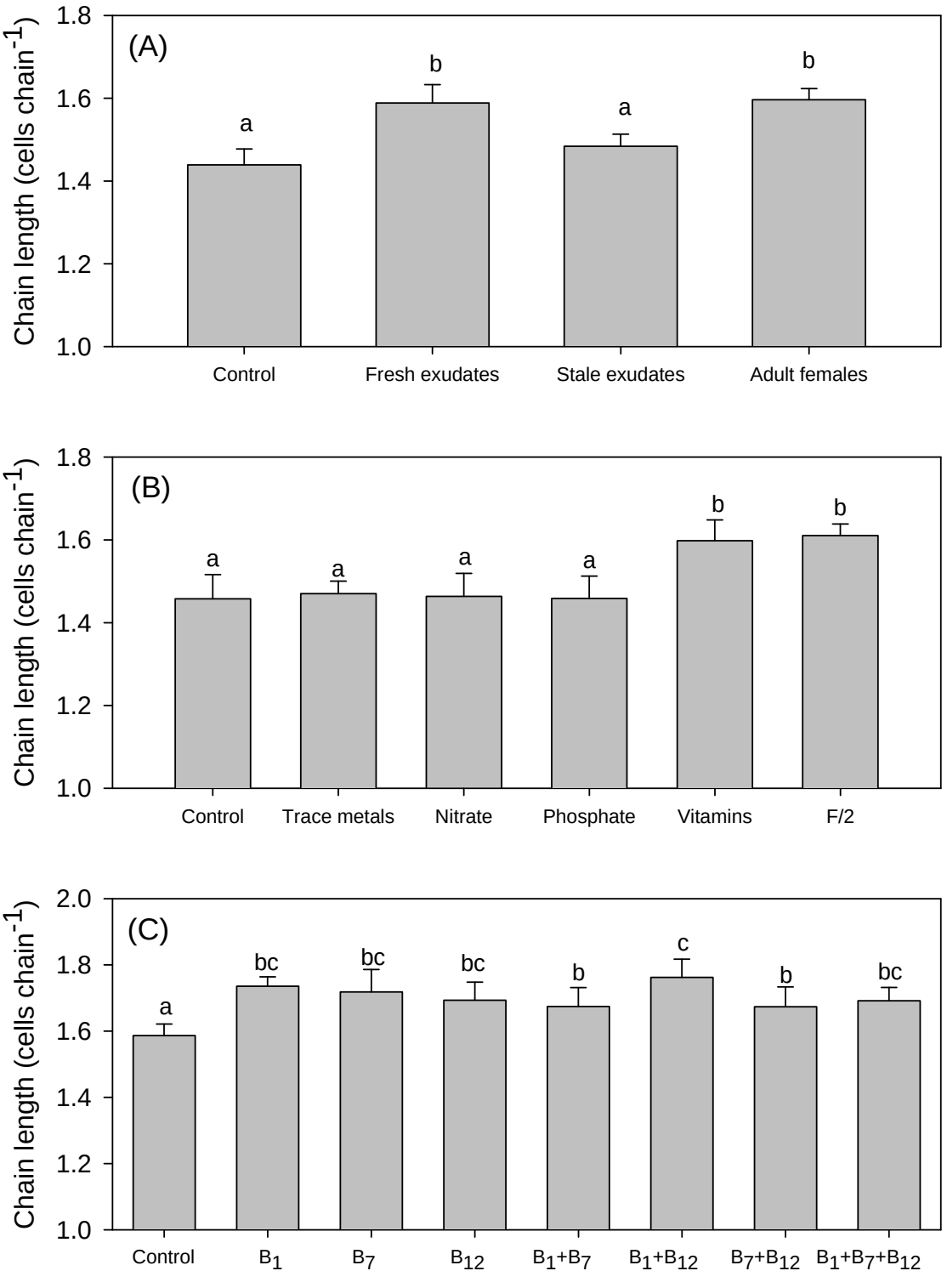

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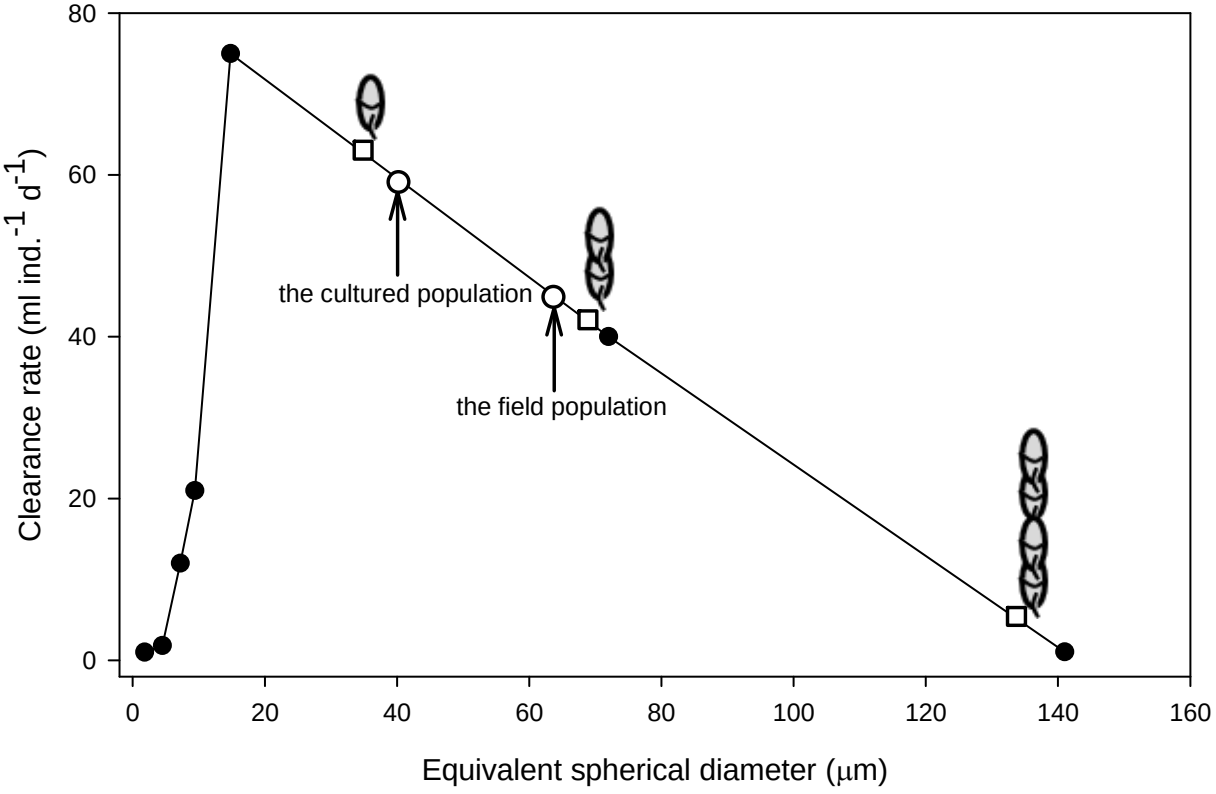

Fig. 7 Jiang et al.

Chapter nine: The role of nitrogenous nutrients in the occurrence of the harmful dinoflagellate blooms caused by *Cochlodinium polykrikoides* in Long Island estuaries (NY, USA)

In preparation for Harmful Algae

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Abstract: The harmful dinoflagellate *Cochlodinium polykrikoides* is well known for forming ichthyotoxic blooms in coastal regions of Asia and North America, but the nutritional factors supporting and promoting these blooms have not been well studied. To better understand the nutritional ecology of the harmful dinoflagellate blooms caused by *Cochlodinium polykrikoides* in Long Island estuaries (NY, USA), laboratory and field studies of this species were conducted. I documented the spatial and temporal dynamics of nutrients, *C. polykrikoides* cells, and co-occurring phytoplankton within two New York estuaries from 2006 - 2008. I quantified the growth response of *C. polykrikoides* and co-occurring phytoplankton during experimental enrichments with different nitrogen sources. Furthermore, I quantified growth rates of *C. polykrikoides* clonal isolates on a variety of nitrogen sources (urea, ammonium, glutamic acid, nitrate) and over a range of concentrations (2-200 μM). Finally, I quantified the uptake rates of various N compounds in both the field and laboratory using ^{15}N-enriched compounds. *C. polykrikoides* cultures grown on glutamic acid displayed significantly faster growth and higher rates of N uptake compared to cultures grown on urea, ammonium, and nitrate. From 2006 – 2008, blooms of *C. polykrikoides* occurred in where N concentrations and the dominant N compound varied, but blooms were only monospecific (in the >20 μm size range) when concentrations of nitrate and ammonium were < 2 μM. During blooms, the addition of different N compounds (urea, ammonium, glutamic acid, nitrate) significantly increased the growth of *C. polykrikoides* more frequently than other phytoplankton groups suggesting blooms were N-limited. Finally, the dominant N compounds assimilated by bloom communities differed between sites, with nitrate and nitrite being taken up fastest at the most eutrophic locations and urea and glutamic acid being assimilated quickest at mesotrophic sites. The sum of these observations suggests that *C. polykrikoides* is a nutritionally flexible species, capable of

assimilating a variety of N compounds, with the compound yielding maximal growth or uptake depending on prevailing nutrient conditions.

INTRODUCTION:

Harmful algal blooms (HABs) are a significant threat to fisheries, public health, and economies worldwide. HABs are classified harmful for a suite of reasons including the ability of many HAB-forming dinoflagellates to produce potent biotoxins which can sicken or kill humans. While many HABs do not directly harm marine life, others can be lethal (Landsberg, 2002; Sunda et al. 2006). In Asian waters, the red tide forming dinoflagellate *Cochlodinium polykrikoides* is well known for its harmful effects on marine organisms (Yuki and Yoshimatsu, 1989; Yamatogi et al., 2002; Huang and Dong, 2000; Lee, 2006; Kim, 1998). Several studies have demonstrated the fish killing capabilities of *Cochlodinium sp.* (Onoue et al., 1985; Yuki and Yoshimatsu, 1989; Guzmán et al., 1990; Qi et al., 1993; Gárrate-Lizárraga. et al., 2004; Whyte et al., 2001; Kim et al., 1999; Gobler et al., 2008) and *C. polykrikoides* blooms have been responsible for hundreds of millions of USD in fisheries losses in Korea alone (Kim, 1998). At Vancouver Island, British Columbia, fishery losses exceeding $3 million USD were attributed to a 1999 bloom of *C. polykrikoides* (Whyte et al., 2001).

Cochlodinium polykrikoides and other closely related species in the genus are catenating dinoflagellates approximately 20 μm in size, athecate, and known to vertically migrate on diel cycles (Kudela et al., 2008). The life history of *C. polykrikoides* has not been well studied but Kim et al. (2002) have found that cultured cells can form resting cysts.

Cochlodinium polykrikoides was first identified in Phosphorescent Bay, Puerto Rico by Margalef (1961) but blooms have now been reported from many locations across North America and Asia. Blooms of *Cochlodinium sp.* have been reported in the Gulf of California (Garate-Lizárraga et al., 2004) and within coastal waters of the United States, including Rhode Island (Hargraves and Maranda, 2002; Tomas and Smayda, 2008), California (Kudela et al.,

2008; Curtiss et al., 2008), New Jersey (Sousa e Silva, 1976), and the lower Chesapeake Bay system (Marshall, 1995; Mulholland et al 2009). *Cochlodinium polykrikoides* has formed dense blooms in the Peconic Estuary and Shinnecock Bay of Long Island, NY during late summer early fall months annually since 2004 (Gobler et al., 2008). Originally described there as nuisance blooms (Nuzzi, 2004), recent experiments have shown blooms of *C. polykrikoides* in NY and VA can be lethal to fish and shellfish (Gobler et al., 2008; Mulholland et al., 2009). Contact with bloom waters or clonal isolates having densities greater than 1 x 10^3 cells ml^{-1} resulted in rapid mortality in fish (i.e. hours) and shellfish (i.e. days; Gobler et al., 2008; Mulholland et al., 2009 Tang and Gobler, 2009).

Globally, nutrient over-enrichment is generally considered a prime promoter of HABs (Anderson et al 2008;Heisler et al. 2008). However, the manner in which nutrients may promote blooms of *C. polykrikoides* is not well understood. Jeong et al. (2004, 2005) have reported that *C. polykrikoides* isolates from Southeast Asia can be mixotrophic, making its nutritional options diverse. Kim et al. (2001) reported that the Korean strain of *C. polykrikoides* showed a preference for ammonium over nitrate. Kudela et al. (2008) studied *Cochlodinium fulvescens* (Iwataki et al., 2008) blooms on the west coast of the US and found that at elevated nutrient concentrations, ammonium and urea uptake rates exceeded those of nitrate. While *C. polykrikoides* blooms have become common along the US east coast (Marshall, 1995; Hargraves and Maranda, 2002; Gobler et al., 2008; Mulholland et al 2009), the nutrient sources promoting these blooms are unknown. In the lower Chesapeake Bay system, a broad spectrum of inorganic and organic N compounds (nitrate, nitrite, ammonium urea and dissolved free amino acids) contributed to the N demand during blooms with no clear nutrient preference and organic N compounds contributed 25 to 69% of the total measured N uptake (Mulholland et al.,

2009). Understanding how nutrient quantity and/or quality promotes or controls HABs is crucial to developing strategies for their management and remediation. It is the intention of this study, therefore, to understand the nutritional ecology of *C. polykrikoides* in New York estuaries.

To better understand the nutritional ecology of the harmful dinoflagellate blooms caused by *Cochlodinium polykrikoides* in Long Island estuaries (NY, USA), laboratory and field studies of this species were conducted. I documented the spatial and temporal dynamics of *C. polykrikoides* cells, nutrient concentrations, and co-occurring phytoplankton within two New York estuaries from 2006 to 2008. I quantified of the growth response of *C. polykrikoides* and co-occurring phytoplankton during enrichment with different nitrogen sources. Furthermore, I quantified of growth rates of culture isolates of *C. polykrikoides* on a variety of nitrogen sources (urea, ammonium, glutamic acid, nitrate) supplied at a range of concentrations (2-200 μM). Finally, I quantified the uptake rates of various N compounds in both field and laboratory conditions using ^{15}N-enriched nitrate, nitrite, urea, ammonium, and glutamic acid.

METHODS:

Culture Based Experiments:

***Cochlodinium polykrikoides* growth on various sources and concentrations of N:**

Cochlodinium polykrikoides strain CP1 was isolated from a 2006 bloom in Flanders Bay, Long Island, New York, USA. These cultures were grown on GSe medium (Doblin et al., 1999) made from artificial salts and supplemented with 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, VA) added to the medium immediately before inoculation at a final

concentration of 1-2% to minimize contamination by bacteria and fungi. Periodic DAPI-staining of cultures indicated the absence of bacteria during experiments. Cultures were maintained at 21° C on a 14:10 light:dark cycle, illuminated by a bank of fluorescent lights that provided ~100 μmol quanta m^{-2} sec^{-1}. These conditions approximated temperature and light exposures found in Long Island estuaries during late summer months when *C. polykrikoides* blooms (Gobler et al., 2008).

The growth of *C. polykrikoides* on different species and concentrations of nitrogen was examined during simultaneously implemented experiments using four different nitrogen compounds supplied at each of six concentrations in modified GSe medium (the N composition and concentration was altered). Cultures were grown in triplicate Pyrex test tubes (50 ml) with 2, 5, 10, 25, 50, 100, and 200 μM N as nitrate, ammonium, urea, or glutamic acid. To assure that nutrient saturation would be reached, the highest nitrogen concentrations were near those of standard phycological media, but exceeded ranges found in *C.* bloom-prone embayments (Gobler & Boneillo 2003; this study). Initial sets of tubes received an inoculum from a single microalgal culture grown under the conditions described above. Accumulation of cell biomass over time was estimated by *in vivo* fluorescence, measured the same time each day (to avoid diel fluctuations in cell fluorescence) using a Turner Designs TD-700 fluorometer. Previous research has demonstrated that *in vivo* fluorescence is proportional to cell density for a variety of cultured phytoplankton species (Fogg and Thake, 1987; Taylor et al. 2006), and I found this to be the case for *Cochlodinium polykrikoides*. Upon entering late exponential phase growth, cultures were transferred into fresh media with the appropriate treatment concentration and N compound, and diluted to a cell density of ~100 cells ml^{-1}. Cultures were maintained on media with the appropriate N compound and treatment concentration for a minimum of 6 transfers

prior to initiating experiments in order to ensure that cells were fully acclimated to treatment conditions and that N concentrations and nutrient stores from the initial culture medium (full strength GSe) was eliminated.

Once cultures were fully acclimated to experimental conditions, cellular growth rates were calculated for all cultures in two ways. *In vivo* fluorescence was used to generate biomass production rate constants (d^{-1}) during exponential-phase growth. By doing this, cellular chlorophyll *a* quotients would not influence calculations so long as they are relatively constant during early to mid-exponential growth phase. Growth rates based on cell biovolume (μm^3) were also determined on 100 µl aliquots of Lugol's iodine-preserved samples using a Beckman-Coulter© Multisizer 3.0. All growth rates were calculated daily during exponential phase growth using the formula $\mu = \ln (B_t/B_0)/t$, where B_o and B_t are the initial and final biovolume, and t is the incubation duration in days. Growth rates were averaged over the entire exponential phase, which typically persisted for 3 – 6 days, depending on the concentration of N in the media. Growth curves from changes in cell volume and *in vivo* fluorescence were nearly identical and not statistically different.

The Michealis-Menton kinetic terms μ_{max} (maximum growth rate) and K_S (half saturation constant) were derived using Lineweaver-Burk transformations and an affinity coefficient, α, was calculated from μ_{max} / K_S. This provides a more descriptive picture of nutrient affinity at sub-saturating concentrations ($<K_s$) and might better predict competitive outcomes when interspecies competition for nutrients is likely to occur (Harrison et al 1989). Differences in growth rates betweem treatments were examined by means of a two-way analysis of variance, where nitrogen concentrations and nitrogen compound were the main treatments. Multiple comparisons among treatments were also examined using Tukey test tests.

Uptake rates of nitrogenous nutrients:

To quantify the rate of uptake of the different N compounds in cultures grown at different N concentrations, ^{15}N tracer experiments were conducted. Nitrogen uptake was measured using tracer additions (20 ± 11%) of highly enriched (98%) ^{15}N-labeled compounds (Mulholland et al., 2002 and 2009). Cultures were grown through seven transfers on each N compound tested at concentrations of 2 μM and 20 μM N, which are similar to mean and maximal levels of nitrate and ammonium present during blooms (Table 1). In late exponential phase growth, cultures growing on 2 μM and 20 μM glutamic acid, urea, nitrate and ammonia were amended with the tracer addition of ^{15}N-labeled glutamic acid, urea, nitrate or ammonia (10%) plus 2 μM or 20 μM addition of each ^{14}N compound. Incubations were performed under normal culture conditions for 60 minutes, after which cultures were filtered onto pre-combusted (2 h @ 450ºC) GF/F glass fiber filters. The natural abundance of ^{15}N in particulate organic nitrogen (PON) prior to enrichment was also determined. Samples were palletized in tin discs and were analyzed for at the U.C. Davis Stable Isotope Facility on a ……. Uptake rates were calculated according to the mixing model of Montoya et al. (2002) and using equations from Orcutt et al. (2001). Rates were considered net uptake as they not corrected for the effects of isotope dilution (Glibert et al., 1982) although these are expected to be minimal in cultures due to the short incubation times and absence of zooplankton and bacteria. A one-way analysis of variance with post-hoc Tukey test multiple comparison tests was performed to determine differences in uptake between the 2 μM and 20 μM N treatments.

Field Experiments:

Field Sampling:

During this study, estuaries in which *C. polykrikoides* blooms were previously reported (Shinnecock Bay and the Peconic Estuary; Gobler et al., 2008) were accessed using small vessels from the Stony Brook-Southampton Marine Science Center. Specific sampling sites included: Old Fort Pond (a tributary connecting to Shinnecock Bay, 40.8621° N, 72.4396° W) and Shinnecock Bay proper (40.8621° N, 72.4734° W), Great Peconic Bay (40.9252°N, 72.5614°W) and Flanders Bay (40.9255°N, 72.5928°W) in the western extent of the Peconic Estuary, and Meetinghouse Creek (40.9210° N, 72.6245°W), a tributary connecting to the north shore of Flanders Bay. Weekly sampling of all sites was performed in late summer (July - August), prior to the development of blooms, and continued into the fall when blooms had ended (October). During blooms, both dense bloom patches (surface swarms) and non-patch areas were sampled (Gobler et al., 2008).

Surface and bottom salinity, temperature, and dissolved oxygen were measured at each sampling site using a hand-held YSI© 556 sonde. Surface water was collected in 20-L, acid-cleaned carboys. Whole water from each station sampled was filtered through pre-combusted (2 hrs @ 450°C) glass fiber filters and frozen for nutrient analysis. Ammonium, nitrate, nitrite, phosphate, urea, silicate, total dissolved nitrogen and total dissolved phosphorus concentrations were determined colorimetrically using wet chemical techniques and a spectrophotometric microplate reader (Valderma, 1981,; Jones, 1984; Parsons et al., 1984; Price and Harrison, 1987). Selected samples were analyzed for individual dissolved free amino acids in duplicate by high performance liquid chromatrography (HPLC; (Cowie and Hedges 1992). Chlorophyll *a* samples were collected by filtering whole water onto 0.7 µm GF/F filters and 5 µm and 20 µm polycarbonate filters and analyzed using standard fluorometric techniques (Welschmeyer,

1994). Whole seawater samples were preserved in Lugol's iodine solution and species identification and enumeration was performed using an inverted light microscope (Hasle, 1978).

Differences in biological, chemical, and physical parameters between sites and years were assessed by means of one-way analyses of variance (ANOVA) with post-hoc Tukey test multiple comparison tests or Student's T-tests. For comparative purposes, a threshold of 330 cells mL^{-1} was used to define 'bloom' conditions, as this is the minimal density of this species capable of killing fish (Tang and Gobler 2009). The degree to which individual variables were correlated was evaluated by a Spearman's Rank Order Correlation Matrix. In all cases, a significance level of 0.05 was applied to justify statistically significant differences or correlations.

Nutrient amendment experiments:

During the initiation, peak, and demise of *C. polykrikoides* bloom events, nutrient amendment experiments were conducted to determine how enrichment with different N compounds affected the growth of this species relative to other members of the phytoplankton community. Experiments were conducted at various sampling sites during the summers of 2005, 2006, 2007, and 2008. Surface seawater was collected using acid-cleaned 20-L carboys and within two hours was dispensed into 1.1-L acid-cleaned polycarbonate bottles. Triplicate bottles were used for each treatment, which included an unamended control, sodium nitrate (10 μM), urea (5 μM = 10 μM N), glutamic acid (10 μM), and ammonium (10 μM). Nutrient stocks were filter-sterilized (0.2 μm) and stored frozen. Bottles were incubated for 48 hours in eastern Shinnecock Bay under ambient light and temperature conditions. Termination of experiments included filtration of water to determine concentrations of total and >5 μm chlorophyll *a* concentrations and preservation in Lugol's iodine solution for microscopic

quantification of *C. polykrikoides* and co-occurring phytoplankton, which were broadly grouped as 'diatoms' and 'other dinoflagellates'. Net growth rates (d^{-1}) of each component of the algal community were calculated as $\mu = [\ln(B_t/B_o)]/t$ where B_o and B_t are the initial and final biomass (pigment or cell density) of each algal population, respectively, and t is the incubation duration in days. One-way analyses of variance with post-hoc Tukey test multiple comparison tests were performed to determine significant differences in growth rates among treatments for each algal population: *C. polykrikoides*, diatoms, other dinoflagellates, and small phytoplankton (< 5 μm).

Uptake rates of nitrogenous nutrients during bloom events:

^{15}N tracer experiments were conducted in 'bloom patches' (surface water cell swarms) following the methods described for cultures, to assess the source of N assimilated by *C. polykrikoides* bloom populations. Differences included an assessment of nitrite uptake, shorter incubations (30 minutes), and incubations were carried out under ambient light and temperature conditions. Since bloom patches of *C. polykrikoides* contained few other phytoplankton (65-97% of cells > 20μm were *C. polykrikoides* during this study), ^{15}N-amended experimental water was filtered on pre-combusted (2 h @ 450ºC) GF/F glass fiber filters with and without pre-filtration with a 20μm mesh to remove *C. polykrikoides* cells. Isotope dilution was not measured and uptake by bacteria and production or release of N compounds during incubations may have influenced our rate estimates. The difference in uptake observed in the total and < 20 μm size fraction was ascribed to cells > 20 μm, and microscopic quantification was used to assess the relative abundance of *C. polykrikoides* cells in this size fraction during each experiment. A one-way analysis of variance with post-hoc Tukey test multiple comparison tests was performed to determine significant differences among uptake rates for each compound from each experiment.

RESULTS:

Growth rates of C. polykrikoides on differing sources and concentrations of N

Cultures of *Cochlodinium polykrikoides* strain CP1 grown on nitrate, ammonium, urea, or glutamic acid displayed standard Monod growth kinetics over the range of N concentraions used (2 – 200 μM; Fig 1). Growth rates were similar among N compounds at the low levels of N, at ~0.15-0.2 d^{-1} with growth rates on glutamic acid being somewhat higher (Fig. 1). Growth rates seemed to saturate above 25 μM for all N species (Fig. 1). Maximal growth rates (μ_{max}) achieved by *C. polykrikoides* on glutamic acid (0.50 ± 0.10 d^{-1}) were significantly (Tukey test, $p<0.05$) higher than those for nitrate, ammonium, and urea (0.41 ± 0.10, 0.41 ± 0.07 and 0.42 ± 0.10 d^{-1}, respectively; Table 2). Half-saturation constants (K_s) were lower for glutamic acid and urea (1.84 ± 0.60 and 2.18 ± 0.51 μM, respectively) when compared to the higher ammonium and nitrate (2.60 ± 0.49, and 2.94 ± 0.70 μM, respectively; Table 2).

Affinity coefficients indicated *C. polykrikoides* has the highest affinity for glutamic acid (α = 0.27 $d^{-1}\mu M^{-1}$; Table 2), followed by urea and ammonium (0.19, and 0.16 $d^{-1}\mu M^{-1}$, respectively; Table 2). *C. polykrikoides* has the lowest affinity for nitrate (α = 0.14; Table 2). Both the chemical form and concentration of N were significant treatment effects for *C. polykrikoides* growth rates ($p < 0.001$; Two-way analysis of variance). *C. polykrikoides* growth rates were significantly greater when growing with glutamic acid as an N source relative to growth on all other N sources (Tukey test test, $p<0.05$). There were also significant differences between growth rates of C. polykrikoides on media with high versus low N concentraions (e.g. 200 μM significantly greater than 2- 25 μM; Tukey test test, $p<0.05$). For all sources of N, increasing concentraions of N predictably yielded longer periods of exponential growth and

higher final cell densities (data not shown). N concentrations were generally < 1μM at the end of expoential phase growth (data not shown).

N assimilation by C. polykrikoides growing on different chemical forms and concentrations of N

Uptake rates of N differed among *C. polykrikoides* cultures grown on different N compounds and concentrations. At both low and high nitrogen concentrations, glutamic acid-N uptake rates were significantly higher than those measured for all other N compounds ($p<0.001$; Tukey test Test; Fig 2). Ammonium uptake rates were significantly higher than those of urea and nitrate ($p<0.001$; Tukey test Test; Fig 2), which did not differ from each other. Glutamic acid was the only nutrient with significantly faster uptake rates in cultures grown at higher N concentrations (Tukey test, $p<0.05$).

For comparative purposes, N uptake rates measured with ^{15}N compounds in culture were compared to the N demand of this species estimated from its N quota, cell densities, and growth rates. Based on concentrations of PON measured in *C. polykrikoides* cultures of known cell densities, we estimate that this species contains $2.3 \pm 0.3 \times 10^{-11}$ mol N cell^{-1}. This value is within the range estimated previously based on cell biovolume and a Refield C:N ratio (1.9×10^{-11} mol N cell^{-1}; Stoecker et al. 1994; Jiang et al, in press). For laboratory cultures grown on low levels of N (2 μM N), the amount of N uptake measured was almost identical to the estimated demand for urea and nitrate based on its growth rate and the N biomass (Table 3). At higher concentrations (20 μM N), the measured N uptake from nitrate and urea less than (~44%) that estimated based on it's cell quota. For *C. polykrikoides* grown on glutamic acid and ammonium uptake rates were a factor of two higher than that necessary to meet the calculated N

demand based on cell densities, growth rates, and cellular N quotas (Table 3), suggesting there was luxury uptake or rapid turnover of these two compounds.

Dynamics of phytoplankton and nutrients during C. polykrikoides blooms in NY estuaries, 2006 - 2008

Blooms (defined as >330 cells mL^{-1}) of *C. polykrikoides* occurred in Long Island estuaries during late summer (August) through early fall (September) from 2006 - 2008. Widespread (all study sites) and extended (> 1 month) *C. polykrikoides* blooms occurred in 2006 and 2008, while blooms in 2007 were isolated (Old Fort Pond and Flanders Bay only) and short (1 week). The bloom in 2006 was generally denser than the bloom in 2008 (Table 4). Blooms peaked at 55,000 cells mL^{-1} (Great Peconic Bay, 8/30/06; Table 4) and Great Peconic Bay experienced the densest blooms averaged over all three years (Table 4). Mean nitrogen levels at the sites were generally low with nitrate, ammonium and urea ranging from 0.63-3.79μM, 0.63-2.22 μM, and 0.15-1.11 μM respectively (Tables 5 and 6). Silicate and phosphorous ranged from 32.92-65.01 μM and 1.14-2.04 μM, respectively (Table 5).

Analysis of all bloom and non-bloom conditions from all sites over all years (Table 6), revealed that, as would be expected, blooms had significantly more *C. polykrikoides* cells, total chlorophyll *a* and < 5 μm chlorophyll *a*, than non-bloom water ($p<0.001$). Bloom sites also had significantly higher silicate levels and significantly lower salinity ($p<0.01$; Table 6). There were five-times more diatoms present under non-bloom conditions, compared to blooms (Table 6). Moreover, *C. polykrikoides* abundances were inversely correlated with diatom densities ($p < 0.05$) and significantly correlated with silicate concentrations ($p < 0.01$; Table 6). In addition, *C. polykrikoides* abundances were also significantly correlated with concentrations of dissolved organic nitrogen and phosphorous for all years (DON and DOP; $p < 0.05$ for each). In contrast,

other phytoplankton groups such as other dinoflagellates, diatoms and small phytoplankton (< 5 μm) were not significantly correlated with DON or DOP. Comparisons between the bloom years (2006 and 2008) and the minor-bloom year (2007) revealed significantly higher concentrations of ammonium and urea in 2007 compared to 2006 and 2008 (Table 5, $p<0.05$).

During blooms in Great Peconic Bay and Shinnecock Bay, *C. polykrikoides* cells comprised over 95% of the cells >20 μm, while in Meetinghouse Creek and Old Fort Pond *C. polykrikoides* was a significantly lower percentage of phytoplankton cells >20 μm (50-69% ; $p<0.05$; Table). Comparisons of all field parameters between monospecific (for phytoplankton >20 μm) bloom sites (Great Peconic and Shinnecock Bays) and the mixed bloom sites (Meetinghouse Creek and Old Fort Pond) indicated there was a significantly greater abundance of non-*C. polykrikoides* dinoflagellates at the mixed bloom sites ($p<0.05$). Furthermore, nitrate concentrations were significantly higher at the mixed bloom locations (t-test, $p<0.005$). In contrast, during monospecific blooms higher salinity and urea concentrations were present ($p<0.05$).

N assimilation rates by plankton communities during C. polykrikoides blooms

Ten N-uptake experiments were conducted during August and September of 2008 in four locations: Old Fort Pond, Shinnecock Bay, Flanders Bay, and Great Peconic Bay. During experiments, *C. polykrikoides* cell densities ranged from 480 to 5,484 cells ml^{-1} while total chlorophyll *a* levels ranged from 18.3 to 55.7 μg L^{-1} (Table 6). Of the cells > 20 μm enumerated during experiments, *C. polykrikoides* represented a large majority of the total (72-97%), averaging 89±11% and being greater than 96% on three occasions (Table 6). Total N uptake for all N species ranged from 0.30 to 3.9 μmol N L^{-1} hr^{-1}, and averaged 1.8 ± 1.0 μmol N L^{-1} hr^{-1} (Fig 3a). The > 20μm size fraction accounted for, on average, 34 ± 12% of the total N

uptake, ranging from 12 – 48% (Fig 3). The N compound displaying the greatest uptake in the > 20µm size fraction varied by site and date. Within Old Fort Pond and Flanders Bay, nitrate and nitrite dominated total N uptake (69%; Fig 3). Of the four experiments in Old Fort Pond and Flanders Bay, the >20 µm plankton group displayed significantly greater uptake of nitrate and nitrite compared to glutamic acid in three experiments, significantly greater uptake of nitrate and nitrite compared to urea in two experiments and significantly greater uptake of nitrate and nitrite compared to ammonium in one experiment (Tukey test, $p<0.05$). In contrast, within Shinnecock Bay and Great Peconic Bay, urea was the compound taken up at the highest rates by plankton > 20 µm, ranging from 41 – 83% of the total N-assimilation rate (Fig 3). Of six experiments in Shinnecock Bay and Great Peconic Bay, the >20 µm plankton group displayed significantly greater uptake of urea compared to glutamic acid and nitrite in all experiments, and significantly greater uptake of urea compared to nitrate and ammonium within four experiments (Tukey test, $p<0.05$). Notably, glutamic acid was assimilated at the greatest rate by the larger plankton in Shinnecock Bay on September 16th (20% of total, Fig 3). The N uptake characteristics of the > 20µm size fraction contrasted with those of the smaller plankton (< 20µm), which acquired the majority of their N from ammonium and urea, regardless of location (Fig 3).

For comparative purposes, measured N uptakes rates were compared to the theoretical N demand of bloom populations on the three occasions in 2008 when *C. polykrikoides* was > 94% of the >20µm phytoplankton community: 27 August in Great Peconic Bay, 10 September in Flanders Bay, and 16 September in Shinnecock Bay (Table 4). Cellular N quotas of cultures ($2.3 \pm 0.3 \times 10^{-11}$ mol N cell^{-1}; see culture work for details), were applied to bloom cell densities, and cellular growth rates measured for cultures at the levels of N present during

blooms (0.2 d^{-1} at 2 μM N; Fig 1) on these three dates. The ^{15}N assimilation rates summed for all measured compounds on these dates accounted for 43%, 70% and 111% of the estimated N demand on 27 August in Great Peconic Bay, 10 September in Flanders Bay, and 16 September in Shinnecock Bay (Table 3).

Growth rates of plankton communities in response to nutrient amendment during C. polykrikoides blooms

Twenty-one nutrient amendment experiments were performed from 2005-2008. Enrichment with at least one of the N compounds significantly increased *C. polykrikoides* growth rates in 62% of experiment performed (Tukey test, $p<0.05$; Tables 8 and 9). Enrichment of whole water with nitrate, ammonium, urea, or glutamic acid yielded significantly higher growth rates relative to the control treatment in 57, 53, 39, and 27% of experiments (Tukey test, $p<0.05$; Tables 8 and 9). Other members of the plankton community responded less frequently to N enrichment. For example, growth rates of diatoms, other dinoflagellates and small phytoplankton (< 5 μm) increased significantly in response to at least one form of N in 43, 17, and 38% of experiments conducted (Tukey test, $p<0.05$; Tables 8 and 9). These groups benefited most from nitrate enrichment (significantly increased growth in 36, 11, and 25% of experiments; Tukey test, $p<0.05$; Tables 8 and 9), but responded less frequently to other forms of N. For example, while *C. polykrikoides* experienced significantly increased growth when enriched with glutamic acid in 27% of experiments, this compound elicited a similar response in diatoms, and other dinoflagellates in only 18 and 7 of experiments (Tukey test, $p<0.05$; Tables 8 and 9) and never significantly altered the growth of small phytoplankton (0% of experiments; Tables 8 and 9).

The growth rates of *C. polykrikoides* in unamended control treatments were almost always slower than those of diatoms, dinoflagellates, or small phytoplankton (18 of 21; 86% experiments; Table 8 and 9, Fig 4). However, in nearly 32% of treatment incubations, enrichment by at least one of the N compounds (but not always the same one) resulted in an increased growth rate for *C. polykrikoides* relative to other phytoplankton groups. For example, during experiments conducted in Shinnecock Bay in 2008 and in Old Fort Pond in August and September of 2005, enrichment with nitrate resulted in *C. polykrikoides* growth rates exceeding all other phytoplankton groups (Table 8 and 9, Fig 4). In experiments in Great Peconic Bay (2008) and in Meetinghouse Creek (2007 and 2008), the addition of urea led to *C. polykrikoides* growth rates outpacing all other algal groups (Table 8, Fig 4). Finally, during the 2008 Great Peconic Bay experiment, glutamic acid enrichment resulted in higher growth rates for *C. polykrikoides* compared to other algal groups (Table 8).

DISCUSSION

Harmful algal blooms are an increasingly common phenomenon in coastal waters around the world, and nutrient enrichment is commonly an important contributor to the occurrence of these events (Heisler et al., 2008; Anderson et al., 2008). While *C. polykrikoides* has emerged during the past two decades as an ichthyotoxic HAB species which has caused annual blooms throughout Southeast Asia (Kim, 1998; Kim et al., 1999) and both coasts of North America (Curtiss et al., 2008; Gobler et al., 2008; Kudela et al., 2008' Mulholland et al., 2009), the nutritional regime supporting these blooms has not been deterimined. By combining laboratory and field studies, the present study indicated that *C. polykrikoides* is a nutritionally flexible species, capable of growing well on a variety of organic and inorganic forms of N.).

Growth of C. polykrikoides on differing N sources

During culture experiments, *C. polykrikoides* grew at rates comparable to those reported in prior studies of this species (0.4 d^{-1}; Kim et al 2001) on both organic and inorganic forms of N. While many phytoplankton grow well on urea, robust growth on amino acids is generally less common (Antia et al., 1975; Bronk et al., 2007). However, *C. polykrikoides* cultures grown on glutamic acid attained significantly higher growth rates ($\mu max = 0.50 \pm 0.10$ d^{-1}) and had substantially lower half saturation constants ($Ks = 1.84 \pm 0.60$ μM) compared to all other N-sources tested (μmax = ~0.4; Ks = 2.2 – 2.9 μM; Table 2). Additionally, *C. polykrikoides'* low affinity coefficient (α) for organic N sources indicate that it has a higher affinity for amino acids than for urea or inorganic N compounds tested when compared with other phytoplankton. Consistent with this hypothesis, *C. polykrikoides* densities were significantly correlated with DON concentrations in the field ($p < 0.05$) while other phytoplankton groups were not. *C. polykrikoides'* robust growth on an amino acid is consistent with that of many other dinoflagellates, including HAB species such a *Karenia brevis, Prorocentrum minimum*, and *Lingulodinium polyedrum*, which also grow well on organic forms of nitrogen (Taylor 1987, Smayda 1997, Anderson et al 2008, Heisler et al 2008). The half saturation constants for growth reported here are similar to those measured for N uptake during a bloom of *C. fulvescens* in Monterey Bay (1.0 – 1.6 μM; Kudela et al., 2008) and for cultures of this species isolated from Korea grown on nitrate and ammonium (1.0 and 2.1 μM, respectively; Kim et al., 2001) and support the tenet that this species is adapted to moderate-to-low levels of N.

In addition to the higher growth rates on glutamic acid, *C. polykrikoides* cultures also assimilated this compound at rates significantly higher than those observed in nitrate and urea-grown cultures, particularly when nutrient levels were low (2 μM; Fig 1). Since the growth

rates varied depending on the form of N supplied and varied by smaller amounts than those observed for glutamic acid (Fig 1), the high uptakes rates of glutamic acid by cultures suggests something other than N nutrition may be responsible for the higher uptake rates of these compounds. Consistent with this hypothesis, N uptake rates by cultures grown on glutamic acid exceeded the theoretical demand for N by more than two-fold at both high and low levels of N, suggesting the high uptake rates of this compound represented luxury uptake perhaps as a means to obtain extra organic carbon. Mulholland et al. (2009) recently demonstrated that bloom populations of *C. polykrikoides* are capable of obtaining both N and C from amino acids. This could be used to supplement their photosynthesis (Droop, 1974; Lewitus and Kana, 1995) and could establish a mechanism for 24-h C acquisition. In an ecosystem setting, exploiting such biochemical pathways could give this species a competitive advantage over algae obtaining C exclusively by means of photosynthesis. Interestingly, glutamic acid was the most abundant amino acid in selected (n = 10) seawater samples analyzed during *C. polykrikoides* blooms, present at a concentration of 0.2 ± 0.1 μM. Finally, during blooms, diel vertical migration of may allow *C. polykrikoides* to access such amino-acids and other DON sources from sediments (MacIntyre et. al., 2004; Kudela et. al., 2008).

***C. polykrikoides* bloom dynamics**

During this study, large (present at all study sites) extended (> 1 month) *C. polykrikoides* blooms occurred in 2006 and 2008, while blooms in 2007 were isolated (Old Fort Pond and Flanders Bay only) and brief (1 week). Blooms of *C. polykrikoides* occurred over a fairly wide range of nutrient conditions, including tributaries with high nutrient levels, such as Old Fort Pond and Meetinghouse Creek (mean DIN = 2.2 ± 1.1), and open water sites such as Great Peconic Bay and Shinnecock Bay (DIN = 0.8 ± 0.3; Table 5). Organic nutrients such as urea

and amino acid levels during blooms were generally low (< 2 μM) in most locales, although the DON pool was large (mean DON = 21 ± 4.9; Table 5). Interestingly, blooms were generally denser and more monospecific in the open parts of estuaries where nitrate levels were significantly lower ($p<0.05$; Tables 4 and 5). Also, concentrations of ammonium were significantly higher during the 2007 when large blooms were not present ($p<0.05$; Table). Together, these two observations suggest blooms of *C. polykrikoides,* particularly those which are monospecific, are less likely to form when concentrations of ammonium and nitrate are elevated. Since all half-saturation constants for growth for this species were relatively low (~ 2 μM), it seems that persistent, monospecific *C. polykrikoides* blooms can develop and persist at even moderate levels of nutrient enrichment.

Patterns in the abundance of diatoms, *C. polykrikoides*, and other dinoflagellates provide some preliminary insights into interspecific competition during *C. polykrikoides* blooms. The five-fold lower concentrations of diatoms during *C. polykrikoides* blooms, particularly in Flanders Bay and Meetinghouse Creek ($p<0.001$; Table 1), suggests that this group either cannot compete with Cochlodinium or it's growth is somehow inhibited by Cochlodinium…. Here one could add info on allelopathic substances produced by Cochlodinium…. This succession of phytoplankton is the likely cause of the significantly higher concentrations of silicate during blooms (i.e. fewer diatoms = less Si-uptake = higher concentrations; Table 1, 4). The significantly greater abundance of non-*C. polykrikoides* dinoflagellates at mixed bloom sites ($p<0.05$; Table 1) indicates that other dinoflagellates are able to co-exist within more eutrophic tributary sites, but not within open, estuarine sites and suggesting *C. polykrikoides* occupies a broader ecological niche than these other species.

Effects of nutrient enrichment on bloom populations

During the months in which *C. polykrikoides* blooms occurred, phytoplankton populations in the Peconic and Shinnecock Bays were frequently stimulated by N enrichment. *C. polykrikoides*, diatoms, dinoflagellates and small (<5 µm) phytoplankton were stimulated by N enrichment in 62%, 43, 17, and 38% of experiments, indicating the growth of *C. polykrikoides* and everything else was limited by N-supply during blooms; more so than other algal populations. The low DIN:DIP ratios present during *C. polykrikoides* blooms (2.5 ± 0.4; Table 4) further supports the hypothesis that phytoplankton populations are N limited during blooms. Half-saturation constants for individual nutrients are often utilized as proxies for nutrient limitation in marine ecosystems and N concentrations below half-saturation constants are often considered limiting (Caperon and Meyer, 1972; Fisher et al., 1992). The concentrations of nitrate, ammonium, urea, and glutamic acid were generally near or below the half-saturation constants of *C. polykrikoides* cultures for these nutrients, further supporting the hypothesis that *C. polykrikoides* blooms are limited by N in NY estuaries. While *C. polykrikoides* grew slower than other phytoplankton in all but 14% of experimental control treatments, N-enrichment led to *C. polykrikoides* displaying growth rates faster than other phytoplankton groups in at least one N treatment in 32% of experiments (Table 9), suggesting that N enrichment can, at times, promote *C. polykrikoides* dominance. Interestingly, this species has been shown to be allelopathic to other phytoplankton (Tang and Gobler, in prep) and is most toxic when in exponential phase growth (Tang and Gobler, 2009). As such, the fastest growth rates in some N treatments could be due to both faster growth by *C. polykrikoides* and slower net growth rates by other phytoplankton due to allelopathic effects.

In a manner paralleling laboratory experiments, this species grew well when enriched with both organic and inorganic forms of N during experimental bloom conditions. However, *C.*

polykrikoides was not always the best competitor for N during amendment experiments, as other groups such as diatoms and other dinoflagellates displayed higher growth rates in many treatments with enriched levels of N (Table 8). This finding is consistent with low half saturation constants for nutrients displayed by cultures (1.8 – 2.9 μM; Table 2), suggesting the 10 μM N used in experiments were sometimes more favorable for species with higher growth rates and presumably higher half saturation coefficients. The faster growth rate of other phytoplankton during 10 μM N enrichment is also consistent with field observation of monospecific blooms occurring when DIN levels were generally lower (Table 5). Therefore, I conclude that growth of *C. polykrikoides* monospecific blooms are associated with modest, but not heavy nutrient enrichment.

N-uptake characteristics of C. polykrikoides blooms

Within near-monspecific microplankton (> 20 μm) blooms of *C. polykrikoides* (70 – 100% of cells), both organic and inorganic forms of N were assimilated although the dominant N source taken up varied by location. The former result is consistent with prior N-uptake experiments on bloom populations which were not size fractionated (Kudela et al., 2008; Mulholland et al., 2009) and with prior studies of HABs in general (Mulholland et al., 2002; Bronk et al., 2007). The N uptake by blooms seemed partly dependent on location. Within the two most eutrophic and enclosed sites studied (Old Fort Pond and Flanders Bay), nitrate and nitrite were the primary forms of N assimilated. These two sites had the highest levels of nitrate during this study, and are known to be heavily loaded by nitrate-contaminated groundwater (Schubert, 1998; Motulcon and Sañudo-Wilhelmy, 2001). In contrast, within open water locations where levels of nitrate were lower and urea and DON were significantly more abundant, organic nitrogen compounds (urea and glutamic acid) were assimilated at higher rates

(Table 8). *C. polykrikoides* densities were significantly correlated with DON concentrations during this study. As such, in open estuarine waters where is species was monospecific among the microphytoplankton (> 20 μm), the dominance of *C. polykrikoides* may be partly due to its ability to grow faster on DON sources such as glutamic acid, but also its ability to assimilate and grow rapidly on organic N when DIN levels are low. Although *C. polykrikoides* grew fastest on glutamic acid in culture, this compound never comprised more than 20% of its N assimilation in the field. This discrepancy was likely due to the lower glutamic acid concentrations during blooms (mean = 0.2 ± 0.1μM) which are below the half-saturation of *C. polykrikoides*, but likely within ideal concentrations for smaller, heterotrophic bacteria (Kirchman et al., 1994). Regardless, the overall pattern of differing nutrient sources being exploited by *C. polykrikoides* based on local environmental conditions is consistent with both my laboratory experiments and my field incubation experiments and suggests *C. polykrikoides* employs a flexible nutrient strategies to form blooms in both eutrophic and mesotrophic regions of estuaries.

The phagotrophic abilities of *C. polykrikoides* have been demonstrated within laboratory cultures of this species isolated from South Korea, and could circumvent the need to assimilate dissolved nutrients during blooms. However, during this study, dissolved N uptake rates were high, being similar to those measured during non-phagotrophic algal blooms in NY waters (*A. anophagefferens*; Berg et al., 1997; Mulholland et al., 2002). As such, dissolved nutrient acquisition is likely important pathways for NY bloom populations of *C. polykrikoides*. However, the shortfall of N uptake from dissolved compounds on two dates during this study (8/27/08, GPB, and 9/10/08, Flanders Bay; Table 3) could be due to phagotrophic acquisition of

N, the assimilation of dissolved N compounds not measured during this study (e.g. amino acids besides glutamic acid), an overestimate of N demand, or some combination of these factors.

In summary, *C. polykrikoides* was observed to grow rapidly on, and assimilate, both organic and inorganic forms of N. During field experiments, growth of this species was frequently stimulated by the enrichment of both organic and inorganic forms of N. Densities of *C. polykrikoides* were significantly correlated with concentrations DON and concentrations of urea were significantly higher is locations where *C. polykrikoides* was monospecific among the microphytoplankton (> 20 μm). In contrast, nitrate levels were significantly higher in regions where *C. polykrikoides* were mixed with other dinoflagellates. Finally, the dominant source of N assimilated by bloom populations of *C. polykrikoides* changed with location, with inorganic forms as nitrate and nitrite being the primary forms of N assimilated in the more eutrophic locations, while organic N was more commonly assimilated within mesotrophic locations where levels of DIN were lower. Overall, this species displays nutritional flexibility which may facilitate its ability to form large (> 50km) and extended (> 1 month) blooms on the US Atlantic coast. It is more likely to form monospecific blooms in regions with higher DON and lower DIN, but can also bloom along with other dinoflagellates in regions with higher DIN concentrations. Finally, the generally low growth rates displayed by this species in culture (μ_{max} = 0.4 – 0.5 d^{-1}) and in the field (0.1 – 1.0 d^{-1}; commonly 0.2 d^{-1}) compared other phytoplankton (Tables 8) suggests that other processes such as allelopathy and predator deterrence are likely to be important for bloom formation.

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	Total	Bloom	Non-Bloom
C. polykrikoides (mL^{-1})	4830 ± 1570	**6890 ± 2980**	**65.6 ± 89.1**
Diatoms (mL^{-1})	177 ± 203	82.3 ± 107	421 ± 605
Dinoflagellates (mL^{-1})	331 ± 320	470 ± 548	107 ± 21.6
Chlorophyll *a* (µg L-1) Total	53.3 ± 6.31	**69.5 ± 9.36**	**20.4 ± 33.7**
Chlorophyll *a* (µg L-1) <5µm	26.6 ± 8.83	26.4 ± 8.55	22.4 ± 30.0
Chlorophyll *a* (µg L-1) >5µm	33.0 ± 3.60	**41.3 ± 6.27**	**8.92 ± 5.71**
Salinity (psu)	27.9 ± 1.17	**27.5± 1.62**	**29.0 ± 0.480**
Temperature (°C)	24.2 ± 0.580	24.0 ± 0.720	24.6 ± 0.410
Dissolved Organic Nitrogen (µM)	21.1 ± 8.67	21.9 ± 8.20	19.5 ± 9.60
Dissolved Organic Phosphorous (µM)	1.46 ± 1.07	1.58 ± 1.16	1.04 ± 0.488
Nitrate (µM)	1.35 ± 0.890	1.55 ± 1.30	1.13 ± 1.02
Ammonium (µM)	1.30 ± 0.350	1.33 ± 0.570	1.20 ± 0.170
Urea (µM)	0.540 ± 0.350	0.54 ± 0.350	0.490 ± 0.330
Sillicate (µM)	40.7 ± 11.8	**44.9 ± 13.1**	**30.6 ± 14.1**
Phosphate (µM)	1.48 ± 0.260	1.61 ± 0.320	1.27 ± 0.230

Tables:

Table 1. Mean ± standard deviation (SD) for biological and chemical parameters measured from all sites from all years for bloom, non-bloom and both (total) periods. Parameters with statistically significant differences (t-test, $p<0.008$) between bloom and non-bloom are bolded.

	Glutamic acid	**Ammonium**	**Urea**	**Nitrate**
μ_{max} (d^{-1})	0.50 ± 0.10	0.41 ± 0.07	0.42 ± 0.10	0.41 ± 0.10
K_s (µM)	1.84 ± 0.60	2.60 ± 0.49	2.18 ± 0.51	2.94 ± 0.70
α (d^{-1} μM^{-1})	0.27	0.16	0.19	0.14

Table 2. Maximal growth rates (μ_{max}), half-saturation constants (K_s) and competition coefficients (d^{-1} μM^{-1}) for growth curves of *C. polykrikoides* cultures grown on glutamic acid, ammonium, urea, and nitrate.

Treatment	Measured Cells L^{-1}	Measured Growth rate d^{-1}	Measured N content	Estimated µM N L^{-1} d^{-1}	Estimated µM N L^{-1} h^{-1}	Measured µM N L^{-1} h^{-1}	Measured / Estimated
Glutmic acid, 2µM	246,064	0.3	2.3E-11	1.70E-06	7.07E-08	2.00E-07	2.83
Ammonium, 2µM	232,751	0.2	2.3E-11	1.07E-06	4.46E-08	1.00E-07	2.24
Nitrate, 2µM	222,397	0.2	2.3E-11	1.02E-06	4.26E-08	5.00E-08	1.17
Urea, 2µM	251,980	0.2	2.3E-11	1.16E-06	4.83E-08	5.00E-08	1.04
Glutmic acid, 20µM	453,151	0.5	2.3E-11	5.21E-06	2.17E-07	5.00E-07	2.30
Ammonium, 20µM	288,960	0.4	2.3E-11	2.66E-06	1.11E-07	2.50E-07	2.26
Nitrate, 20µM	299,315	0.4	2.3E-11	2.75E-06	1.15E-07	5.00E-08	0.44
Urea, 20µM	302,273	0.4	2.3E-11	2.78E-06	1.16E-07	5.00E-08	0.43
GPB, 8/27/08	5,484,000	0.2	2.3E-11	2.52E-05	1.05E-06	4.55E-07	0.43
FB, 9/10/08	3,376,000	0.2	2.3E-11	1.55E-05	6.47E-07	4.48E-07	0.69
SB, 9/16/08	2,884,000	0.2	2.3E-11	1.33E-05	5.53E-07	6.11E-07	1.11

Table 3. A comparison of N demand of *C. polykrikoides* cultures and field populations estimated from its N quota, cell densities, and growth rates to N uptake rates measured with ^{15}N. Cell densities and growth rates from cultures were quantified in situ. Field growth rates were extrapolated from low N cultures. N content was determined from PON measurements of cultures with known cell densities. Measured, field uptake rates were summed for all compounds, while measured uptake rates for cultures were for a single compound.

Site	Year	Date	*C. polykrikoides* (mL^{-1})	*C. polyrikoides* as % of cells >20 μm	Diatoms (mL^{-1})	Other Dinoflagellates (mL^{-1})	Chlorophyll *a* (μg L^{-1}) Total	Chlorophyll *a* (μg L^{-1}) >5μm
Flanders Bay	2006	22-Aug	20700 ± 2790	----	----	----	58.8 ± 1.66	42.6 ± 8.49
		25-Aug	3650 ± 116	----	----	----	30.4 ± 2.22	23.7 ± 0.30
		30-Aug	30000 ± 9490	----	----	----	269 ± 105	182 ± 24.8
	2007	22-Aug	13.0 ± 1.50	1	1810 ± 23.8	176 ± 12.5	7.32 ± 0.28	6.13 ± 0.52
		28-Aug	10.0 ± 1.50	0	2330 ± 99.4	29.0 ± 2.31	3.66 ± 0.19	2.79 ± 0.13
		30-Aug	8.00 ± 0.660	9	75.8 ± 9.87	5.50 ± 2.10	14.6 ± 1.03	6.28 ± 0.39
		4-Sep	974 ± 17.4	91	40.0 ± 5.02	54.0 ± 7.58	93.7 ± 1.08	49.5 ± 6.25
	2008	27-Aug	1050 ± 322	57	16.0 ± 0.00	786 ± 33.9	22.7 ± 1.36	10.3 ± 1.45
		28-Aug	5930 ± 554	96	4.00 ± 1.66	248 ± 33.2	53.5 ± 1.27	18.2 ± 6.04
		29-Aug	5770 ± 1090	95	4.00 ± 1.67	280 ± 22.6	61.1 ± 3.29	26.5 ± 7.64
		4-Sep	608 ± 147	70	124 ± 50.9	140 ± 5.66	8.80 ± 0.81	3.40 ± 0.80
		10-Sep	4950 ± 56.6	95	132 ± 16.9	120 ± 56.6	68.8 ± 5.07	25.6 ± 2.79
		17-Sep	4120 ± 690	94	12.0 ± 5.66	256 ± 102	55.8 ± 3.68	12.5 ± 2.25
		24-Sep	480 ± 45.3	72	80.0 ± 22.6	104 ± 56.6	18.3 ± 0.25	18.0 ± 1.14
Great Peconic Bay	2006	22-Aug	13.7 ± 5.31	----	----	----	7.12 ± 0.18	2.09 ± 0.52
		30-Aug	5590 ± 9450	----	----	----	242 ± 1.13	204 ± 17.7
	2007	22-Aug	0.00 ± 0.00	0	14.0 ± 2.31	120 ± 40.0	4.75 ± 0.57	4.04 ± 0.23
		28-Aug	0.00 ± 0.00	0	0.00 ± 0.00	66.7 ± 23.1	1.50 ± 0.13	0.83 ± 0.05
		30-Aug	0.00 ± 0.00	0	0.00 ± 0.00	80.0 ± 17.3	5.41 ± 1.04	3.13 ± 0.20
		4-Sep	0.00 ± 0.00	0	0.00 ± 0.00	160 ± 26.5	6.38 ± 1.35	4.48 ± 0.19
	2008	27-Aug	9140 ± 2460	94	16.0 ± 2.63	548 ± 77.2	51.2 ± 1.66	37.8 ± 4.07
		28-Aug	3690 ± 475	97	0.00 ± 0.00	108 ± 16.9	56.4 ± 1.98	22.4 ± 2.16
		29-Aug	3960 ± 869	95	8.00 ± 0.00	216 ± 56.6	55.0 ± 4.41	24.5 ± 3.71
		4-Sep	6920 ± 492	93	0.00 ± 0.00	492 ± 50.9	66.7 ± 2.20	35.6 ± 2.55
		10-Sep	1860 ± 49.2	96	44.0 ± 5.66	36.0 ± 5.66	45.5 ± 1.66	17.7 ± 1.80
		11-Sep	2290 ± 90.5	99	24.0 ± 0.00	0.00 ± 0.00	49.7 ± 1.11	24.1 ± 1.88
Meeting House Creek	2006	21-Aug	159 ± 27.8	----	----	----	242 ± 1.13	204 ± 17.7
		22-Aug	910 ± 82.3	----	----	----	42.6 ± 0.53	20.5 ± 2.17
		30-Aug	20800 ± 1670	----	----	----	62.8 ± 1.50	37.4 ± 2.81
		5-Sep	1950 ± 213	----	----	----	48.9 ± 1.92	21.6 ± 3.73
		7-Sep	13700 ± 5700	----	----	----	102 ± 7.64	67.8 ± 1.78
	2007	22-Aug	26.6 ± 3.09	20	13.0 ± 2.31	93.3 ± 6.11	3.30 ± 0.50	2.28 ± 0.08
		28-Aug	10.0 ± 1.50	1	656 ± 39.1	96.0 ± 17.3	26.2 ± 2.62	12.9 ± 4.19
		30-Aug	23.0 ± 2.00	11	54.3 ± 4.32	124 ± 9.67	37.2 ± 0.51	27.2 ± 1.09
		4-Sep	133 ± 61.0	31	107 ± 42.2	187 ± 46.2	93.9 ± 2.21	10.0 ± 2.75
	2008	27-Aug	1120 ± 117	18	0.00 ± 0.00	5270 ± 361	75.2 ± 2.83	64.5 ± 12.8
		28-Aug	4160 ± 147	99	0.00 ± 0.00	58.5 ± 4.95	79.9 ± 3.02	65.7 ± 12.2
		29-Aug	1270 ± 93.9	46	40.0 ± 11.3	1460 ± 238	21.9 ± 0.76	9.85 ± 0.95
		4-Sep	1220 ± 31.7	55	604 ± 17.0	384 ± 11.3	21.9 ± 0.38	19.4 ± 1.10
		11-Sep	952 ± 102	51	572 ± 130	336 ± 67.9	41.5 ± 0.20	22.6 ± 1.18
		24-Sep	1120 ± 56.6	48	0.00 ± 0.00	1190 ± 106	55.2 ± 0.94	46.4 ± 1.56
Old Fort Pond	2006	24-Aug	868 ± 22.6	----	----	----	48.8 ± 14.39	25.7 ± 4.19
		31-Aug	33300 ± 9090	----	----	----	----	----
		1-Sep	12000 ± 1760	----	----	----	----	----
		7-Sep	984 ± 52.6	----	----	----	----	----
	2007	22-Aug	1210 ± 266	43	0.00 ± 0.00	1600 ± 1220	45.5 ± 2.36	41.9 ± 1.31
		28-Aug	1270 ± 55.6	46	0.00 ± 0.00	1470 ± 254	54.8 ± 4.83	52.9 ± 9.90
		30-Aug	430 ± 20.7	24	40.0 ± 2.62	1290 ± 244	35.3 ± 4.56	19.8 ± 1.25
		4-Sep	160 ± 14.4	12	0.00 ± 0.00	1160 ± 69.3	27.7 ± 4.27	5.92 ± 0.38
	2008	13-Aug	892 ± 16.9	24	1930 ± 136	896 ± 113	28.9 ± 0.98	18.1 ± 0.36
		22-Aug	276 ± 107	7	1220 ± 10.0	2430 ± 90.5	34.4 ± 2.94	20.0 ± 1.66
		3-Sep	476 ± 39.6	69	180 ± 73.5	36.0 ± 2.83	31.9 ± 0.87	24.4 ± 2.50
		5-Sep	9630 ± 741	100	0.00 ± 0.00	8.00 ± 1.13	172 ± 17.33	90.7 ± 11.7
		23-Sep	2772.00 ± 254.55	97	32.0 ± 2.26	56.0 ± 4.53	53.2 ± 3.67	36.9 ± 3.10
Shinnecock Bay	2006	5-Sep	5110 ± 97.9	----	----	----	173 ± 9.07	----
	2007	22-Aug	107 ± 23.1	50	13.3 ± 2.30	93.3 ± 6.11	3.49 ± 0.29	----
		28-Aug	0.00 ± 0.00	0	53.3 ± 3.09	160 ± 40.0	2.36 ± 0.09	----
		30-Aug	0.00 ± 0.00	0	26.7 ± 6.42	93.3 ± 14.6	5.30 ± 0.14	----
		4-Sep	0.00 ± 0.00	0	80.0 ± 10.5	120 ± 40.0	4.91 ± 0.81	----
	2008	22-Aug	2390 ± 668	80	44.0 ± 5.10	552 ± 0.00	25.3 ± 1.33	20.8 ± 1.25
		28-Aug	8540 ± 735	99	4.00 ± 2.66	116 ± 16.9	45.9 ± 2.72	38.3 ± 3.46
		29-Aug	8130 ± 328	98	16.0 ± 2.62	184 ± 47.1	59.7 ± 2.13	37.7 ± 1.79
		4-Sep	7580 ± 56.6	95	64.0 ± 9.05	372 ± 62.2	53.7 ± 5.52	56.4 ± 3.45
		5-Sep	5300 ± 1320	97	36.0 ± 16.9	120 ± 0.00	121 ±14.58	47.3 ± 0.83
		16-Sep	2880 ± 226	97	16.0 ± 4.31	60.0 ± 19.9	40.7 ± 2.07	35.8 ± 4.37
		23-Sep	2550 ± 192	95	72.0 ± 22.6	64.0 ± 11.3	54.0 ± 4.17	36.1 ± 5.20

Table 4. Cell abundances mL^{-1} and chlorophyll *a* biomass (μg L^{-1}) at all sampling sites from 2006-2008.

Site	Year	Date	Salinity (psu)	Temperature (°C)	Dissolved Organic Nitrogen (µM)	Dissolved Organic Phosphorous (µM)	Nitrate (µM)	Ammonia (µM)	Urea (µM)	Sillicate (µM)	Phosphate (µM)	DIN:DIP
Flanders Bay	2006	22-Aug	24.0	20.6	----	2.72 ± 0.04	0.41 ± 0.14	2.32 ± 0.24	----	80.6 ± 4.94	1.46 ± 0.11	1.87
		25-Aug	25.8	22.3	----	1.38 ± 0.30	0.20 ± 0.19	1.57 ± 0.11	----	82.9 ± 4.49	1.42 ± 0.06	1.25
		30-Aug	24.0	20.7	----	1.47 ± 1.14	4.50 ± 0.82	1.48 ± 0.10	----	45.5 ± 0.24	1.63 ± 0.81	3.67
	2007	22-Aug	28.9	23.6	21.9 ± 3.10	0.93 ± 0.50	5.44 ± 2.78	1.33 ± 0.43	0.37 ± 0.30	34.1 ± 6.49	1.16 ± 0.30	5.84
		28-Aug	28.1	24.5	10.9 ± 0.19	0.59 ± 0.02	1.38 ± 1.31	1.10 ± 0.30	0.08 ± 0.03	27.6 ± 1.30	1.07 ± 0.26	2.32
		30-Aug	29.1	24.0	8.48 ± 3.19	0.64 ± 0.00	1.15 ± 0.65	1.02 ± 0.20	0.37 ± 0.42	20.5 ± 6.70	0.86 ± 0.39	2.52
		4-Sep	28.5	24.2	25.3 ± 4.72	1.42 ± 0.10	0.511 ± 0.36	8.30 ± 0.21	0.81 ± 0.51	35.2 ± 7.42	1.38 ± 0.43	5.37
	2008	27-Aug	23.9	23.6	27.6 ± 4.03	1.87 ± 0.35	0.49 ± 0.23	1.23 ± 0.20	0.04 ± 0.01	54.0 ± 3.65	2.35 ± 0.16	0.73
		28-Aug	27.4	24.6	30.2 ± 6.80	3.37 ± 1.60	0.72 ± 0.26	1.26 ± 0.44	0.06 ± 0.02	50.7 ± 7.58	2.10 ± 0.40	0.94
		29-Aug	27.9	24.3	26.1 ± 2.86	2.17 ± 0.47	0.41 ± 0.12	0.88 ± 0.35	0.05 ± 0.02	40.8 ± 7.80	1.57 ± 0.22	0.82
		4-Sep	26.8	25.9	25.8 ± 1.44	1.37 ± 0.38	0.80 ± 0.61	0.68 ± 0.24	0.04 ± 0.02	50.2 ± 3.05	1.37 ± 0.11	1.08
		10-Sep	27.4	23.8	16.4 ± 2.52	1.75 ± 0.06	0.47 ± 0.13	1.06 ± 0.30	0.04 ± 0.03	30.0 ± 7.40	1.35 ± 0.45	1.13
		17-Sep	27.6	23.1	24.9 ± 7.20	0.00 ± 0.19	0.30 ± 0.09	1.12 ± 0.53	0.06 ± 0.03	25.1 ± 3.23	2.04 ± 0.21	0.70
		24-Sep	27.6	19.3	44.0 ±7.03	4.26 ± 0.15	0.32 ±0.11	0.22 ± 0.16	0.12 ±0.23	39.9 ± 1.90	1.22 ± 0.22	0.44
Great Peconic Bay	2006	22-Aug	28.0	24.9	----	----	----	----	----	----	----	----
		30-Aug	27.7	25.0	----	----	----	----	----	----	----	----
	2007	22-Aug	30.0	24.2	12.1 ± 7.18	0.52 ± 0.30	1.37 ± 1.52	1.80 ± 0.18	0.46 ± 0.16	9.11 ± 0.74	1.72 ± 0.14	1.84
		28-Aug	30.1	24.4	13.7 ± 1.75	0.78 ± 0.05	0.22 ± 0.16	1.27 ± 0.19	0.27 ± 0.17	16.4 ± 3.33	0.83 ± 0.09	1.80
		30-Aug	30.3	24.5	12.5 ±3.39	1.55 ± 0.30	0.53 ± 0.29	1.26 ± 0.07	0.17 ± 0.05	21.5 ± 0.64	0.78 ± 0.09	2.29
		4-Sep	30.6	23.7	7.92 ± 5.41	1.35 ± 0.60	0.17 ± 0.14	1.33 ± 0.40	0.18 ± .011	16.9 ± 5.29	1.20 ± 0.18	1.25
	2008	27-Aug	28.3	24.2	16.4 ± 3.91	2.06 ± 0.46	0.60 ± 0.32	0.27 ± 0.27	0.36 ± 0.17	47.3 ± 5.48	1.79 ± 0.25	0.49
		28-Aug	28.0	24.2	12.8 ± 2.71	1.37 ± 0.50	0.70 ± 0.20	1.12 ± 1.03	0.36 ±0.08	45.0 ± 8.81	2.14 ± 0.96	0.85
		29-Aug	28.4	24.7	17.0 ± 0.61	1.81 ± 0.69	0.90 ± 0.39	0.77 ± 0.28	0.46 ±0.12	39.3 ± 6.31	1.29 ± 0.25	1.29
		4-Sep	28.4	25.2	17.8 ± 3.96	2.22 ± 0.60	0.70 ± 0.10	0.35 ± 0.23	0.42 ± 0.12	53.8 ± 1.86	1.56 ± 0.23	0.67
		10-Sep	28.0	23.9	14.9 ± 3.95	1.36 ± 0.37	0.45 ± 0.10	0.99 ± 0.56	0.44 ± 0.08	33.6 ± 11.2	1.02 ± 0.38	1.41
		11-Sep	28.3	23.5	15.3 ± 2.34	0.79 ± 0.30	0.74 ± 0.09	0.28 ± 0.07	0.68 ± 0.36	45.8 ± 1.21	1.48 ± 0.17	0.69
Meeting House Creek	2006	21-Aug	25.4	26.1	12.2 ± 1.48	0.27 ± 0.01	1.45 ± 0.15	1.91 ± 0.09	----	110 ± 5.95	2.66 ± 0.09	1.26
		22-Aug	24.9	25.4	9.29 ± 2.74	0.85 ± 0.10	2.07 ± 0.46	2.91 ± 0.73	----	88.7 ± 6.80	3.60 ± 0.36	1.38
		30-Aug	21.8	20.8	22.9 ± 0.50	0.14 ± 0.01	1.84 ± 0.02	5.55 ± 1.95	----	51.5 ± 6.84	3.17 ± 0.63	2.33
		5-Sep	24.4	20.2	19.6 ± 5.12	3.51 ±0.80	2.02 ± 0.49	6.00 ± 0.40	----	55.3 ± 4.55	2.46 ± 0.87	3.26
		7-Sep	24.7	20.1	37.5 ± 17.7	1.93 ± 0.47	2.25 ± 0.26	3.34 ± 0.70	----	45.4 ± 3.93	0.72 ± 0.09	7.76
	2007	22-Aug	29.8	24.0	41.9 ± 17.8	1.18 ± 1.07	0.02 ± 0.006	1.38 ± 0.34	0.25 ± 0.03	31.1 ± 6.64	2.67 ± 0.15	0.52
		28-Aug	30.2	24.4	32.7 ± 17.6	2.26 ± 0.77	1.20 ± 0.85	0.91 ± 0.09	0.86 ± 0.19	47.3 ± 1.01	0.44 ± 0.16	4.80
		30-Aug	28.2	25.3	25.9 ± 5.03	1.46 ± 0.09	0.10 ± 0.005	0.59 ± 0.06	0.23 ± 0.14	22.8 ± 0.43	0.17 ± 0.03	4.06
		4-Sep	26.8	25.7	22.4 ± 5.35	0.81 ± 0.20	0.46 ± 0.19	1.08 ± 0.06	0.45 ± 0.15	37.1 ± 5.20	1.69 ± 0.88	0.91
	2008	27-Aug	26.3	24.7	31.1 ± 6.26	3.69 ± 0.36	1.37 ± 0.34	2.68 ± 1.28	0.20 ± 0.16	73.3 ± 7.44	2.29 ± 0.46	1.77
		28-Aug	26.4	25.6	32.1 ± 6.50	3.57 ± 1.17	0.96 ± 0.48	1.13 ± 0.53	0.97 ± 0.20	63.9 ± 13.4	2.05 ± 0.64	1.02
		29-Aug	26.5	24.6	26.7 ± 3.46	0.59 ± 0.20	0.90 ± 0.36	0.16 ± 0.08	0.26 ± 0.06	74.9 ± 5.91	1.67 ± 0.70	0.63
		4-Sep	26.3	25.7	16.2 ± 0.82	0.20 ± 0.05	0.65 ± 0.15	0.12 ± 0.10	0.36 ± 0.03	53.8 ± 9.57	1.37 ± 0.79	0.56
		11-Sep	25.4	23.5	15.5 ± 0.91	1.19 ± 0.03	0.97 ± 0.42	0.15 ± 0.07	0.34 ± 0.15	79.4 ± 2.25	1.69 ± 0.52	0.66
		24-Sep	26.2	20.9	14.2 ± 1.63	0.47 ± 0.03	1.08 ± 0.49	0.18 ± 0.09	0.39 ± 0.19	64.0 ± 6.68	1.40 ± 0.41	0.90
Old Fort Pond	2006	24-Aug	----	----	33.4 ± 2.29	0.3 ± 0.03	0.27 ± 0.13	1.62 ± 0.16	----	37.2 ± 1.18	2.02 ± 0.07	0.94
		31-Aug	----	----	36.8 ± 8.02	0.55 ± 0.15	0.27 ± 0.16	1.46 ± 0.03	----	45.4 ± 1.66	2.83 ± 0.33	0.61
		1-Sep	----	----	----	----	----	----	----	----	----	----
		7-Sep	----	----	19.9 ± 3.73	0.19 ± 0.17	0.10 ± 0.06	1.71 ± 0.16	----	72.4 ± 2.58	1.71 ± 0.03	1.06
	2007	22-Aug	30.2	25.0	29.7 ± 8.26	0.90 ± 0.59	7.92 ± 1.20	1.85 ± 0.69	0.36 ± 0.08	32.4 ± 5.33	2.95 ± 0.63	3.31
		28-Aug	29.8	25.1	13.5 ± 5.22	1.20 ± 0.40	9.61 ± 2.50	2.62 ± 0.03	1.13 ± 0.43	26.8 ± 14.1	2.96 ± 2.15	4.13
		30-Aug	30.0	25.1	8.27 ± 2.05	1.09 ± 0.00	8.82 ± 0.90	0.99 ± 0.03	0.28 ± 0.01	41.3 ± 4.37	0.54 ± 0.14	18.2
		4-Sep	30.6	24.6	16.9 ± 4.13	1.20 ± 0.19	0.21 ± 0.02	1.94 ± 0.03	1.78 ± 0.20	22.8 ± 0.72	2.69 ±1.80	0.80
	2008	13-Aug	28.6	25.0	16.5 ± 1.02	1.24 ± 0.14	1.91 ± 1.02	0.65 ± .31	0.46 ± 0.44	13.2 ± 3.53	0.29 ± 0.14	8.83
		22-Aug	26.7	25.5	18.2 ± 0.41	1.21 ± 0.21	1.10 ± 1.07	0.09 ± 0.06	0.15 ± 0.13	36.1 ± 1.58	0.07 ± 0.04	17.0
		3-Sep	30.2	23.1	24.9 ± 0.09	0.87 ± 0.18	1.27 ± 1.26	0.44 ± 0.27	0.31 ± 0.26	16.8 ± 0.83	0.55 ± 0.11	3.11
		5-Sep	22.8	26.2	20.9 ± 0.35	1.72 ± 0.12	1.42 ± 1.14	0.65 ± 0.31	0.63 ± 0.30	37.5 ± 13.6	0.90 ± 0.49	2.30
		23-Sep	30.3	19.1	15.3 ± 0.06	0.20 ± 0.09	0.54 ± 0.30	0.23 ± 0.17	0.08 ± 0.07	12.5 ± 4.50	0.90 ± 0.14	0.86
Shinnecock Bay	2006	5-Sep	----	----	----	----	----	----	----	----	----	----
	2007	22-Aug	----	----	----	----	----	----	----	----	----	----
		28-Aug	----	----	----	----	----	----	----	----	----	----
		30-Aug	----	----	----	----	----	----	----	----	----	----
		4-Sep	----	----	----	----	----	----	----	----	----	----
	2008	22-Aug	28.3	25.6	11.0 ± 1.12	0.71 ± 0.18	0.72 ± 0.25	0.82 ± 0.24	0.42 ± 0.14	33.7 ± 14.0	0.64 ± 0.16	2.41
		28-Aug	28.8	24.2	23.1 ± 2.04	2.37 ± 0.14	0.73 ± 0.21	1.26 ± 0.37	0.87 ± 0.32	32.1 ± 9.23	1.64 ± 0.39	1.21
		29-Aug	29.4	24.2	26.3 ± 6.71	2.18 ± 0.23	0.67 ± 0.16	1.85 ± 0.59	0.90 ± 0.38	21.3 ± 2.63	1.53 ± 0.23	1.65
		4-Sep	28.9	24.5	25.5 ± 2.95	0.38 ± 0.10	0.10 ± 0.09	0.84 ± 0.47	0.85 ± 0.23	39.1 ± 2.59	1.44 ± 0.28	0.65
		5-Sep	27.7	26.9	21.7 ± 1.45	4.87 ± 0.19	0.51 ± 0.12	0.96 ± 0.56	1.09 ± 0.42	55.4 ± 3.20	0.85 ± 0.17	1.73
		16-Sep	26.9	21.7	23.1 ± 3.92	2.18 ± 0.15	0.84 ± 0.18	0.52 ± 0.29	0.77 ± 0.58	31.1 ± 15.3	1.01 ± 0.15	1.35
		23-Sep	30.7	19.0	11.7 ± 0.38	1.24 ± 0.09	0.83 ± 0.17	1.96 ± 0.24	2.85 ± 0.51	23.5 ± 1.63	0.87 ± 0.14	3.21

Table 5. Salinity (psu), temperature (°C), and dissolved nutrient concentrations (µM) at all sampling sites from 2006-2008.

Site		*C. polyrikoides* as % of cells >20 µm	*C. polykrikoides* (mL^{-1})	Diatoms (mL^{-1})	Other Dinoflagellates (mL^{-1})	Chlorophyll *a* (µg L-1) Total	Chlorophyll *a* (µg L-1) >5µm	Salinity (psu)	Temperature (°C)	Dissolved Organic Nitrogen (µM)	Dissolved Organic Phosphorous (µM)	Nitrate (µM)	Ammonia (µM)	Urea (µM)	Sillicate (µM)	Phosphate (µM)	DIN:DIP
Flanders Bay	Total	----	5590 ± 8850	421 ± 825	200 ± 215	54.8 ± 67.5	30.5 ± 45.8	27.0 ± 1.83	23.3 ± 1.72	23.8 ± 9.68	1.84 ± 1.07	1.22 ± 1.63	1.68 ±1.96	0.185 ± 0.242	44.1 ± 18.8	1.49 ± 0.42	2.12 ± 1.91
	Bloom	96	7120 ± 9480	51.5 ± 53.5	248 ± 232	67.4 ± 71.4	37.5 ± 49.8	26.5 ± 1.78	22.9 ± 1.92	27.5 ± 7.74	1.98 ± 1.14	0.462 ± 0.175	1.83 ± 2.21	0.153 ± 0.267	48.6 ± 18.6	1.63 ± 0.369	1.50 ± 1.66
	Non-Bloom	<1	10.3 ± 2.53	1410 ± 1180	70.2 ± 02.4	8.54 ± 5.59	5.07 ± 1.97	28.7 ± 0.541	24.2 ± 0.517	13.7 ± 7.12	0.720 ± 0.184	2.66 ± 2.41	1.15 ± 0.161	0.273 ± 0.167	27.4 ± 6.83	1.03 ± 0.154	3.56 ± 1.97
Great Peconic Bay	Total	----	10500 ± 18600	21.2 ± 13.9	201 ± 200	49.4 ± 65.7	31.8 ± 55.9	28.8 ± 1.04	24.4 ± 0.515	14. 1 ± 2.91	1.38 ± 0.561	0.638 ± 0.344	0.944 ± 0.516	0.380 ± 0.151	32.9 ± 15.7	1.38 ± 0.439	1.26 ± 0.592
	Bloom	97	11900 ± 19600	23.0 ± 15.4	296 ± 261	81.0 ± 71.5	52.4 ± 67.5	28.2 ± 0.32	24.4 ± 0.59	15.7 ± 1.78	1.60 ± 0.53	0.682 ± 0.149	0.630 ± 0.379	0.453 ± 0.118	44.1 ± 6.95	1.55 ± 0.389	0.901 ± 0.371
	Non-Bloom	10	13.7 ± 0.103	14.0 ± 0.321	107 ± 42.2	5.03 ± 2.17	2.91 ± 1.48	29.7 ± 1.13	24.3 ± 0.453	11.6 ± 2.51	1.05 ± 0.481	0.573 ± 0.555	1.42 ± 0.259	0.270 ± 0.134	15.9 ± 5.13	1.13 ± 0.434	1.79 ± 0.428
Meeting House Creek	Total	----	3170 ± 5970	292 ± 300	919 ± 160	63.7 ± 56.8	42.2 ± 49.7	26.2 ± 2.45	24.1 ± 1.97	24.0 ± 9.69	1.47 ± 1.25	1.16 ± 0.690	1.87 ± 1.89	0.431 ± 0.268	59.9 ± 23.0	1.87 ± 0.977	2.12 ± 2.05
	Bloom	72	4730 ± 6890	405 ± 316	1450 ± 1950	55.3 ± 25.6	37.6 ± 22.0	25.1 ± 1.65	23.7 ± 2.32	22.5 ± 9.12	1.61 ± 1.46	1.41 ± 0.58	2.22 ± 2.25	0.42 ± 0.28	65.0 ± 13.8	2.04 ± 0.869	2.03 ± 2.19
	Non-Bloom	17	70.3 ± 69.9	207 ± 301	125 ± 43.4	80.6 ± 96.4	51.4 ± 86.1	28.9 ± 1.83	24.9 ± 0.787	27.0 ± 11.2	1.19 ± 0.743	0.646 ± 0.648	1.17 ± 0.501	0.448 ±0.292	49.7 ± 35.1	1.53 ± 1.19	2.31 ± 1.97
Old Fort Pond	Total	----	4950 ± 9330	679 ± 855	994 ± 833	53.3 ± 43.1	33.6 ± 24.1	28.6 ± 2.87	24.9 ± 1.06	21.2 ± 8.48	0.889 ± 0.484	2.79 ± 3.68	1.19 ± 0.791	0.576 ± 0.548	32.9 ± 16.6	1.53 ± 1.12	5.09 ± 6.27
	Bloom	87	5810 ± 9950	111 ± 86.8	795 ± 718	58.9 ± 47.0	38.8 ± 24.1	28.6 ± 3.24	24.8 ± 1.31	21.9 ± 9.18	0.826 ± 0.509	3.56 ± 3.98	1.22 ± 0.753	0.464 ± 0.338	33.5 ± 18.0	1.57 ± 1.07	4.32 ± 5.45
	Non-Bloom	8	218 ± 82.0	608 ± 859	1790 ± 899	31.0 ± 4.77	12.9 ± 9.96	28.7 ± 2.72	25.0 ±0.636	17.6 ± 0.849	1.21 ± 0.007	0.655 ±0.629	1.02 ± 1.31	0.965 ± 1.15	29.5 ± 9.46	1.39 ± 1.85	8.89 ± 11.5
Shinnecock Bay	Total	----	4730 ± 2950	38.7 ± 25.9	175 ± 152	49.2 ± 51.9	38.9 ± 10.9	28.7 ± 1.21	24.1 ± 2.71	20.3 ± 6.33	1.99 ± 1.49	0.629 ± 0.258	1.17 ± 0.547	1.11 ± 0.795	33.7 ± 11.3	1.14 ± 0.391	1.74 ± 0.839
	Bloom	96	5310 ± 2550	36.0 ± 25.7	209 ± 185	71.8 ± 49.9	38.9 ± 10.9	28.7 ± 1.21	24.1 ±2.71	20.3 ± 6.33	1.99 ± 1.49	0.629 ±0.258	1.17 ± 0.547	1.11 ± 0.795	33.7 ± 11.3	1.14 ± 0.391	1.74 ± 0.839
	Non-Bloom	14	26.7 ± 53.3	43.3 ± 29.6	117 ± 31.5	4.02 ± 1.35	----	----	----	----	----	----	----	----	----	----	----

Table 6. Mean ± standard deviation (SD) of cell abundances, chlorophyll *a* levels, salinity, temperature and ambient dissolved nutrients during bloom, non-bloom and both (total) periods at all sites during 2006-2008. A bloom is defined as > 330 cells mL^{-1}.

Site	Date	Salinity (psu)	Temperature (°C)	Total chlorophyll *a* (μg L^{-1})	*C. polykrikoides* (mL^{-1})	*C. polykrikoides* as % of total cells >20 μm	PON (μg N) of matter >20 μM	Ambient Concentrations (μM)				
								DFAA	Ammoinium	Urea	Nitrate	Nitrite
Old Fort Pond	9/3/2008	30.2	23.1	32.0 ± 0.87	476 ± 40	68	13.1 ± 3.26	0.423 ± 0.017	0.442 ± 0.27	0.308 ± 0.26	1.27 ± 1.2	0.602 ± 0.24
Shinnecock Bay	8/22/2008	28.3	25.6	25.3 ± 1.3	2390 ± 670	80	13.2 ± 6.78	0.151 ± 0.006	0.816 ± 0.24	0.418 ± 0.14	0.400 ± 0.16	0.680 ± 0.16
	8/25/2008	29.4	23.7	27.4 ± 3.0	1200 ± 280	78	7.18 ± 2.23	0.519 ± 0.021	0.446 ± 0.15	0.807 ± 0.33	0.216 ± 0.16	0.445 ± 0.20
	9/5/2008	27.7	26.9	48.2 ± 5.9	2020 ± 240	76	42.5 ± 5.41	0.824 ± 0.033	0.958 ± 0.56	1.08 ± 0.42	0.070 ± 0.06	0.569 ± 0.19
	9/16/2008	26.9	21.7	40.7 ± 2.1	2880 ± 51	97	36.5 ± 9.67	0.147 ± 0.006	0.522 ± 0.29	0.768 ± 0.58	0.268 ± 0.18	0.469 ± 0.13
Great Peconic Bay	8/27/2008	28.3	24.2	55.4 ± 4.0	5480 ± 85	96	19.9 ± 3.58	0.569 ± 0.023	0.431 ± 0.27	0.358 ± 0.17	0.598 ± 0.32	0.608 ± 0.15
	9/4/2008	28.4	25.2	55.7 ± 2.8	2710 ± 110	81	35.5 ± 2.15	0.572 ± 0.023	0.350 ± 0.23	0.422 ± 0.12	0.690 ± 0.20	0.336 ± 0.10
Flanders Bay	8/29/2008	27.9	24.3	33.1 ± 1.0	1040 ± 23	83	12.8 ± 2.96	0.516 ± 0.021	0.883 ± 0.35	0.100 ± 0.02	0.721 ± 0.26	0.636 ± 0.26
	9/10/2008	27.4	23.8	71.1 ± 1.5	3380 ± 290	94	28.7 ± 0.823	1.50 ± 0.060	1.06 ± 0.30	0.100 ± 0.03	0.474 ± 0.13	0.445 ± 0.16
	9/24/2008	27.6	19.3	18.3 ± 0.25	480 ± 45	78	13.3 ± 2.03	0.280 ± 0.011	0.222 ± 0.16	0.214 ± 0.14	0.323 ± 0.11	0.326 ± 0.04

Table 7. Salinity (psu), temperature (°C), total chlorophyll *a* (μg L^{-1}), cell density of *C. polykrikoides* (mL^{-1}) . Particulate oraginic nitrogen (PON, ug N L^{-1}) and ambient concentratins (μM) of dissolved free amino-acids (DFAA) and other N source and percentage of total cells >20 μm that were *C. polykrikoides* from field ^{15}N experiments.

Site and Date	*C. polykrikoides* (μ)					Other dinoflagellates (μ)					Diatoms (μ)					Chlorophyll *a* <5 μm (μ)				
Old Fort Pond	Control	Nitrate	Urea	Glutamic acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia
9/3/2008	0.37 ± 0.22	**0.75 ± 0.10**	**0.64 ± 0.13**	0.32 ± 0.13	**0.64 ± 0.13**	0.69 ± 0.11	0.73 ± 0.32	0.70 ± 0.16	0.11 ± 0.35	0.45 ± 0.21	-0.93 ± 0.49	-0.54 ± 0.19	-0.61 ± 0.54	-0.33 ± 0.07	-0.33 ± 0.47	-0.51 ± 0.25	-0.36 ± 0.32	-0.44 ± 0.73	-1.06 ± 0.00	**0.14 ± 0.01**
9/8/2008	0.15 ± 0.20	0.23 ± 0.24	0.36 ± 0.33	0.60 ± 0.14	0.05 ± 0.25	-0.46 ± 0.31	-0.18 ± 0.25	-0.18 ± 0.50	-0.13 ± 0.35	-0.54 ± 0.00	0.35 ± 0.15	0.51 ± 0.10	0.47 ± 0.37	-0.28 ± 0.00	-0.63 ± 0.00	0.00 ± 0.00	0.13 ± 0.06	0.02 ± 0.20	0.03 ± 0.14	-0.45 ± 1.01
10/2/2008	0.39 ± 0.39	-0.03 ± 0.20	0.07 ± 0.08	0.42 ± 0.37	0.47 ± 0.15	0.38 ± 0.12	0.43 ± 0.15	-0.14 ± 0.23	0.57 ± 0.16	0.41 ± 0.39	0.75 ± 0.45	1.47 ± 0.15	10.03 ± 0.30	'.46 ± 0.04	1.58 ± 0.51	0.03 ± 0.12	**0.54 ± 0.06**	**0.52 ± 0.06**	0.25 ± 0.10	0.14 ± 0.18
8/30/2007	-0.08 ± 0.02	**0.02 ± 0.01**	**0.03 ± 0.01**	-0.09 ± 0.04	**0.19 ± 0.04**	0.53 ± 0.04	**0.74 ± 0.01**	0.43 ± 0.03	**0.67 ± 0.01**	0.51 ± 0.05	1.20 ± 0.05	0.63 ± 0.17	1.11 ± 0.04	1.01 ± 0.05	1.18 ± 0.06	-0.29 ± 0.07	0.03 ± 0.13	-0.05 ± 0.03	-1.15 ± 0.36	0.20 ± 0.05
8/30/2005	-0.09 ± 0.35	**0.63 ± 0.21**	NA	NA	NA	-0.001 ± 0.03	0.06 ± 0.09	NA	NA	NA	-0.005 ± 0.09	0.01 ± 0.04	NA	NA	NA	NA	NA	NA	NA	NA
9/13/2005	-0.001 ± 0.04	0.06 ± 0.04	NA	NA	NA	0.00 ± 0.04	-0.03 ± 0.04	NA	NA	NA	-0.004 ± 0.08	**0.85 ± 0.05**	NA	NA	NA	NA	NA	NA	NA	NA
9/27/2005	0.08 ± 0.34	**0.29 ± 0.09**	NA	NA	NA	-0.002 ± 0.05	-0.004 ± 0.04	NA	NA	NA	-0.0004 ± 0.03	**0.29 ± 0.01**	NA	NA	NA	NA	NA	NA	NA	NA
	C. polykrikoides (μ)					Other dinoflagellates (μ)					Diatoms (μ)					Chlorophyll *a* <5 μm (μ)				
Great Peconic Bay	Control	Nitrate	Urea	Glutamic acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia
9/4/2008	-0.06 ± 0.07	0.14 ± 0.12	0.10 ± 0.02	**0.11 ± 0.05**	**0.21 ± 0.07**	-0.07 ± 0.04	-0.18 ± 0.10	-0.14 ± 0.07	-0.23 ± 0.03	-0.26 ± 0.07	NA	NA	NA	NA	NA	-0.19 ± 0.33	-0.28 ± 0.13	-0.20 ± 0.01	-0.28 ± 0.15	-0.13 ± 0.08
9/11/2008	-0.07 ± 0.04	-0.18 ± 0.10	-0.14 ± 0.07	-0.04 ± 0.13	-0.23 ± 0.03	0.64 ± 0.14	0.42 ± 0.48	0.58 ± 0.08	0.45 ± 0.14	0.52 ± 0.09	NA	NA	NA	NA	NA	-1.36 ± 0.24	**-0.27 ± 0.33**	-0.82 ± 9.42	-0.28 ± 0.16	-1.24 ± 1.19
	C. polykrikoides (μ)					Other dinoflagellates (μ)					Diatoms (μ)					Chlorophyll *a* <5 μm (μ)				
Meeting House Creek	Control	Nitrate	Urea	Glutamic acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia
10/3/2008	0.59 ± 0.35	**0.81 ± 0.21**	**0.85 ± 0.19**	**0.75 ± 0.38**	**0.78 ± 0.18**	0.64 ± 0.24	0.73 ± 0.08	0.70 ± 0.04	0.66 ± 0.01	0.70 ± 0.08	0.87 ±0.57	0.89 ± 0.15	0.83 ± 0.32	1.05 ± 0.23	1.07 ± 0.06	0.57 ± 0.07	0.61 ± 0.02	0.64 ± 0.02	0.66 ± 0.01	0.62 ± 0.02
8/30/2007	0.13 ± 0.03	**0.93 ± 0.11**	**1.15 ± 0.05**	**0.43 ± 0.05**	**1.08 ± 0.03**	-1.35 ± 0.07	-1.86 ± 0.19	-1.25 ± 0.07	-1.50 ± 0.10	-1.50 ±0.12	0.58 ± 0.05	**1.10 ± 0.14**	**1.34 ± 0.01**	0.50 ± 0.04	0.65 ± 0.04	NA	NA	NA	NA	NA
9/5/2006	0.13 ± 0.05	**0.25 ± 0.03**	**0.34 ± 0.07**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-0.08 ± 0.06	**0.20 ± 0.08**	**0.10 ± 0.03**	NA	NA
	C. polykrikoides (μ)					Other dinoflagellates (μ)					Diatoms (μ)					Chlorophyll *a* <5 μm (μ)				
Shinnecock Bay	Control	Nitrate	Urea	Glutamic acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia
8/25/2008	0.06 ± 0.04	**0.15 ± 0.14**	0.04 ± 0.09	0.01 ± 0.03	**0.12 ± 0.02**	0.53 ± 0.02	0.53 ± 0.12	0.33 ± 0.10	0.32 ± 0.26	0.49 ± 0.21	-0.37 ± 0.64	0.00 ± 0.00	-0.56 ± 0.96	-1.11 ± 0.96	-1.00 ± 0.88	1.61 ± 0.00	1.60 ± 0.32	1.54 ± 0.17	1.37 ± 0.48	1.63 ± 0.07
9/5/2008	0.05 ± 0.08	**0.18 ± 0.10**	0.02 ± 0.06	0.03 ± 0.12	0.02 ± 0.04	-0.06 ± 0.11	-0.04 ± 0.12	-0.14 ± 0.13	-0.28 ± 0.17	-0.22 ± 0.11	NA	NA	NA	NA	NA	-0.74 ± 0.13	-0.97 ± 0.42	-1.23 ± 0.34	-0.30 ± 0.00	-0.83 ± 0.18
9/23/2008	-0.35 ± 0.08	-0.59 ± 0.27	-0.42 ± 0.16	-0.39 ± 0.47	-0.50 ± 0.20	-0.20 ± 0.18	-0.10 ± 0.45	**0.97 ± 0.32**	-0.34 ± 0.66	-0.92 ± 9.40	-0.78 ± 0.56	-0.10 ± 0.18	-0.21 ±0.09	-0.55 ±0.38	-0.11 ±0.53	-0.86 ± 0.00	-1.25 ± 0.52	-0.61 ± 0.34	-0.83 ± 1.00	-0.75 ± 0.15
	C. polykrikoides (μ)					Other dinoflagellates (μ)					Diatoms (μ)					Chlorophyll *a* <5 μm (μ)				
Flanders Bay	Control	Nitrate	Urea	Glutamic acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia	Control	Nitrate	Urea	Glutamic Acid	Ammonia
8/29/2008	0.14 ± 0.02	**0.33 ± 0.10**	**0.29 ±0.08**	0.14 ± 0.20	**0.42 ± 0.11**	0.65 ± 0.08	0.56 ± 0.08	0.46 ± 0.07	0.71 ± 0.13	0.49 ± 0.16	NA	NA	NA	NA	NA	-0.89 ± 0.81	-1.23 ± 1.05	-0.58± 0.13	-0.59 ± 0.41	-0.44 ± 0.05
9/24/2008	0.20 ± 0.08	0.25 ± 0.14	0.18 ± 0.07	-0.05 ± 0.31	0.15 ± 0.09	1.08 ± 0.12	1.07 ± 0.47	1.03 ± 0.21	0.75 ± 0.44	0.99 ± 0.34	0.26 ± 0.45	1.52 ± 0.31	0.39 ± 0.68	1.20 ± 0.29	0.64 ± 0.20	-0.10 ± 0.03	**0.23 ± 0.08**	-0.11 ± 0.02	0.09 ± 0.03	0.06 ± 0.12
8/22/2007	-0.09 ± 0.03	**0.14 ± 0.01**	0.06 ± 0.07	**0.24 ± .0.1**	**0.13 ± 0.05**	0.05 ± 0.08	**0.29 ± 0.01**	**0.29 ± 0.07**	0.04 ± 0.03	0.19 ± 0.09	-0.11 ± 0.11	**0.82 ± 0.05**	0.05 ± 0.10	**0.41 ± 0.15**	**0.65 ± 0.02**	0.15 ± 0.14	0.20 ± 0.10	0.13 ± 0.15	0.36 ± 0.14	0.22 ± 0.06
9/4/2007	0.14 ± 0.07	-0.16 ± 0.31	0.07 ± 0.04	-0.18 ± 0.15	-0.33 ± 0.37	0.79 ± 0.02	0.64 ± 0.09	0.65 ± 0.07	0.60 ± 0.05	0.70 ± 0.05	-1.30 ± 0.08	**-0.11 ± 0.12**	**-0.54 ± 0.22**	**-0.41 ± 0.18**	**-0.67 0.22**	NA	NA	NA	NA	NA
8/23/2006	-0.08 ± 0.01	**0.13 ± 0.05**	**0.14 ± 0.05**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-0.07 ± 0.10	-0.63 ± 0.19	**0.27 ± 0.06**	NA	NA
9/11/2006	-0.27 ± 0.13	0.17 ± 0.10	0.03 ± 0.15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-0.02 ± 0.93	0.04 ± 0.18	0.26 ± 0.17	NA	NA

Table 8. Net growth rates of all nutrient amendment experiments from 2008, 2007, 2006 and 2005 from all sites under all treatments. Significantly increased net growth rates (Tukey test, $p<0.05$) when compared to control are highlighted red.

	C. polykrikoides	**Other dinoflagellates**	**Diatoms**	**Small phytoplankton**
Any N compound	62% (13/21)	17% (3/18)	43% (6/14)	38% (6/16)
Nitrate	57% (12/21)	11% (2/18)	36% (5/14)	25% (4/16)
Ammonium	53% (8/15)	0% (0/15)	18% (2/11)	8% (1/13)
Urea	39% (7/18)	13% (2/15)	18% (2/11)	19% (3/16)
Glutamic acid	27% (4/15)	7% (1/15)	18% (2/11)	0% (0/13)

Table 9. The percentage of experiments in which N compounds significantly increased the net growth rate of four phytoplankton groups relative to control treatments ($p<0.05$) during nutrient amendment experiments. Percentages and number of significant treatments out of total number of experiments (in parentheses) shown

FIGURES

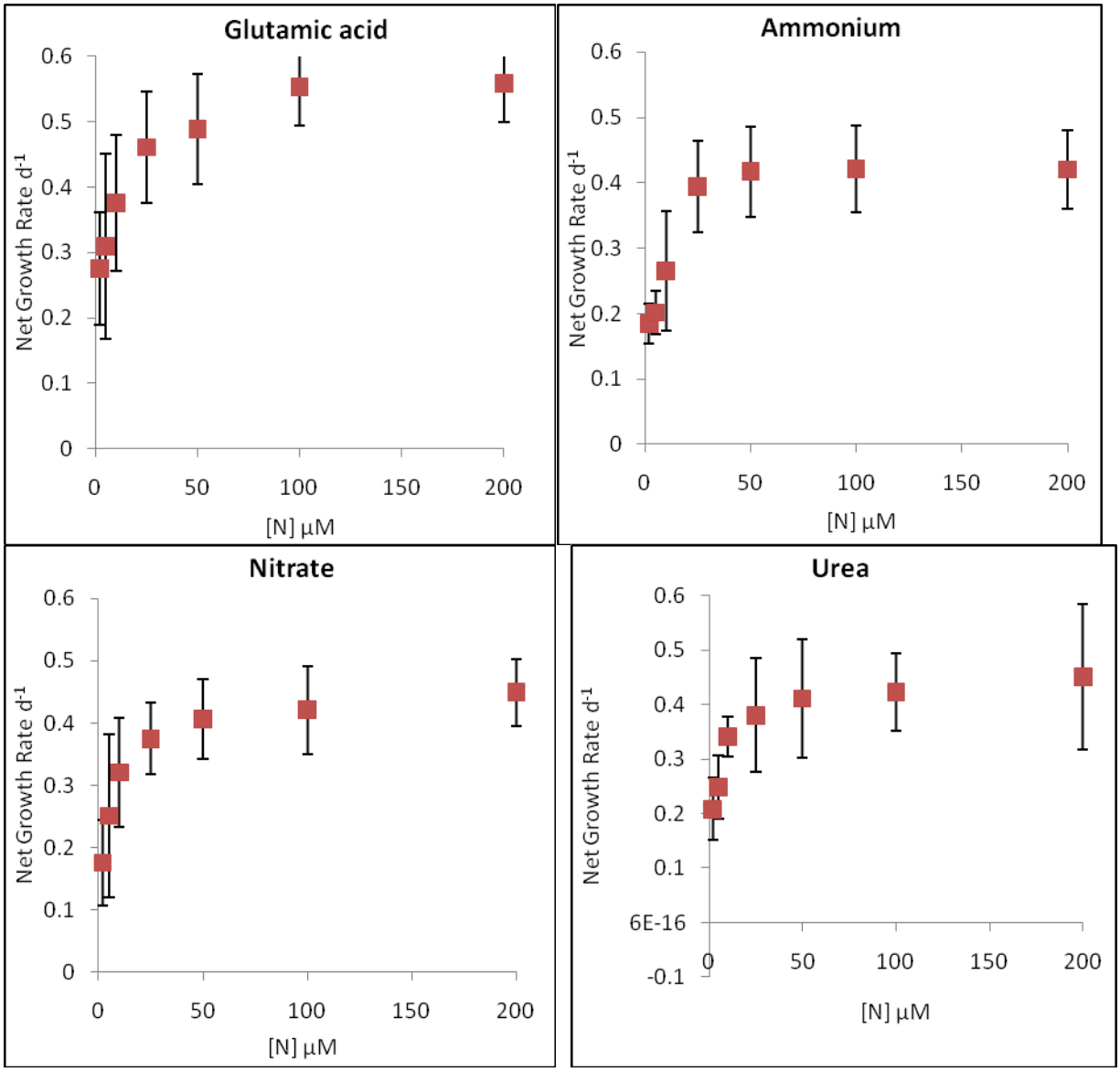

Figure 1. Growth rates (±SD) *C. polykrikoides* cultures grown on multiple concentrations (2, 5, 10, 25, 50, 100, 200 µM) of glutamic acid, ammonium, urea and nitrate.

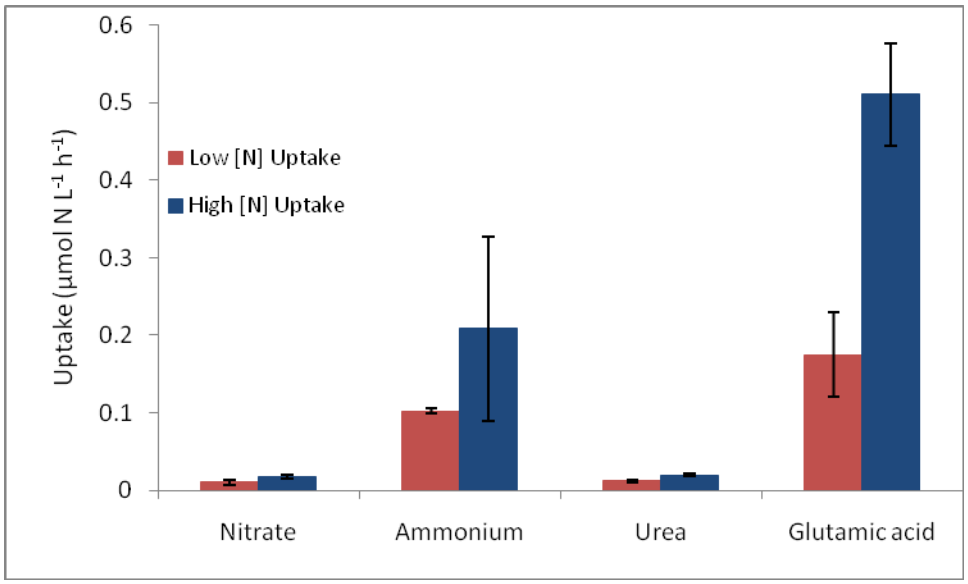

Figure 2. Nitrogen uptake of cultures grown on nitrate, urea, glutamic acid, and ammonium at high concentrations (20µM) and low concentrations (2µM) of N. Labeled nitrogen was the same as nitrogen species in which culture was grown.

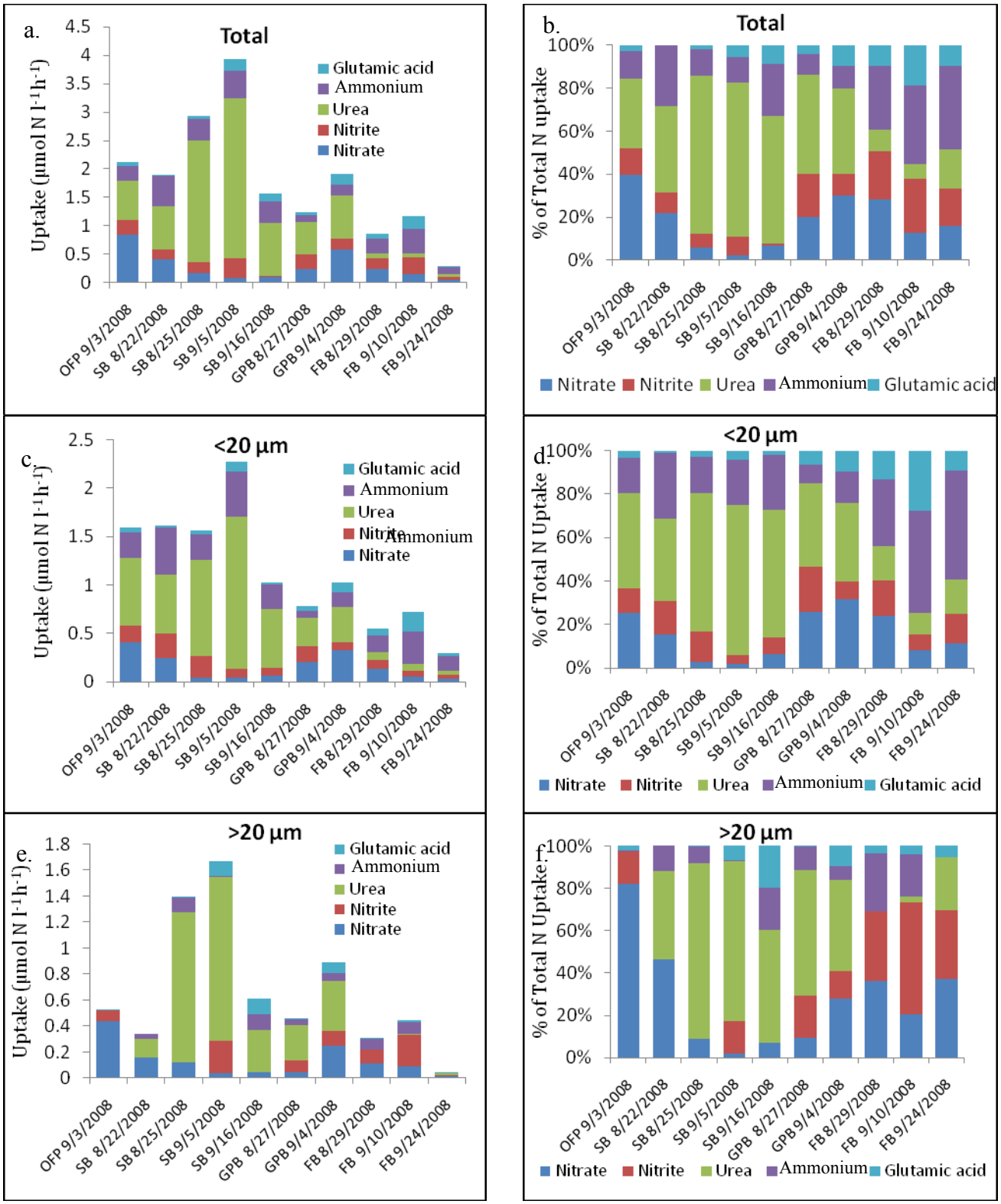

Figure 3 a-f. Uptake (µmol N l^{-1} h^{-1}) and % of total uptake of ^{15}N-labeled nitrogen compounds by three plankton size fractions (total, <20 µm, and >20 µm) in *C. polykrikoides* bloom water. Water was obtained from Old Fort Pond (OFP), Shinnecock Bay (SB), Great Peconic Bay (GPB) and Flanders Bay (FB). Mean RSD of uptake rates for all experiments was 0.27.

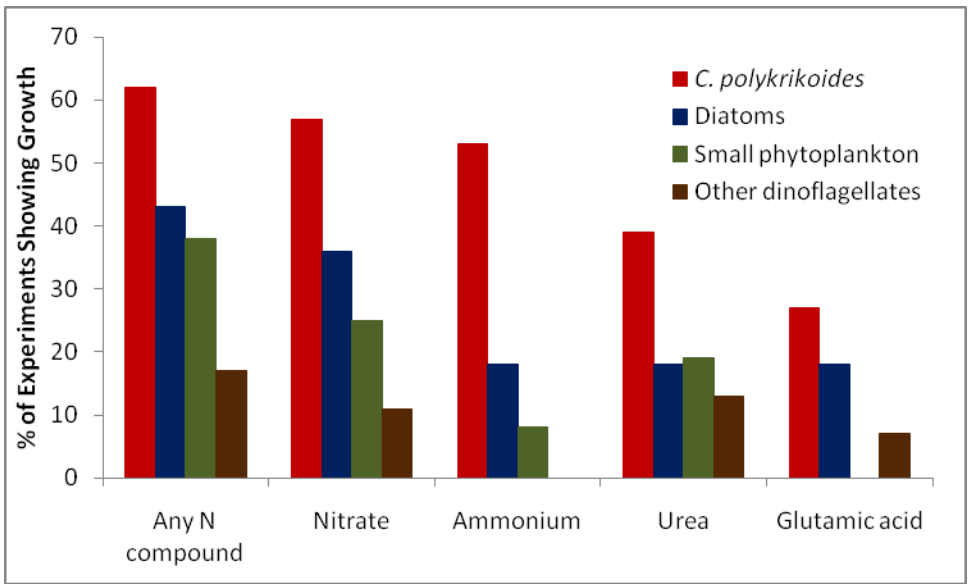

Figure 4. Percentage of growth experiments where specific phytoplankton group (*C. polykrikoides,* other dinoflagellates, diatoms and small phytoplankton) showed growth under specific N source (any N compound, nitrate, ammonium, urea, and glutamic acid).