MONITORING AND UNDERSTANDING TOXIC CYANOBACTERIA AND *COCHLODINIUM POLYKRIKOIDES* BLOOMS IN SUFFOLK COUNTY

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TABLE OF CONTENTS:

EXECUTIVE SUMMARY

This project, Monitoring and Understanding Toxic Cyanobacteria and *Cochlodinium polykrikoides* Blooms in Suffolk County, was funded by Suffolk County Capital Project 8224, Harmful Algal Blooms, and was initiated to address ongoing blooms of toxic cyanobacteria and *Cochlodinium polykrikoides* in Suffolk County waters.

Cyanobacteria

Cyanobacteria, also known as blue-green algae, are microscopic organisms found in both marine and fresh water environments. Under favorable conditions of sunlight, temperature, and nutrient concentrations, cyanobacteria can form massive blooms that discolor the water and often result in a scums and floating mats on the water's surface. Because some species of cyanobacteria produce toxins, these blooms represent a serious threat to the health of humans, animals and aquatic ecosystems. Globally, the frequency and intensity of toxic cyanobacteria blooms have increased greatly during the past decade and toxin concentrations during many blooms often surpass the World Health Organization (WHO) safe drinking water and recreational water limits.

Past studies conducted in Suffolk County have documented cyanobacteria blooms in a number of freshwater systems, including some that contain public bathing beaches. A twenty lake survey conducted in 2004 by the Gobler lab at Stony Brook University (also funded by CP-8224, Harmful Algal Blooms) found that all of the lakes studied contained potentially toxic cyanobacteria and detectable levels of the toxin microcystin. Fifteen of the lakes showed toxin levels exceeding World Health Organization (WHO) levels permissible for drinking water, with five lakes having levels that posed a moderate to high risk to human health for recreation. One of these five locations is the site of a very popular public bathing beach on Lake Ronkonkoma.

During this study, most Suffolk County bathing beaches did not experience toxic cyanobacteria blooms. The only exception was the Peconic Dunes County Park beach, which had levels of the cyanotoxin microcystin ranging from 1 to 7 $\mu g\ L^{-1}$, significantly exceeding the World Health Organization's (WHO) guidelines for drinking water (1 µg

L^{-1}). Importantly, during prior studies, Peconic Dunes County Park had not experienced cyanobacterial blooms while other systems (such as Lake Ronkonkoma) did. This demonstrates that conclusions with regard to the vulnerability of water bodies to cyanotoxins cannot be made based on a single year of sampling, and emphasizes the importance of maintaining an ongoing monitoring program.

Among the other freshwater sites included in the study, Lake Agawam on eastern Long Island exhibited dense cyanobacterial blooms through the spring, summer, and fall of 2012 and had microcystin levels exceeding WHO guidelines for drinking water by an order of magnitude on all dates tested, as well as exceeding the recreational limit (20 µg L^{-1}) on four occasions with values ranging from 10.5 to 44.7 µg L^{-1}. In prior years, other systems such as Mill Pond also exhibited similarly high toxin levels. As such, these findings collectively reiterate the need for continued and frequent monitoring of these and other Long Island water bodies in order to protect citizens from incidental exposure to these toxins.

Cochlodinium

Cochlodinium polykrikoides is an emerging "red tide" algal bloom first noted in Suffolk County waters in 2004. It causes intense and widespread reddish-brown blooms that have been coined locally as the "rust tide". The organism has been implicated as causing toxic algal blooms in coastal waters worldwide, and has been found to be lethal to multiple species and life stages of fish and shellfish. Locally, the initial bloom occurred throughout the Peconic Estuary and in eastern Shinnecock Bay in 2004, and has occurred at the same locations every year since. In 2011, blooms also occurred in Great South Bay.

Prior studies conducted by the Gobler lab (also funded by CP-8224) have demonstrated that the organism is highly toxic, capable of killing other phytoplankton, zooplankton and fish, as well as juvenile and larval shellfish including bay scallops, hard clams and oysters, in a matter of hours to days. Observed impacts during blooms have included the complete mortality of captive finfish and the mortality of caged and wild shellfish.

During the blooms of 2008 and 2009, fishermen reported the mass mortality of fish held in pound nets both in the Peconic Estuary and in Shinnecock Bay. During 2009, a mass mortality of scallops that occurred in Little Peconic Bay and Noyack Bay was similarly attributed to a bloom of *Cochlodinium polykrikoides*.

A significant result of this study was the visual confirmation of the production of resting cysts by *C. polykrikoides* in laboratory cultures isolated from Suffolk County waters. Evidence included sexually mating cell pairs, planozygotes with two longitudinal flagella, formation of both pellicular (temporary) cysts and resting cysts, and a time series of the cyst germination process. This definitive evidence of resting cyst production by *C. polykrikoides* provides a mechanism to account for the recurrence of annual blooms in given locales as well as the expansion of *C. polykrikoides* blooms across Suffolk County during the past decade. This finding also suggests that the identification of cyst beds in specific regions could be useful for future mitigation activities associated with sediments such as degrading or dredge spoil disposal.

Also of significance in this study, was the finding that cellular growth rates of C. polykrikoides increased monotonically from 13 to 25°C, suggesting that the early onset blooms in Suffolk County waters when temperatures are warmest may result in the most expansive blooms and that may also be the most toxic to fisheries. While this project explored many aspects of the toxicity of *C. polykrikoides*, most chemical analyses produced negative results with regard to the production of specific reactive oxygen and nitrogen species. Although the ability of peroxidases to mitigate the toxicity of *C. polykrikoides* continued to implicate reactive oxygen and nitrogen species as the toxic principle, the identification of the precise compound remains elusive. Regardless, compounds such as peroxidases could be considered for future small-scale mitigation projects.

Finally, regarding the vulnerability of fish populations to *C. polykrikoides*, the algae was found to have caused significantly elevated mortalities in both embryos and eleutheroembryos of multiple species of forage fish, with *Menidia* species (Siversides)

noted to be more vulnerable to toxicity than Sheepshead minnows. Although embryos were somewhat resistant to *C. polykrikoides* toxins until they hatched, once they hatched, they experienced rapid mortality and impaired swimming ability. These findings suggest the impacts of *C. polykrikoides* blooms on Suffolk County fisheries may be more severe than previously anticipated, as mortality in these early life stages may be not be visually obvious, but may be significant given the important trophic position these forage fish hold in marine food webs. Future studies may consider modeling the economic and food web impacts of these blooms on Long Island fisheries.

In summary, studies done on this and prior research studies, have highlighted the chronic existence of toxins made by cyanobacteria in Suffolk County lakes, and in some cases, have documented toxin levels which pose a high health risk for waters used for drinking purposes, while in others, have found a moderate-to-high health risk to citizens involved in recreational beach activities. Regarding *Cochlodinium polykrikoides*, the organism has been found to be highly lethal to fish and shellfish, and has bloomed annually since 2004 in the Peconic Estuary as well as in a number of locations in the county's south shore bays system.

In terms of basic public health protection at bathing beaches affected by cyanobacteria blooms, and in consideration of the economic value of fisheries impacted by *Cochlodinium* blooms, the continued monitoring of Suffolk County waters to document the occurrence and severity of these blooms, in conjunction with studies which elucidate bloom causation and the effectiveness of bloom remediation practices, is vital.

TASK 1. – LITERATURE AND REGULATORY REVIEW

BACKGROUND:

Cyanobacteria are a large and diverse group of prokaryotic photosynthetic organisms, which can be found in marine, freshwater, and terrestrial environments. Cyanobacteria blooms in freshwater systems are typically associated with eutrophic and poorly flushed waters (high residence time; Paerl, 1988, 2001; Philipp *et al*, 1991, Carmichael, 1994; Rapala, 1997; Oliver & Ganf 2000). While it is clear that the occurrence of toxic cyanobacteria blooms around the world have increased during recent decades (Chorus & Bartram, 1999), the underlying causes of such blooms and their increased frequency are poorly understood.

Blooms formed by toxic cyanobacteria can include the genera *Anabaena*, *Aphanizomenon*, *Nodularia*, *Oscillatoria*, and *Microcystis* (Carmichael; 1994; Fleming *et al*, 2001; Carmichael 1993; Chorus & Bartram, 1999). Cyanotoxins associated with these genera fall into two broad categories. Microcystins and cylindrospermopsins are cyclic peptides (hepatotoxins) which inhibit eukaryotic protein serine/threonine phosphatases (Honkanen *et al*., 1990; Chorus & Bartram, 1999). Anatoxin-a, anatoxin-a(s), and saxitoxins are three types of neurotoxins which block neuronal signal transmission and can lead to the paralysis of the heart and lungs resulting in rapid death in terrestrial vertebrates (Duy *et al.* 2000; Briand *et al*, 2003).

Of all the known cyanotoxins, microcystins are the most widespread and common. They are produced in the majority of blooms formed by *Microcystis* spp., but also can be produced by *Anabaena* spp., *Oscillatoria*/*Planktothrix* spp., and *Nodularia* spp. (Chorus & Bartram, 1999). Microcystin toxicity can accumulate within the liver of vertebrates causing chronic liver damage (Fitzgeorge *et al*, 1994; Falconer *et al*, 1988). They bind irreversibly to protein phosphatases 1 and 2A which play a key role in maintaining homeostasis in the cell (Cohen, 1989; Chorus & Bartram, 1999). The inhibition of these compounds can lead to increased phosphoralation of tumor suppressor proteins which can result in increased signaling and promote cell proliferation, transformation, and tumor promotion (Fujikia & Suganuma, 1993; Chorus & Bartram, 1999).

Human interaction with cyanotoxins is primarily from drinking water; however recreational activities such as swimming or boating can also lead to exposure (Turner *et al*, 1990; Carmichael, 1994; Falconer, 1999). Although direct consumption of lake water in the United States is rare, a number of cases of cyanotoxin contamination of raw and treated drinking water have been reported within a number of municipalities (Boyer *et al*, 2004). Moreover, in developing nations, due to a lack of water treatment plants, raw lake water may be consumed. Symptoms of short term recreational exposure to microcystin can include vomiting, diarrhea, central abdominal pain, sore throats, and blistering of the lips (Turner *et al.*, 1990). On the other hand, long term exposure to cyanotoxins can be associated with severe health effects. Worldwide, reports of human deaths, liver and colorectal cancers, neurological disorders, such as Alzhiemer's disease, and other illnesses have been associated with water contaminated with cyanotoxins (Falconer *et al*, 1988;Carmichael & Falconer, 1993; Bell & Codd, 1994; Carmichael, 1994; Chorus & Bartram, 1999; Zegura *et al*, 2003; Cox *et al*, 2005). For example, in 1994 and 1995 in rural China, where residents depend on surface water contaminated with cyanotoxins for their drinking water, Primary Liver Cancer (PLC) was one of the most common cancers reported in those regions (Yu, 1995). It is likely that the ingestion of microcystin along with aflatoxin exposure from corn and the hepititus B virus acted to initiate and promote PLC (Yu, 1995).

More commonly reported, especially in countries that do not rely on surface water for their primary source of drinking water (e.g. the United States), are animal deaths relating to microcystin poisoning. The first report of animal poisonings due to cyanotoxins was in Australia in 1878 when multiple farm animals died after ingesting water from a lake contaminated with a bloom of *Nodularia spumigena* (Francis, 1878) Over the past 130 years, cyanotoxin poisonings have been reported worldwide in animals of various sizes ranging from ducks to rhinoceros (Charmichael, 1992). However, in the United States most reports of cyanotoxin poisonings involve water fowl, cattle, and domestic pets which are exposed to cyanotoxins by ingestion of contaminated water. For example, there have been five reported cases of canine death due to the ingestion of cyanobacterial scum on the shores of Lake Champlain over the past few years (Rosen *et al*, 2001; Rolland *et al*, 2005).

Cyanotoxins such as microcystin and anatoxin are generally more harmful to mammals and birds then they are to aquatic animals (Oberholster *et al*, 2006). However, fish deaths during multiple cyanobacterial blooms have been reported (Davidson, 1959; Koon, 1960; Ochumba, 1990; Sevrin-Reyssac & Pletikosic, 1990). A study conducted in the United Kingdom suggested that damage to gills, digestive tract, and liver were responsible for fish kills during cyanobacteria blooms (Rodger *et al*, 1994; Oberholster *et al*, 2006). Moreover, recent findings suggest cyanotoxins accumulate throughout the food web and within in fish (Ibelings *et al*, 2005).

In addition to high concentrations of potentially lethal toxins, blooms of cyanobacteria can have numerous other negative impacts on aquatic environments. Dense blooms can cause a dramatic decrease in light penetration which can shade out benthic aquatic plants (Paerl *et al*, 2001). The demise of dense cyanobacteria blooms can rapidly increase bacterial respiration. I observed this phenomenon in Lake Agawam, NY (Figure 2) in 2006 when a dense cyanobacteria bloom ended abruptly, DO levels decreased to less than 1mg L^{-1} and thousands of fish died while others were "gulping" for air at the surface. Toxic cyanobacteria blooms can also alter the structure of the food web. Research has demonstrated that cyanobacteria are often not readily grazed upon by zooplankton (Paerl *et al*, 2001; Gobler *et al*, 2007). Whether it is because of the toxin synthesis, size or shape, or poor nutritional value, cyanobacteria do not seem to be an optimal pray item for most zooplankton (Paerl *et al*, 2001; Wilson *et al*, 2006). This shift in the structure of the base of the food web can disrupt the flow of energy and carbon to the higher trophic levels (Paerl *et al*, 2001). Lastly, another negative, yet probably less detrimental, impact these blooms can have on an environment are the foul odors that can be associated with them (Chorus & Bartram, 1999; Carmichael, 2001; Paerl *et al*, 2001). Since many cyanobacteria have the ability to control their buoyancy and congregate in surface waters, during high wind events they can be carried to the leeward (or downwind) shore of lakes where an algal scum line can occur (Chorus & Bartram, 1999). These cells eventually die and begin to get broken down creating noxious and sulfidic odors (Chorus & Bartram, 1999; Carmichael, 2001; Paerl *et al*, 2001).

Toxic and non-toxic strains of cyanobacteria:

One complexity in the study of cyanobacteria blooms in the field has been the existence and often co-existence of toxic and non-toxic strains of the same species which are morphologically and taxonomically indistinguishable. Nearly every major species of toxic cyanobacteria has both toxic and non-toxic strains (Chorus & Bartram, 1999). Historically, scientists have used light microscopy to determine the density of cyanobacteria. While this technique is sometimes adequate for determining overall population density, it is insufficient for determining the densities of the toxic and non-toxic subpopulations that comprise the overall population. Advances in molecular biology such as polymerase chain reaction (PCR), and the ability to quantify specific DNA sequences using quantitative polymerase chain reaction (qPCR) have provided the means to study these potentially harmful blooms in greater detail (Scholin, 1998; Coyne *et al*, 2001; Popels 2003). Historically, the dynamics of toxic and nontoxic strains of cyanobacteria blooms are poorly understood (Rapala & Sivonen, 1998). The variation in toxicity of blooms is probably influenced by environmental conditions, the presence of toxic or nontoxic cells, the physiological condition of the cells and the overall plankton community composition (Vėzie *et al*, 2002). Blooms of microcystin producing cyanobacteria are usually comprised of toxic and non-toxic strains (Ohtake *et al*,1989; Vėzie *et al*, 1998; Welker *et al* 2003; Welker *et al* 2007; Kardinaal *et al*, 2007). Recent molecular advances have identified the genes responsible for the cellular synthesis of the hepatotoxin microcystin (the *mcy* genes (A-J); Moore *et al*, 1991; Arment & Carmichael, 1996; Tillett *et al*, 2000; Christiansen *et al*, 2003; Rouhiainen *et al*, 2004) have allowed for the detection of toxic and non-toxic cyanobacteria species as only toxic strains of microcystin producing genera contain the mcy gene cluster (Rinta-Kanto, 2005).

Health Regulatory Guidelines

Cyanotoxins can represent a serious health risk to animals and humans. There are multitudes of examples of sicknesses and deaths associated with chronic, or even sporadic, consumption of water contaminated with cyanotoxins (Carmichael and Falconer 1993; Bell and Codd 1994; Carmichael 1995; Chorus and Bartham 1999). Because of these health concerns, the World Health Organization (WHO) has also established

guidelines for municipalities regarding levels of cyanotoxins in natural water bodies. The guideline for safe drinking water is 1 μg L^{-1}, assuming the water body is used as a primary source of drinking water (Table 1). Since Long Island obtains its drinking water from groundwater, this guideline is less relevant to the current study than the WHO's recreational guideline of 2 – 4 μg L^{-1} for low risk, and 20 μg L^{-1} for moderate risk (Table 1). An absolute toxin value has not been set for high risk, but rather the WHO indicates that cyanobacteria scums which can form near shore may be high risk and should be avoided. It is in the light of these general guidelines the results of the current study will be interpreted. A second general guideline used for comparative purposes are the USEPA's categorization of freshwater ecosystems based on their levels of chlorophyll a, which are presented in Table 2. Historically, Suffolk County's lakes have varied from being safe to drink to being moderate recreational risks (Fig 1).

Table 1a. World Health Organization Guidelines for microcystin

•1 ug / L is the guideline level for safe drinking water (Suffolk County residents do not rely on lakes for drinking water).

•2 – 4 ug / L is the guideline level for a low recreational risk.

•20 ug / L is the guideline level for a moderate recreational risk.

•Surface "scum" accumulation of cyanobacteria are considered a high risk

Table 1b. World Health Organization Guidelines based on cyanobacterial cells and chlorophyll levels

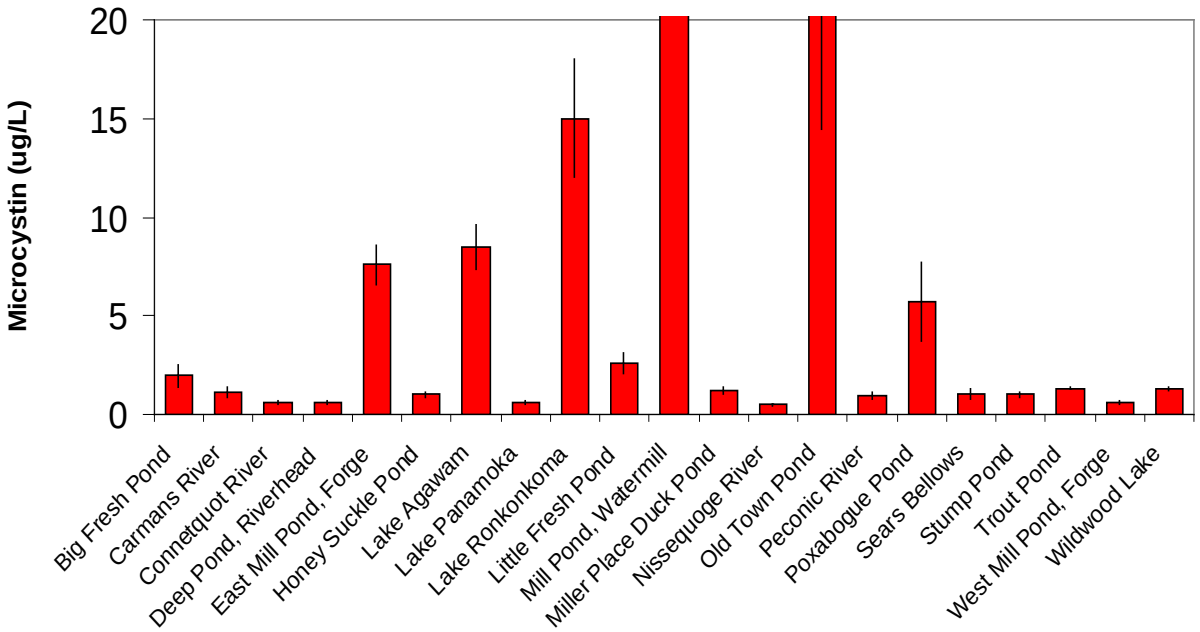
TABLE 8.3. GUIDELINES FOR SAFE PRACTICE IN MANAGING RECREATIONAL WATERS[a]

Guidance level or situation	How guidance level derived	Health risks	Typical actions[b]
Relatively low probability of adverse health effects			
20 000 cyanobacterial cells/ml *or* 10 µg chlorophyll-a/litre with dominance of cyanobacteria	• From human bathing epidemiological study	• Short-term adverse health outcomes, e.g., skin irritations, gastrointestinal illness	• Post on-site risk advisory signs • Inform relevant authorities
Moderate probability of adverse health effects			
100 000 cyanobacterial cells/ml *or* 50 µg chlorophyll-a/litre with dominance of cyanobacteria	• From provisional drinking-water guideline value for microcystin-LR[c] and data concerning other cyanotoxins	• Potential for long-term illness with some cyanobacterial species • Short-term adverse health outcomes, e.g., skin irritations, gastrointestinal illness	• Watch for scums or conditions conducive to scums • Discourage swimming and further investigate hazard • Post on-site risk advisory signs • Inform relevant authorities
High probability of adverse health effects			
Cyanobacterial scum formation in areas where whole-body contact and/or risk of ingestion/aspiration occur	• Inference from oral animal lethal poisonings • Actual human illness case histories	• Potential for acute poisoning • Potential for long-term illness with some cyanobacterial species • Short-term adverse health outcomes, e.g., skin irritations, gastrointestinal illness	• Immediate action to control contact with scums; possible prohibition of swimming and other water contact activities • Public health follow-up investigation • Inform public and relevant authorities

Table 2. Chlorophyll criteria presented by the USEPA for the purposes of classifying the trophic status of lake waters (Gibson, 2000)

	Chlorophyll (ug / L)
Ultraoligotrophic	< 1
Oligotrophic	1- 4
Mesotrophic	4 - 8
Eutrophic	8 - 25
Hypereutrophic	> 25

Figure 1. Mean levels of microcystin detected in Suffolk County lakes, 2003 - 2008



***Cochlodinium polykrikoides* blooms**

Dinoflagellates of the genus *Cochlodinium* were first identified in 1895 by Schütt (1895) and have been forming harmful algal blooms in the coastal waters of Southeast Asia and North America for many decades. The past two decades have seen *Cochlodinium* blooms expanded in their geographic distribution across Asia, Europe, and North America (Figure 1), with fisheries losses associated with blooms in South Korea alone exceeding $100M annually (Kim, 1997). More than 40 species of *Cochlodinium* have been described, although the two primary HAB-forming species are *C. polykrikoides* and *C. fulvescens* (Figure 2). Both of these species are large (~40 µm) athecate dinoflagellates that commonly form chains of 2 to 16 cells. *Cochlodinium* blooms are generally characterized by spatially large (10's to 100's of kilometers) and

dense (>1,000 cells ml^{-1}) cell aggregates that are heterogeneous in their vertical and horizontal distributions. These blooms are strongly ichthyotoxic and can also kill many other marine organisms, although the compound(s) responsible for these impacts have yet to be identified and bloom-associated toxins are not known to affect human health. Partly due to the recent expansion of *Cochlodinium* blooms and the general difficulty in culturing this species, there is far less known about the autecology and toxicity of *Cochlodinium* compared to other HAB species, particularly for the recently described *C. fulvescens*. With this review we seek to characterize the current state of knowledge regarding taxonomy, phylogeny, detection, distribution, ecophysiology, life history, food web interactions, and mitigation of blooms formed by *Cochlodinium* as well as point out pressing questions regarding this increasingly important HAB genus.

The past two decades have witnessed an expansion in the reported occurrences of harmful algal blooms (HABs) caused by the dinoflagellate *Cochlodinium*. Prior to 1990, blooms had been primarily reported in Southeast Asia, with South Korea alone reporting more than $100M USD in annual fisheries losses during the 1990s. Since this time blooms have expanded across Asia, Europe, and North America, with recognition of multiple species and ribotypes that exhibit similar ecophysiological and harmful characteristics. Although *Cochlodinium* is comprised of more than 40 species, the most common is the HAB-forming species native to Suffolk County, *C. polykrikoides*. This alga has been shown to be acutely toxic to a range of fish species, shellfish, zooplankton, and even other phytoplankton. Its flexible nutrient acquisition strategies, inhibition of grazing by inducing rapid mortality in a diverse set of predators, and allelopathic inhibition of a broad range of competing phytoplankton all contribute toward bloom

formation and maintenance. On eastern Long Island, this species had never been observed prior to 2004 despite robust HAB monitoring programs established in the 1980s. This species was never seen on Long Island prior to 2004. However, it has formed dense blooms in the Peconic Estuary and Shinnecock Bay since 2004 and has done so every July, August, and September since then (2004-2011 Gobler et al 2008, Y. Z. Tang and Gobler 2010; C. Gobler pers. obs.). In 2011, *Cochlodinium* blooms expanded across Long Island into Great South Bay.

TASK 2. –SUMMER MONITORING OF FRESHWATER BATHING BEACH LAKES IN SUFFOLK COUNTY. SUFFOLK COUNTY BATHING BEACHES

In 2012, bathing beaches monitored by the Suffolk County Department of Health in conjunction with the Gobler Lab displayed moderate levels of chlorophyll and phycocyanin (proxies for algal and cyanobacterial abundance). In general, values for these two proxies increased from low levels at the beginning of the monitoring period to elevated levels at the end of the monitoring period. The lowest observed values documented were for John Schiff Scout Reservation Beach averaging 3.5 ± 0.9 µg L^{-1} and 1.1 ± 0.6 RFU for chlorophyll *a* and phycocyanin respectively (Figure 1). Periodic spikes in these two parameters were observed in July and August for Sears Bellows Count Park Beach (Figure 2) and Peconic Dunes County Park (Figure 3) and in conjunction, elevated concentrations of the toxin microcystin were measured in Peconic Dunes County Park during the latter part of the monitoring period, ranging from 1.3 µg L^{-1} on Jul 9th to 7.1 µg L^{-1} on Sept. 5th (Figure 3) which exceed the World Health Organization's (WHO) guidelines for drinking water (1 µg L^{-1}). Microcystin was also detected in both Lake Ronkonkoma sites (Figure 4) and the John Schiff Scout Reservation Beach (Figure 1), however concentrations were below 1 µg L^{-1} in all cases. The three remaining sites (Victoria Beach, Camp Baiting Hollow Beach, and Rose Elliston Memorial Park Beach) had low levels of chlorophyll *a*, phycocyanin, and microcystin was absent or at extremely low levels (Figures 5, 6, 7).

With regards to the contaminant *Escherichia coli* (e-coli), most lakes displayed low to moderate counts throughout the monitoring period. However, both bathing beaches on

Lake Ronkonkoma had high e-coli levels with the Brookhaven Town Beach displaying counts above 2,000 cfu per 100 mL for most dates monitored. E-coli levels also increased to similar levels at the Peconic Dunes County Park beginning in August through early September (Figure 8).

Note: Figures for task two appear at the end of Task 3

TASK 3. –SEASONAL MONITORING THE MOST TOXIC LAKES IN SUFFOLK COUNTY

Three lakes previously found to have high levels to toxic cyanobacteria were monitored by the Gobler Lab as part of this effort including Lake Agawam, Mill Pond, and Little Fresh Pond. Both Lake Agawam and Mill Pond exhibited high chlorophyll *a* values (averaging 37.2 ± 24.7 µg L^{-1}) and reaching a max of 63.7 ± 0.7 µg L^{-1} in late June for Mill Pond and 128.0 ± 0.6 µg L^{-1} in early October for Lake Agawam (Figures 9A and 10A). Phycocyanin, a proxy for cyanobacterial abundance, was also elevated for these two lakes, averaging 32.5 ± 18.6 RFU throughout the monitoring period with peaks towards the latter part of the monitoring period (Figures 9A and10A). In contrast, Little Fresh Pond had very moderate levels of both chlorophyll and phycocyanin (averaging 5.6 ± 2.6 µg L^{-1} and 1.3 ± 0.8 RFU, respectively; Figure 11).

Microcystin concentrations in Lake Agawam and Mill Pond tended to mirror phycocyanin and *Microcystis* abundance. Concentrations of microcystin were the highest toxin concentrations measured in all of the lakes monitored in 2012. Lake Agawam exceeded the World Health Organization's (WHO) guidelines for drinking water (1 µg L^{-1}) by an order of magnitude in all dates tested and exceeding the recreational limit (20 µg L^{-1}) on four occasions with values ranging from 10.5 to 44.7 µg L^{-1} and averaging 22.9 ± 11.5 µg L^{-1} (Figure 9A). Microcystin was significantly correlated with *Microcystis* colony abundance for Lake Agawam (p = 0.003). Microcystin concentrations in Mill Pond were much less, averaging 1.9 ± 1.5 µg L^{-1} throughout the monitoring period (Figure 10A) and no correlation was found between concentrations and cyanobacterial abundance. Little Fresh Pond was not tested for microcystins in 2012 as phycocyanin

levels remained low throughout the sampling period. Lake Agawam cyanobacterial populations began at relatively low levels in May and increased to their highest concentrations in mid-October (Figure 9B). In contrast, Mill Pond had high abundances of cyanobacteria throughout the sampling period (Figure 10B). Lake Agawam was largely dominated by *Microcystis* sp. with a brief shift to *Aphanizomenon* sp. in mid to late August (Figure 9B) whereas the dominant cyanobacteria in Mill Pond was *Aphanizomenon* sp. throughout the sampling period with a shift to dominance by *Microsystis* sp. in late September (Figure 10B).

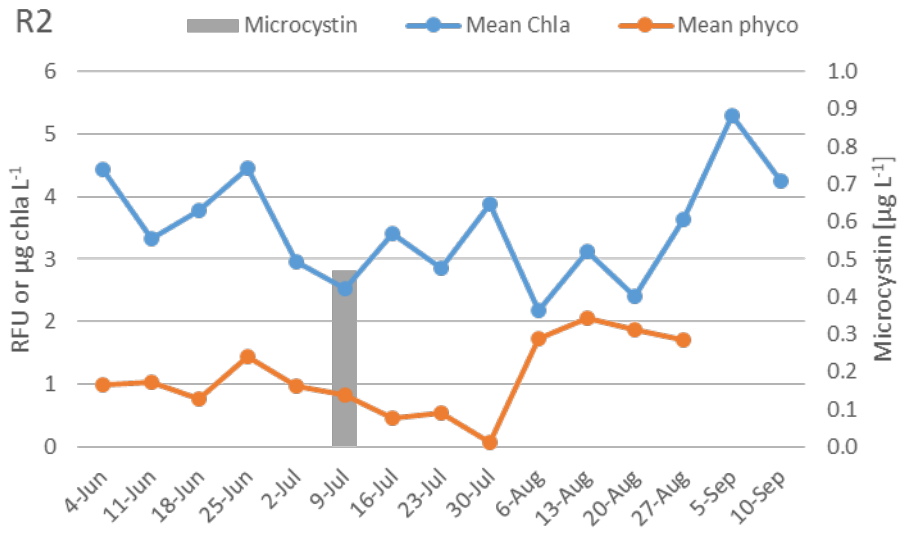


Figure 1 Temporal dynamics at John Schiff Scout Reservation Beach for chlorophyll *a* (Chla in µg L^{-1}), phycocyanin (phyco in RFU), and microcystin concentration during the 2012 sampling season.

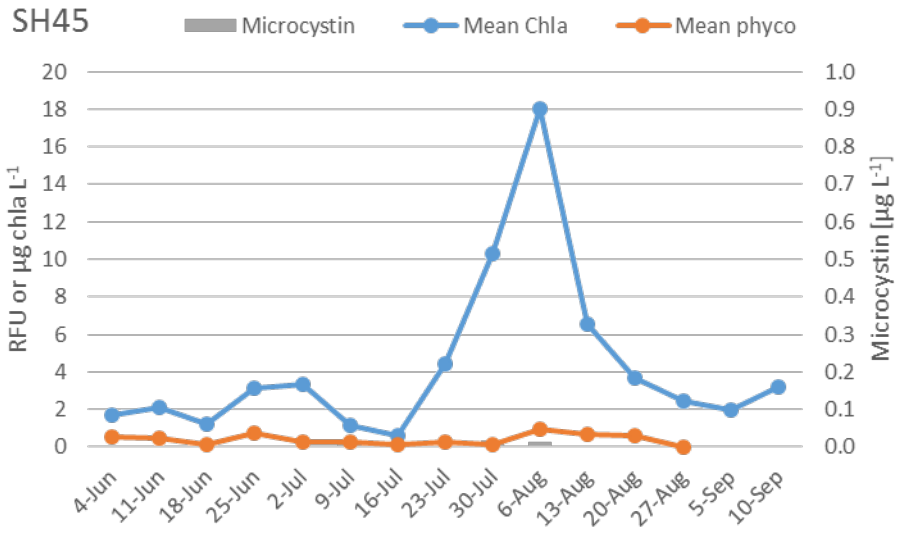


Figure 2 Temporal dynamics at Sears Bellows County Park Beach for chlorophyll *a* (Chla in µg L^{-1}), phycocyanin (phyco in RFU), and microcystin concentration during the 2012 sampling season.

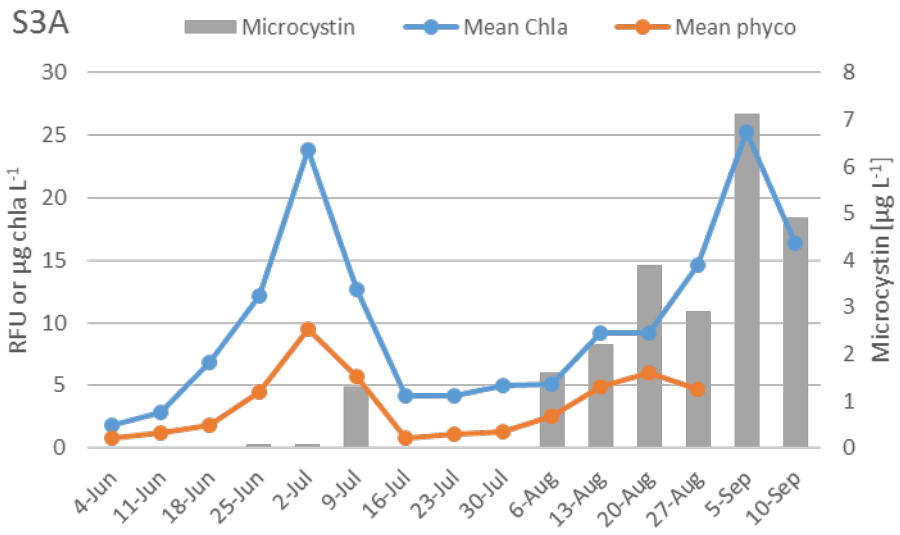


Figure 3 Temporal dynamics at Peconic Dunes County Park for chlorophyll *a* (Chla in µg L^{-1}), phycocyanin (phyco in RFU), and microcystin concentration during the 2012 sampling season.

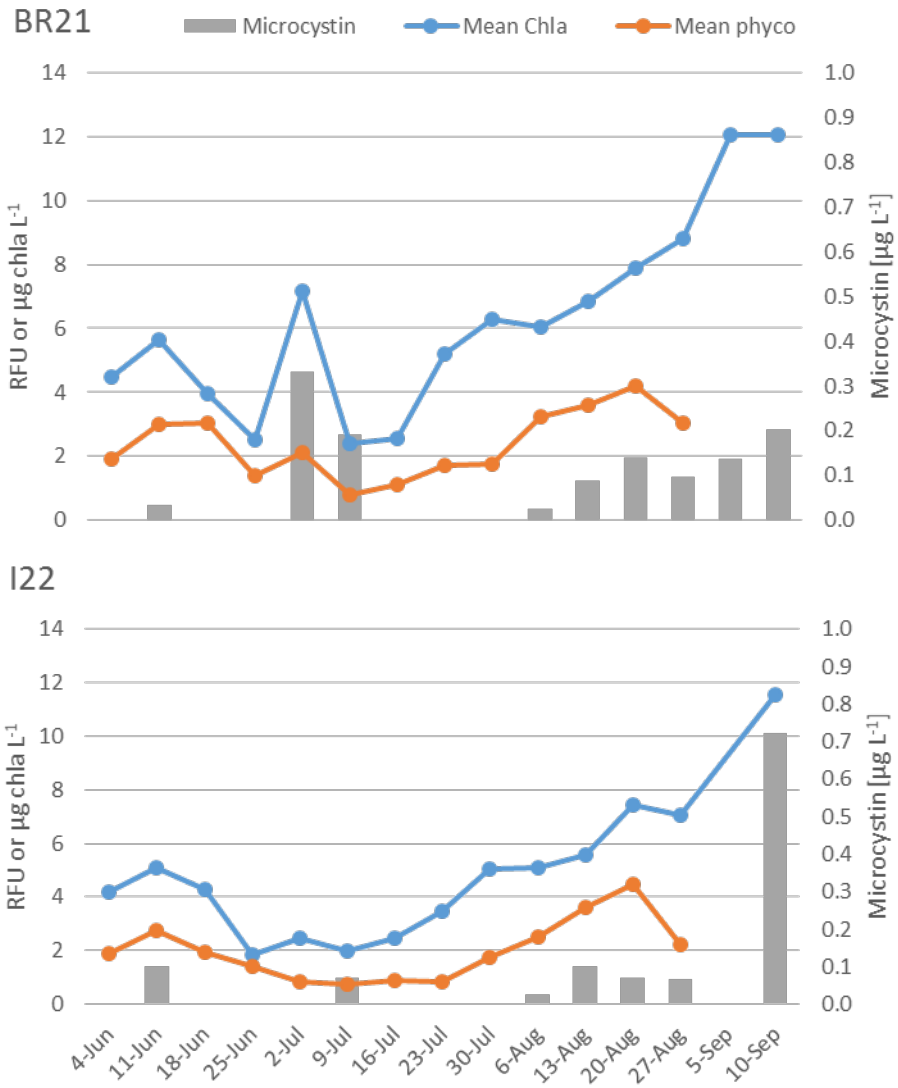


Figure 4 Temporal dynamics at Lake Ronkonkoma bathing beaches for chlorophyll *a* (Chla in µg L^{-1}), phycocyanin (phyco in RFU), and microcystin concentration during the 2012 sampling season.

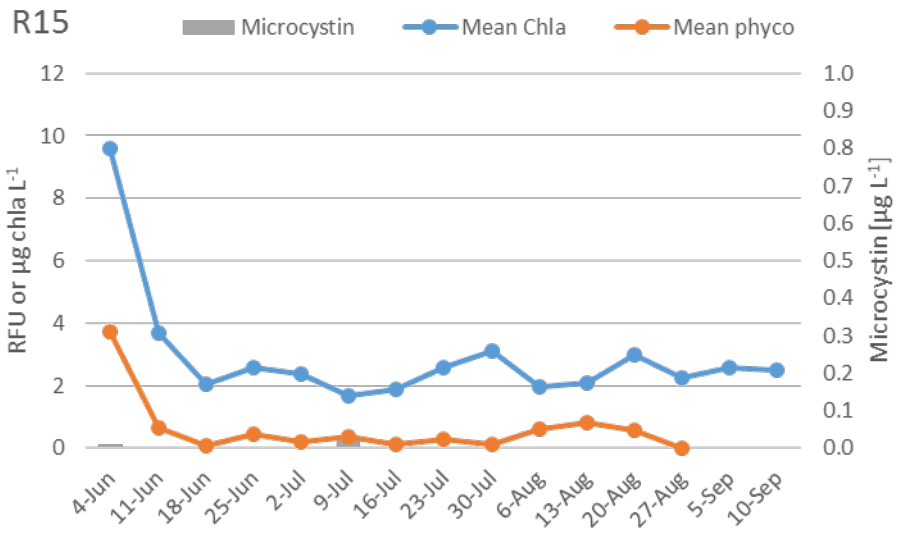


Figure 5 Temporal dynamics at Victoria Beach for chlorophyll *a* (Chla in µg L^{-1}), phycocyanin (phyco in RFU), and microcystin concentration during the 2012 sampling season.

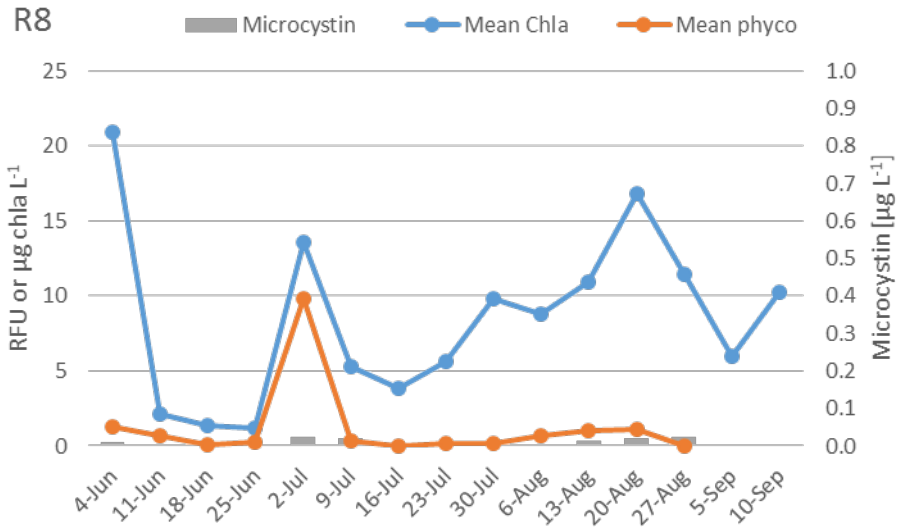


Figure 6 Temporal dynamics at Camp Baiting Hollow Beach for chlorophyll *a* (Chla in µg L^{-1}), phycocyanin (phyco in RFU), and microcystin concentration during the 2012 sampling season.

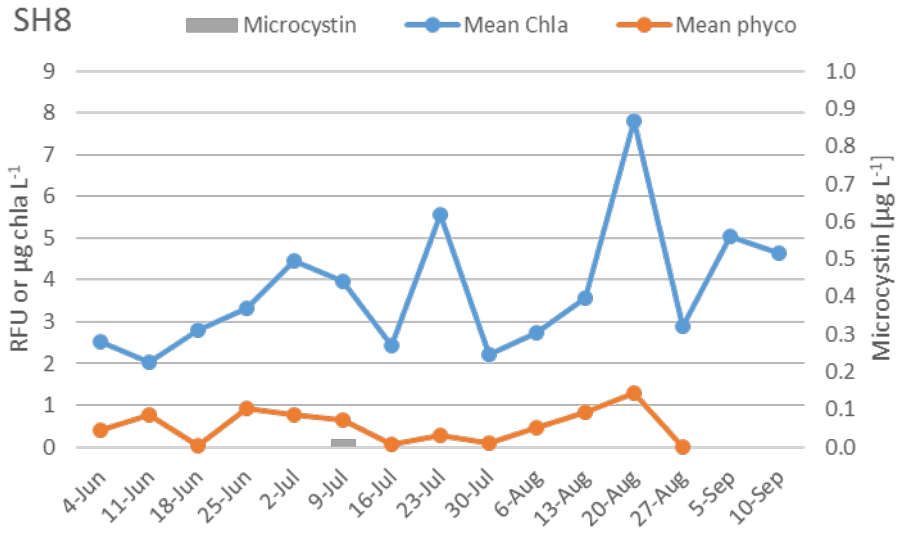


Figure 7 Temporal dynamics at Emma Rose Elliston Memorial Park Beach for chlorophyll *a* (Chla in µg L^{-1}), phycocyanin (phyco in RFU), and microcystin concentration during the 2012 sampling season.

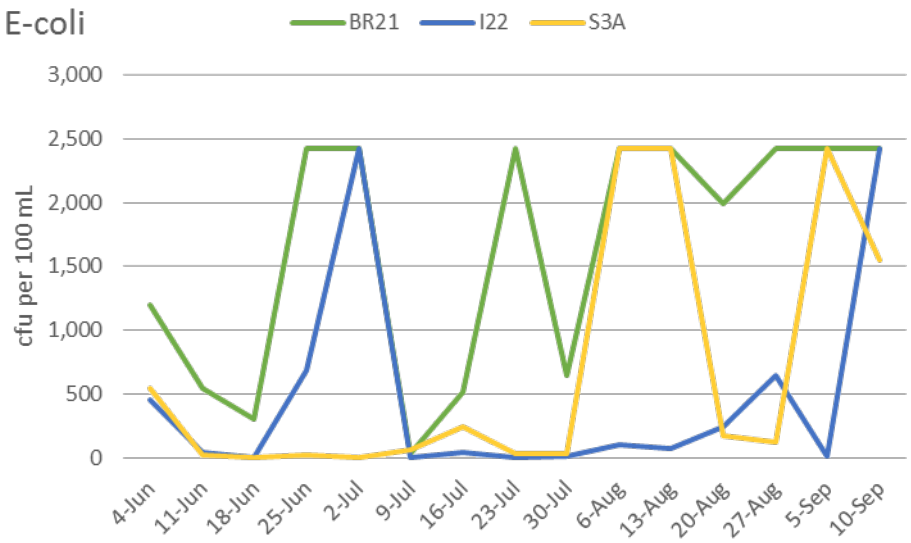


Figure 8 Temporal dynamics of *Escherichia coli* (E-coli) at Ronkonkoma bathing beachs (BR21 and I22) and Peconic Dunes County Park (S3A) during the 2012 sampling season.

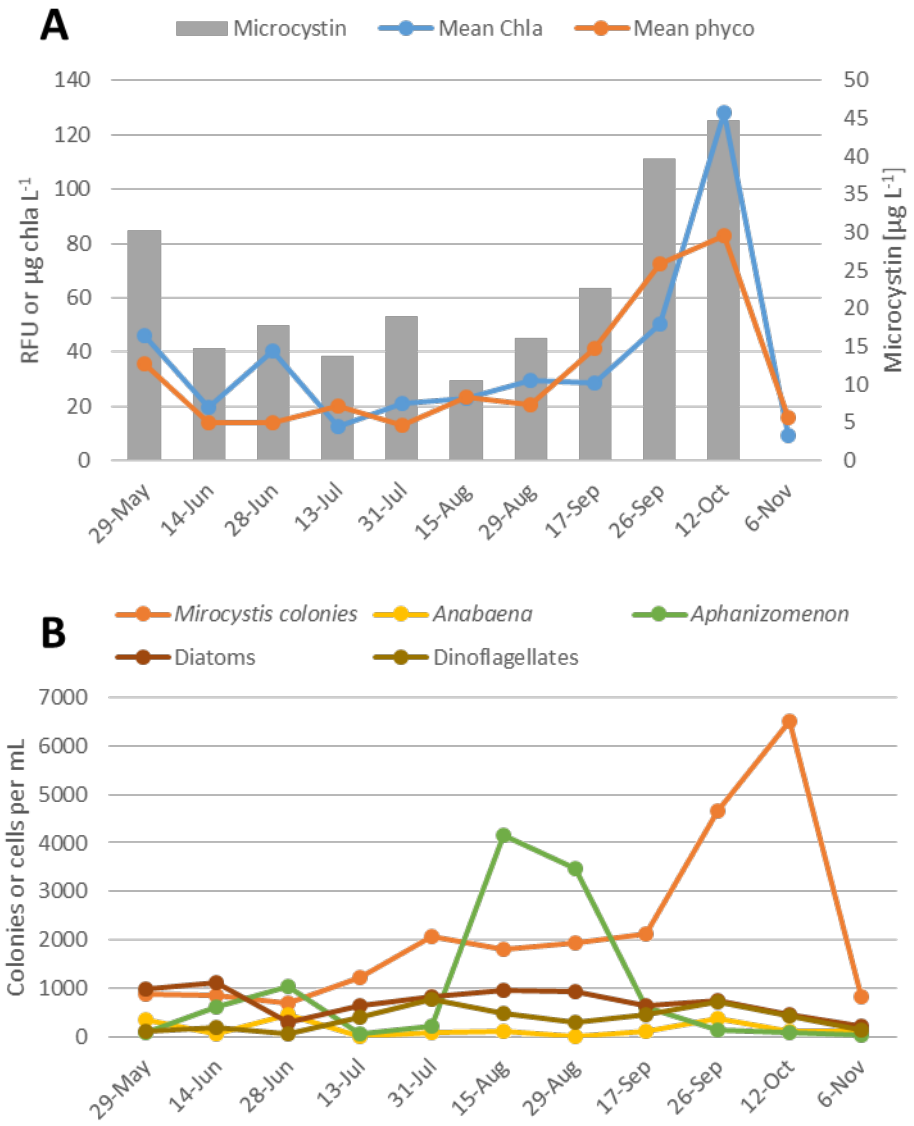


Figure 9 A) Temporal dynamics at Lake Agawam for chlorophyll *a* (Chla in µg L^{-1}), phycocyanin (phyco in RFU), and microcystin concentration during the 2012 sampling season. **B)** Temporal dynamics of dominant phytoplankton species and classes during the 2012 sampling season.

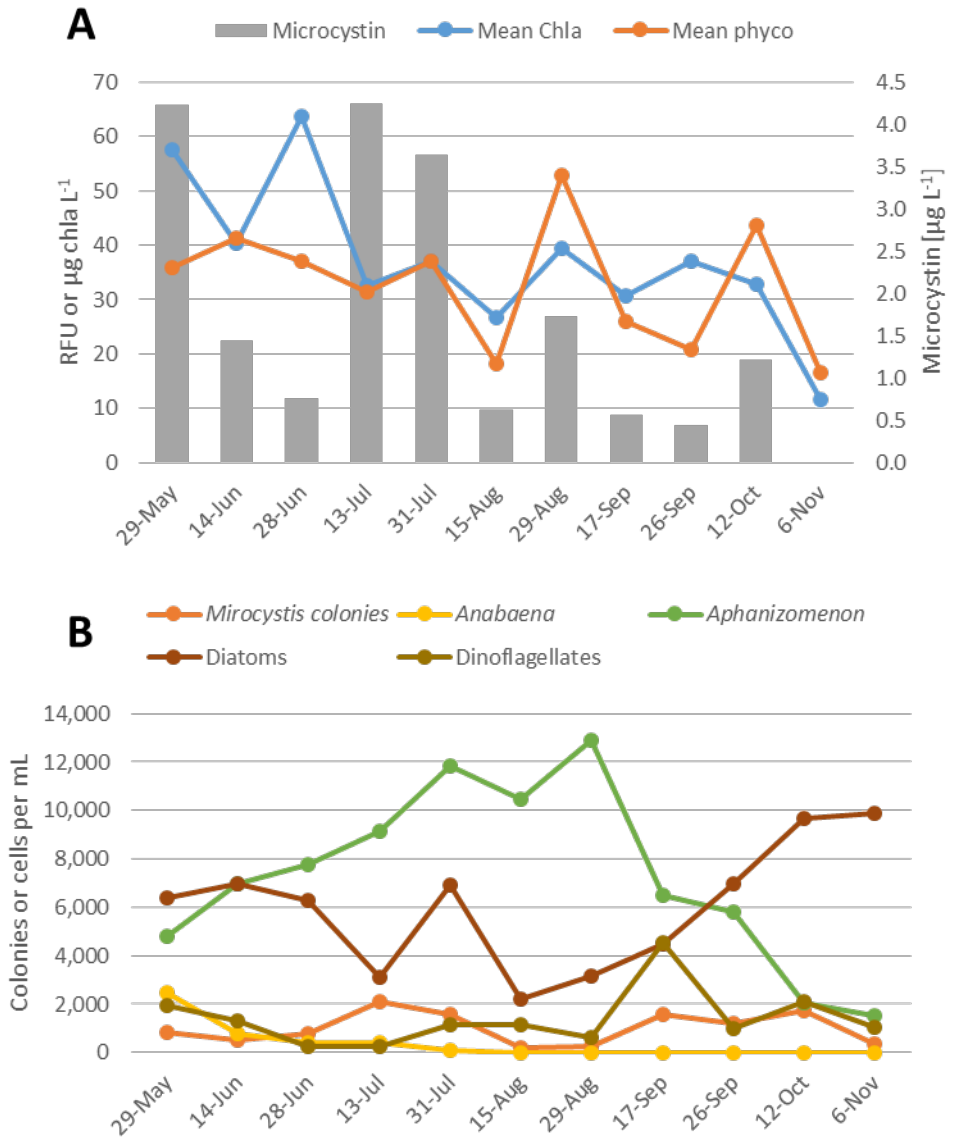


Figure 10 A) Temporal dynamics at Mill Pond for chlorophyll *a* (Chla in µg L^{-1}), phycocyanin (phyco in RFU), and microcystin concentration during the 2012 sampling season. **B)** Temporal dynamics of dominant phytoplankton species and classes during the 2012 sampling season.

TASK 4. CYANOTOXIN FINDINGS AND FINAL REPORT.

This task is addressed by this report.

TASK 5 & 6. ASSESS THE ABILITY OF *COCHLODINIUM POLYKRIKOIDES* TO FORM CYSTS; QUANTIFY THE PRODUCTION AND DENSITIES OF *COCHLODINIUM POLYKRIKOIDES* CYSTS BEFORE, DURING AND AFTER BLOOMS:

Summary of tasks 5 and 6:

While harmful algal blooms (HABs) caused by the toxic dinoflagellate *Cochlodinium polykrikoides* have been known to science for more than a century, the past two decades have witnessed an extraordinary expansion of these events across Asia, North America, and even Europe. Although the production of resting cysts and subsequent transport via ships' ballast water or/and the transfer of shellfish stocks could facilitate this expansion, confirmative evidence for cyst production by *C. polykrikoides* is not available. Here, we provide visual confirmation of the production of resting cysts by *C. polykrikoides* in laboratory cultures isolated from North America. Evidence includes sexually mating cell pairs, planozygotes with two longitudinal flagella, formation of both pellicular (temporary) cysts and resting cysts, and a time series of the cyst germination process. Resting cyst germination occurred up to one month after cyst formation and 2 – 40% of resting cysts were successfully germinated in cultures maintained at 18 - 21˚C. Importantly, cyst formation may take up 1 – 2 months to occur and rates are likely to be slowed and ceased during cool fall and winter temperatures after blooms. Hence, these cysts may overwinter. Pellicular cysts with hyaline membranes were generally larger than resting cysts, displayed discernable cingulum and/or sulcus, and reverted to

vegetative cells within 24 hours to ~ a week of formation. A putative armored stage of *C. polykrikoides* was not observed during any life cycle stage in this study. This definitive evidence of resting cyst production by *C. polykrikoides* provides a mechanism to account for the recurrence of annual blooms in given locales as well as the global expansion of *C. polykrikoides* blooms during the past two decades.

1. INTRODUCTION

Resting cysts of dinoflagellates can be associated with genetic recombination, maintenance of blooms, termination of blooms, recurrence of annual blooms, resistance against unfavorable environmental conditions, protection from viruses, grazers or parasite attacks, and geographical expansion of populations (Anderson and Wall, 1978; Anderson and Morel, 1979; Anderson, 1989; Hallegraeff and Bolch, 1991; Nehring, 1993; Matsuoka and Fukuyo, 2002; Zingone et al., 2002; Figueroa et al., 2010; Anglès et al. 2012). Resting cysts, therefore, play an important role in the ecology of harmful algal blooms (HABs) of dinoflagellates (Matsuoka and Fukuyo, 2003; Figueroa et al., 2010) and have been considered a fundamental attribute of dinoflagellate life cycles (Elbrächter, 2003). About 100 marine and freshwater dinoflagellates have been shown to produce resting cysts, a small number relative to ~ 2,000 extant species of dinoflagellates (Nehring, 1993; Matsuoka and Fukuyo, 2003). More than 20 of these cyst-producing dinoflagellates are known to cause HABs (Nehring, 1993).

Cochlodinium polykrikoides is an unarmored dinoflagellate that has caused catastrophic HABs in the Caribbean Sea, eastern and western Pacific Ocean, the eastern Atlantic Ocean, Indian Ocean, Mediterranean Sea, and the Arabian Gulf (Margalef, 1961; Matsuoka et al., 2008; Richlen et al., 2010; Kudela and Gobler, 2012). The initiation and development of *C. polykrikoides* blooms have been shown to be related to diurnal migration behavior (Park et al., 2001; Y.-S. Kim et al., 2010), mixotrophy (Jeong et al., 2004), light quantity and quality (Oh et al., 2006), the production of toxins lethal to grazers (Jiang et al., 2009; Tang and Gobler, 2009a & b), allelopathic effects on competing phytoplankton (Tang and Gobler, 2010), transport by large-scale currents

(Onitsuka et al., 2010), and stimulation by nutrients such as nitrogen and vitamins (Tang et al., 2010; Gobler et al., 2012). None of these factors, however, can explain the rapid and large geographic expansion of the species across Asia, North America, and even Europe during the past two decades. While the production of resting cysts could contribute toward such an expansion, whether *C. polykrikoides* produces resting cysts has been an open question until this study (Fukuyo, 1982; Matsuoka, 1985, 1987; C.-H. Kim et al., 2002; C.-J. Kim et al., 2007; Richlen et al., 2010).

Prior studies have reported the identification of resting cysts of *C. polykrikoides* or *Cochlodinium* sp. from sediments (Rosales-Loessener et al., 1996; Matsuoka and Fukuyo, 2000; Matsuoka and Fukuyo, 2002; Orlova et al., 2004; Seaborn and Marshall, 2008; Rubino et al., 2010; Mohamed and Al-Shehri, 2011), but the identity of the observed cysts was not fully confirmed in these studies. For example, Orlova et al. (2004) identified cysts in sediments from the East coast of Russia as *C*. cf. *polykrikoides* based on micrographs in Fukuyo (1982, as *Cochlodinium* sp. 1) and Matsuoka and Fukuyo (2000; as *Cochlodinium* sp. 1, or *C*. cf. *polykrikoides*). Seaborn and Marshall (2008) identified *C. polykrikoides* cysts from sediments in a US east coast estuary but micrographs were not provided, germination experiments were not performed, and a detailed description and identification of cysts was not included. Rubino et al. (2010) and Mohamed and Al-Shehri (2011) reported the identification and germination of *C. polykrikoides* cysts from the Red Sea and the Mediterranean, respectively, but, again, the cysts and germlings were not identified unambiguously. Park and Park (2010) detected *C. polykrikoides* by PCR in sediment samples, suggesting the presence of *C. polykrikoides*

in sediments, but no morphological information was provided and hence the detection of vegetative cells or DNA residues of vegetative cells could not be excluded.

C.-H. Kim et al. (2002) reported on the production of hyaline cysts from cultivated *C. polykrikoides* bloom water while C.-J. Kim et al. (2007) and Tomas and Smayda (2008) reported similar hyaline cysts in laboratory cultures and field samples, respectively. These studies all described temporary cysts (or more appropriately called 'pellicles', see Bravo et al. 2010) with hyaline membranes formed by modification of the vegetative cells without sexual mating (C.-H. Kim et al. 2002; C.-J. Kim et al. 2007; Tomas and Smayda 2008). Interestingly, C.-J. Kim et al. (2007) described a life cycle of *C. polykrikoides* comprising two different morphological stages—an armored and an unarmored vegetative swimming stage, with the latter forming long chains of cells that are commonly observed in this taxon and a hyaline cyst as in C.-H. Kim et al. (2002). Still, C.-J. Kim et al. (2007), by their own admission, did not convincingly document the production of resting cysts by *C. polykrikoides*.

Here, we provide clear visual evidence of the production of resting cysts by *C. polykrikoides* from laboratory cultures isolated from the estuaries of Long Island, NY, USA, Cotuit Bay, MA, USA, and Bahia de La Paz, Mexico. Evidence presented includes sexual mating cell pairs, planozygotes with two longitudinal flagella, and time series micrographs of the cyst formation and cyst germination processes. We believe this information provides a mechanism that may account for the recurrence of annual blooms in some locations and the global expansion of *C. polykrikoides* blooms during the past two decades.

2. MATERIALS AND METHODS

2.1. Algal cultures and conditions of cultivation

Culture isolates of *Cochlodinium polykrikoides* strains CPSB-2A, CPNB-40C, CPSB-1A, CPSB-1B, CPSB-1G, CP1, CPPB-17, and CPGSB-1 were obtained by pipetting single cells (except for CPSB-2A and CPNB-40C) to 24-well polystyrene cell culture plates containing sterile GSe culture medium (see below) under an inverted microscope. Cultures were established from bloom water collected between 2006 and 2011 from the estuaries of Long Island, NY, USA (Table 1). The culture CPSB-2A was established from two cells isolated from Shinnecock Bay in 2010, while the culture CPNB-40C was established from 40 cells isolated from the Noyak Bay in 2011, with both cultures purposefully established as multi-cell isolates to explore cyst production. Molecular (large subunit, LSU, rDNA sequencing) and microscopic identification of strain CP1 was reported previously (Gobler et al., 2008; Iwataki et al., 2008). Using the methods described in Gobler et al. (2008), sequencing the large sub-unit rDNA of cultures CPSB-1A, CPSB-1B, CPSB-1G, CPPB-17, and CPGSB-1 from NY as well as strains CPCB-10 and CPPV-1 from Cotuit Bay, Massachusetts, USA, and Bahıa de La Paz, Mexico, respectively, confirmed the identity of each culture as *C. polykrikoides*. Table 1 lists the origins, isolation dates, and isolators for all cultures used in this study. While a clonal culture here refers to a culture established from a single cell (or multiples from the same chain), the possibility of the isolation of a planozygote which is visually similar to vegetative cells cannot be excluded.

All cultures were cultivated in sterile GSe medium with a salinity of 32.5 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. An antibiotic solution (a mixture of 10, 000 I.U. penicillin and10, 000 µg mL^{-1} streptomycin, Mediatech. Inc., Hemdon, VA) was added into the medium immediately before inoculation, with a final concentration of 2% to discourage the growth of bacteria in cultures.

2.2. Production and observation of resting cysts

The production of resting cysts was first observed in CPSB-2A and CPNB-40C, both established from multiple vegetative cells (Table 1). After the production of resting cysts in these cultures was confirmed (see results), pair-wise crossing experiments were conducted with the cultures CPSB-1A, CPSB-1B, CPSB-1G, CP1, CPPB-17, CPCB10, and CPPV-1, using 6-well cell culture plate with each well containing 8 mL GSe medium with 2% antibiotics (Table 2). Crosses were also performed using GSe medium without the addition of nitrogen and phosphorous (GSe-N-P) to observe how the depletion of macronutrients might affect cyst formation, by adding 0.5mL of dense stock cultures (~2,000 cells mL^{-1}) to each well of a plate containing 8mL GSe-N-P medium. These cultures entered stationary growth phase after ~30 days.

Cyst production, cyst morphology, and vegetative cells were observed under a Nikon ECLIPSE TS100 inverted microscope. Micrographs and videos were obtained with a Nikon digital-Sight DS-U2 camera (Nikon Cooperation, Japan) and the NIS Elements BRTM Imaging software 3.22.11 (Build 728), LO, Nikon. Cells within crossing experiments were observed every other day for up to two months and the presence of

planozygotes (i.e. vegetative cells with two longitiudinal flagella), sexually mating cell pairs, and cysts were recorded and/or photographed.

2.3. Germination of resting cysts

Five germination experiments were conducted under a 12:12h light:dark cycle at ~100 µmol quanta m^{-2} s^{-1} at 21˚C except for one experiment at 18˚C (Table 3). Cysts (both resting and pellicular cysts) for germination experiments one, two, and five were isolated from a culture of CPSB-2A, while the cysts for the experiments three and four were isolated from a culture of CPNB-40C (Table 3). Individual cysts were isolated from the cultures by micro-pipetting, were washed with fresh GSe medium in a Petri dish, and placed in individual wells of 24-well cell culture plate containing 2.5 mL fresh GSe medium with a 2% antibiotics solution. For the final experiment (experiment 5), antibiotics were added every the other day to assess the role bacteria may play in cyst germination (Table 3). Cysts were monitored every the other day under the Nikon ECLIPSE TS100 inverted microscope and photographed as described above.

3. RESULTS

3.1. Cyst formation and cyst morphology

Resting cysts of *C. polykrikoides* were formed via the mating of two gametes, which was evidenced from two cells connected or fused in positions perpendicular or non-parallel to each other (Fig. 1B-E). A red accumulation body was typically observed during and/or after the mating process (Fig. 1D, G, I). A fused nucleus could be observed after mating was completed (Fig. 1F and G). Immature cysts were distinguishable from vegetative

cells as well as mature resting cysts and generally displayed a pear-like or elongated shape (Fig. 1H), while matured resting cysts took a perfectly circular shape (Fig. 1I). After mating, planozygotes with two longitudinal flagella and with or without red accumulation body could remain in a vegetative state for several days prior to transforming into cysts (Fig. 2A-F; Supplementary video 1). Cysts were most commonly produced in cultures during late exponential or stationary stage of growth, two-to-three weeks post-inoculation, although cysts were occasionally observed in cultures within one week of inoculation into new media. Cyst production varied among wells of the same culture plate and batches of the same culture (e.g. CPSB-2A) experiencing similar culturing conditions (medium, temperature, light:dark cycle, and light intensity) and many vegetative cells and temporary cysts (pellicles) died and broke into fragments as resting cysts were forming. While this made calculating a precise cyst production rate challenging, rates were generally < one cyst per 1,000 cells. There was no obvious increase in cyst production in cultures grown without nitrogen and phosphorus.

Mature resting cysts of *C. polykrikoides* were 20 to 40 μm, yellowish brown in color, contained one or sometimes two red accumulation bodies, and took a perfect spherical or circular shape but could be ellipsoidal when newly produced. The cyst wall was relatively thin (< 2 μm), possibly comprised of two layers. The cyst surface was smooth, without rough projections seen in cysts of other unarmored dinoflagellates (Fig. 1I, Fig. 5, Day 1, and Fig. 6, Day 1). It was, however, sometimes difficult to discern a newly formed resting cysts from temporary cysts (pellicles) as they both had similar sizes, a round shape, and heavy pigmentation.

Pellicles were observed in both non-clonal (CPSB-2A and CPNB-40C) and some clonal cultures (e.g. CP1) and were distinguishable from mature resting cysts. Pellicles were generally larger (35 to 60 μm) and darker than mature resting cysts, with or without red accumulation bodies, with or without chloroplasts, with many cytoplasmic particles in Brownian motion and features of vegetative cells such as cingulum and sulcus discernible (Fig. 3). Pellicles had thin hyaline membranes (Fig. 3D), consistent with the observations of C.-H. Kim et al. (2002), and could be formed from individual cells as well as from chains of cells (Fig. 3E). Pellicles reverted (or transformed) to vegetative cells (i.e. without shedding the cyst wall after germination) within ~ one week after being transferred into fresh culture medium. Pellicles were generally more fragile than resting cysts and more prone to decomposition if they did not germinate in a short time. Both resting cysts and pellicles were produced from cultures in both the full strength GSe media and media without nitrogen and phosphorous added.

3.2. Resting cyst formation in crossing experiments

During self- and inter-crossing experiments among cultures of *C. polykrikoides* isolated from different locations of North America and different years from Long Island estuaries, both planozygotes with two longitudinal flagella and resting cysts were observed in the non-clonal cultures CPSB-2A and CPNB-40C, the clonal cultures CPSB-1A, CPSB-1G, CPGSB-1, CPPB17, and CPCB10, and most of the crossed culture combinations (e.g. CP1 vs. CPPV-1, CP1 vs. CPSB-1A, CPCB10 vs. CPSB-1A, and CPPB17 vs. CPSB-1A; Table 2). Two of six clonal cultures did not self-cross (CP1 and CPSB-1B) and two cultures did not cross (CP1 vs. CPSB-1G and CPSB-1B vs. CPPB17; Table 2).

3.3. Time series of the cyst germination process

The first germination experiment was conducted under normal culturing conditions (21˚C, 12:12h light:dark cycle, ~100 µmol quanta m^{-2} s^{-1}) with cysts from a three month-old non-clonal culture, CPSB-2A. Five of 24 cysts (21%) completely germinated after 19 to 26 days of incubation (Fig. 4), two cysts ceased germinating in the middle of the process, and the remaining cysts decomposed (Table 3). Experiment two also used cysts isolated from a three-month-old culture of CPSB-2A and only one of 12 cysts (8%) germinated in 8 days, and the remaining cysts decomposed (Table 3).

Germination experiments three and four were conducted with the cysts isolated from a 16 day-old culture and a 61 day-old culture of CPNB-40C (non-clonal culture), respectively. During experiment three, four of 48 resting cysts (8%) germinated in 12-20 days while three pellicular cysts reverted to vegetative stage after 24 h. Most of the cysts (30 of 48) decomposed in two to eight weeks (Table 3). During experiment four, one in 48 cysts germinated in 15 days and one ceased germination in the middle of the process, while all other cysts (96%) decomposed in three weeks (Table 3). The high-rate of cyst decomposition in experiments two, three, and four may have been partly due to the proliferation of bacteria as visualized microscopically (DAPI-staining with epifluorescent microscopy), despite the 2% antibiotics solution added to growth medium at the start of each experiment.

To reduce bacterial growth, experiment five was conducted at a slightly lower temperature (18˚C) and with antibiotics added weekly to cysts isolated from a 40 day-old culture of CPSB-2A. Of 24 total cysts, six pellicular cysts reverted to vegetative stage

within 2-9 days (Fig. 3), eight resting cysts germinated in 15-31 days (Figs. 5, 6), six resting cysts ceased germinating in the middle of the process, while four cysts decomposed over three weeks (Table 3). While the lowered temperature and addition of antibiotics significantly increased the germination rate (Table 3), bacterial growth was still observed (see the decomposed cyst wall of day 22 to 28 in Fig. 5 and day 31 in Fig. 6).

The germination processes of two resting cysts of CPSB-2A over 28 and 31 days are shown in Figs. 5 and 6 as a time series. Unlike the transformation of pellicles into vegetative cells in two days to about a week, the germination of resting cysts occurred within the cyst wall in 8 to 31 days. The cyst gradually developed into a gymnodinoid cell (day 13-16 in Fig. 5, and day 18 in Fig. 6) with the cingulum and sulcus being recognizable first (day 8 in Fig. 5 and day 10 to 15 in Fig. 6). The pigmentation became heavier with the progression of germination (day 18 and onwards in Fig. 6) and the germling sometimes divided before being released (Fig.5) and sometimes did not (Fig. 6). The germling started to move within the cyst wall before the flagella was distinguishable and was visually identical to vegetative cells (Fig. 4C and D, Fig. 5 day 19 to 26; Supplementary video 2).

Differing from the observations of Kim et al. (2007), an armored stage was never observed for any germling in any culture or culture cross during this and prior studies (Tang and Gobler 2009a&b; 2010). According to the observations of multiple germinated cysts, the archeopyle type may belong to the tremic cryptopylic (Matsuoka and Fukuyo, 2002). However, given that the cyst wall is thin and was often decomposed by bacteria

when the cyst completely germinated, the precise archaepyle type requires further confirmation.

4. DISCUSSION

4.1. Morphology of the resting cysts of C. polykrikoides

We have demonstrated that *C. polykrikoides* can produce resting cysts by providing visual evidence of sexual mating cell pairs, immature and mature cysts, planozygotes with two longitudinal flagella, and an up to one month germination process. We have further provided evidence for the production of morphologically and life cycle distinct pellicles/temporary cysts that are consistent with previous literature reports (C.-H. Kim et al., 2002; C.-J. Kim et al., 2007). In contrast to pellicles, which revert or transform to the vegetative stage in a short time (one day to ~ one week) without shedding a cyst wall, the germination of a resting cyst takes place within the cyst over a longer time (about a month) with the germling leaving the cyst through a cryptopylic archaepyle. Since resting cysts were observed in both self-crossed and inter-crossed cultures (Table 2), these resting cysts may be produced homothallically (i.e. by monoclonal cultures; Matsuoka and Fukuyo, 2003) although the initial isolation of planozygotes for cultures cannot be absolutely discounted. Since cysts were successfully germinated at 21˚C after being directly transferred from the culture maintained at 21˚C, a mandatory dormancy at low temperature (Anderson and Keafer, 1987) was not required.

Our observations of the morphology of resting cysts and the life cycle of *C. polykrikoides* differ from previous reports (Fukuyo, 1982; Matsuka and Fukuyo, 2003; C.-J. Kim et al., 2007; Rubino et al., 2010). Fukuyo (1982) and Matsuoka and Lee (1994) (as referred in

Matsuoka and Fukuyo, 2003) described two cyst forms from field sediments identified as *C.* cf. *polykrikoides*. Both forms had prominent surface projections up to a maximum of 7μm and took an irregular shape (subspherical, ovoidal, or ellipsoidal). Since these cysts were isolated from field sediments, the observed membranous surface ornaments or lengthy projections might be caused by biotic or chemical processes on the surface in the sediments or by the sample processing. Otherwise, these extensive surface projections and membranous ornaments distinguish these two cyst forms from those observed in our study, since the surface projections of the cysts observed in the present study were never as dense and lengthy as shown in Matsuoka and Fukuyo (2003). Also, the mature resting cysts observed in our cultures had a perfect round or circular shape, although it was not always possible to discern an immature resting cyst from a pellicle or a temporary cyst and the morphology of the resting cysts may be altered in sediments. Matsuoka and Fukuyo (2003) admittedly 'could not confirm the identification' of cells germinated from putative *C.* cf. *polykrikoides* cysts since only single cells, but not cell chains, were produced. It is noteworthy that Orlova et al. (2004), Seaborn and Marshall (2010), Rubino et al. (2010), and Mohamed and Al-Shehri (2011) all identified cysts of *C. polykrikoides* from sediments by referring to the descriptions of Matsuoka and Fukuyo (2002) or Matsuoka and Fukuyo (2003).

C.-J. Kim et al. (2007) described a life cycle comprised of two different morphological stages of vegetative cells of *C. polykrikoides* —an armored and an unarmored stage. The unarmored stage formed long chains of cells and hyaline 'temporary cysts', while the armored stage produced a non-motile cell type that was presumptively considered as resting cyst. Armored cells were not observed in our cultures during this or any other

prior study of North American cultures (Tang and Gobler, 2009a & b, 2010) and all observed planozygotes with two longitudinal flagella were unarmored.

Considering the substantial phylogenetic differences among populations of *C. polykrikoides* from different geographic regions across the globe, categorized as different 'Ribo-types' (Iwataki et al., 2008), and the recent description of *C. fulvescens* (Iwataki et al., 2007), the abovementioned differences in the morphology of resting cysts and life cycle might be because these 'Ribo-types' and the cysts previously as attributed to *C. polykrikoides* from sediments are in fact different species. This would seem to be more likely for studies of Asian populations of *C. polykrikoides* (Fukuyo, 1982; Matsuka and Fukuyo, 2003; C.-J. Kim et al., 2007) compared to reports of North American populations since the isolates from this region have yet to be shown to be genetically distinct (Iwataki et al., 2008; Mulholland et al., 2009). In any case, it is possible that prior studies incorrectly identified cysts from the field as belonging to *C. polykrikoides*.

4.2. Ecological implication of the production of resting cyst by C. polykrikoides

Prior to 1990, *Cochlodinium polykrikoides* blooms had been documented mainly in Southeast Asia and sporadically on the east coast of North America. However, there has been an extraordinary expansion in the distribution of cells and blooms of *C. polykrikoides* and a similar species, *C. fulvescens*, across Asia, North America, and even Europe during the past two decades (Kudela and Gobler, 2012). The production of resting cysts and subsequent transport via ships' ballast water or the transfer of shellfish stocks is a mechanism that could account for this dramatic expansion (Hallegraeff and Bolch, 1991; Hallegraeff, 1993; Smayda, 1997, 2007). Since confirmative evidence for resting

cyst production in *C. polykrikoides* has not been available, however, seeding the population by cyst germination (excystment) has generally not been considered a mechanism for its bloom initiation and expansion. For example, Matsuoka et al. (2010) suggested that seed populations in the East China Sea might be associated with overwintering vegetative cells rather than cysts. Nagai et al. (2009) also inferred from microsatellite analysis that local populations of *C. polykrikoides* overwinter and survive as vegetative cells in local waters of Japan and Korea. The confirmative evidence of resting cyst production by *C. polykrikoides* in the present study provides both a more convincing basis for those studies reporting identification of *C. polykrikoides* cysts from sediments (e.g. Park and Park, 2010; Mohamed and Al-Shehri, 2011) and a feasible explanation for the expansion of *C. polykrikoides* blooms around the world. For instance, an intensive catastrophic bloom of *C. polykrikoides* suddenly occurred as the first time in the Arabian Gulf region in 2008 and 2009 and phylogenetic analysis confirmed the bloom population was identical to isolates from the northeastern USA, Puerto Rico, Mexico, and Malaysia, known as the ‘‘American/Malaysian’’ ribotype (Richlen et al., 2010). The Arabian Gulf is well known for busy ports hosting large shipping vessels that may discharge copious volumes of cyst-containing ballast water within local water bodies. Therefore, our confirmation of resting cyst production by *C. polykrikoides* strongly supports the hypothesis of Richlen et al. (2010) that the sudden emergence of *C. polykrikoides* blooms in the Arabian Gulf region may have been caused by the recent introduction of this species through ballast water discharge.

Cyst production by *C. polykrikoides* may also account for the annual recurrence of HABs by this species in specific locations. For example, prior to 2004, *C. polykrikoides* blooms

had never been observed in NY waters despite the existence of robust phytoplankton monitoring programs since the 1950s (Ryther, 1954 ; Nuzzi and Waters, 2004) and the highly conspicuous nature of surface blooms of this species. Since 2004, this species has formed blooms every year through 2012 within the same NY estuaries (Shinnecock Bay, Great Peconic Bay; Kudela and Gobler, 2012). This contrasts with other, non-cyst forming HABs that bloom more ephemerally within these same systems (e.g. *Aureococcus anophagefferens*; Gobler and Sunda, 2012). The production of cysts by *C. polykrikoides* that overwinter and germinate to form blooms would account for its ability to form blooms annually in these estuaries.

Park and Park (2010) detected *C. polykrikoides* in surface sediments of the South Sea, Korea, using species-specific real-time polymerase chain reactions (PCR) probes and suggested that *C. polykrikoides* may persist in the form of cyst in sediments where blooms of *C. polykrikoides* occur annually. Our results support this supposition. Further study examining environmental triggers affecting cyst formation and excystment both in the laboratory and in natural populations of *Cochlodinium polykrikoides* are clearly warranted.

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Table 1. Origins, dates of isolation, and isolators for the cultures of *Cochlodinium polykrikoides* used in this study.

Culture	Origins	Date of isolation	Isolator	Clonal?
CPSB-2A	Shinnecock Bay, NY, USA	Aug. 5, 2010	Y.Z. Tang	No
CPNB-40C	Noyak Bay, NY, USA	Aug 16, 2011	Y.Z. Tang	No
CPSB-1A	Shinnecock Bay	Aug. 5, 2010	Y.Z. Tang	Yes
CPSB-1B	Shinnecock Bay	Aug. 5, 2010	Y.Z. Tang	Yes
CPSB-1G	Shinnecock Bay	Aug. 5, 2010	Y.Z. Tang	Yes
CPGSB-1	Great South Bay	Aug. 17, 2011	Y.Z. Tang	Yes
CP1	Flanders Bay, NY, USA	Aug. 31, 2006	Y.Z. Tang	Yes
CPPB-17	Peconic Bay, NY, USA	Sep. 4, 2008	Y.Z. Tang	Yes
CPCB10	Cotuit Bay, MA, USA	Sep 2001	D. Kulis	Unknown
CPPV-1	Bahı´a de La Paz, Mexico	Unknown	L. Morquecho	Unknown

Table 2. Ability of pairs of *C. polykrikoides* cultures to form mating cell pairs and cysts.

	CPSB-2A	CPNB-40C	CPSB-1A	CPSB-1B	CPSB-1G	CP1	CPPB-17	CPCB10	CPPV-1
CPSB-2A	Yes	*	*	*	*	*	*	*	*
CPNB-40C		Yes	*	*	*	*	*	*	*
CPSB-1A			Yes	Yes	Yes	Yes	Yes	Yes	_
CPSB-1B				No	Yes	Yes	No	Yes	_
CPSB-1G					Yes	No	Yes	Yes	_
CP1						No	Yes	Yes	Yes
CPPB-17							Yes	Yes	_
CPCB10								Yes	Yes
CPPV-1									_

‘*’ indicates test not applicable and ‘_’ indicates test not conducted.

Table 3. Conditions and results of five cyst germination experiments.

Germination experiment	1st	2nd	3rd	4th	5th
Culture strain and (age)	CPSB-2A (3 month)	CPSB-2A (3 month)	CPNB-40C (6 day)	CPNB-40C (2 month)	CPSB-2A (40 days)
Germination temperature	21˚C	21˚C	21˚C	21˚C	18˚C
Antibiotics addition	Once	Once	Once	Once	Once a week
Starting no. of resting and pellicular cysts	24	12	48	48	24
No. of pellicles transformed to vegetative cells and time (day)	0	0	3 (1 day)	0	6 (2-9 days)
No. of resting cysts germinated and time (day)	5 (19-26d)	1 (22d)	4 (12-20 days)	1 (15 day)	8 (15-31 days)
No. of resting cysts ceased mid-germination	2	0	0	1	6
No. of cysts broken or decomposed	17	11	41	46	4

Figure Legends

Fig. 1. Formation of resting cysts in *C. polykrikoides* through sexual mating of gametes observed in cultures of CPSB-2A, CPCB10, CPPB17, and CPPB17 × CPSB-1A. (A) a typical vegetative cell; (B-E) mating of gametes with red accumulation body observable during mating (arrow); (F) mating nearly completed with fused nuclei observable (indicated by 'N'); (G) mating completed, with accumulation body; (H) an immature cyst, and (I) a mature resting cyst. Scale bar: 10 μm.

Fig. 2. Planozygotes of *C. polykrikoides* with two longitudinal flagella observed (arrows) in the cultures of (A) CPCB10 (with red body, white arrow); (B) CP1 × CPPV-1; (C) CPSB-1G; (D) CPCB10 × CPSB-1A (with red body, white arrow; with two longitudinal flagella but cannot be seen clearly in the micrograph); (E) CPCB10; and (F) CPGSB-1. See also supplementary video 1.

Fig. 3. Pellicles (temporary cysts) of *C. polykrikoides* observed in the cultures CPSB-2A. Compared to resting cysts, pellicles were larger, germinated faster, left behind no empty cyst, sometimes possessed a discernible cingulum and (A-C, E) and sulcus (B, E), and hyaline membrane. The pellicle in D transformed into a vegetative cell in 2 days.

Fig. 4. Germination of a single resting cyst of *C. polykrikoides* isolated from a 3 month-old non-clonal culture of CPSB-2A. (A-C) different stages of germination within the cyst (A: day 3, B: day 14, C: day 19), (D) the new germling one day after leaving the empty cyst. Scale bar: 10 μm.

Fig. 5. Time series of the germination process of a *C. polykrikoides* resting cyst at 18˚C over 28 days. The cyst at day 1 is newly isolated; the surface became coarser after day 5; the cingulum became discernable on day 8; a division furrow seen on day 16; two divided but connected germlings seen on day 19; and the two new germlings left behind an empty cyst during days 26 - 28. Scale bar: 20μm. See also supplementary video 2.

Fig. 6. Time series of the germination process of a *C. polykrikoides* resting cyst 18˚C over 31 days. The cyst at day 1 is newly isolated; the surface became coarser after day 5; the cingulum became discernable on day 10 and the sulcus discernable on day 15; a gymnodinium-like germling with heavier pigmentation seen on day 18; an enlarged and even more heavily pigmented germling seen on day 22; and the germling left the cyst after day 31. Scale bar: 20μm.

Figures

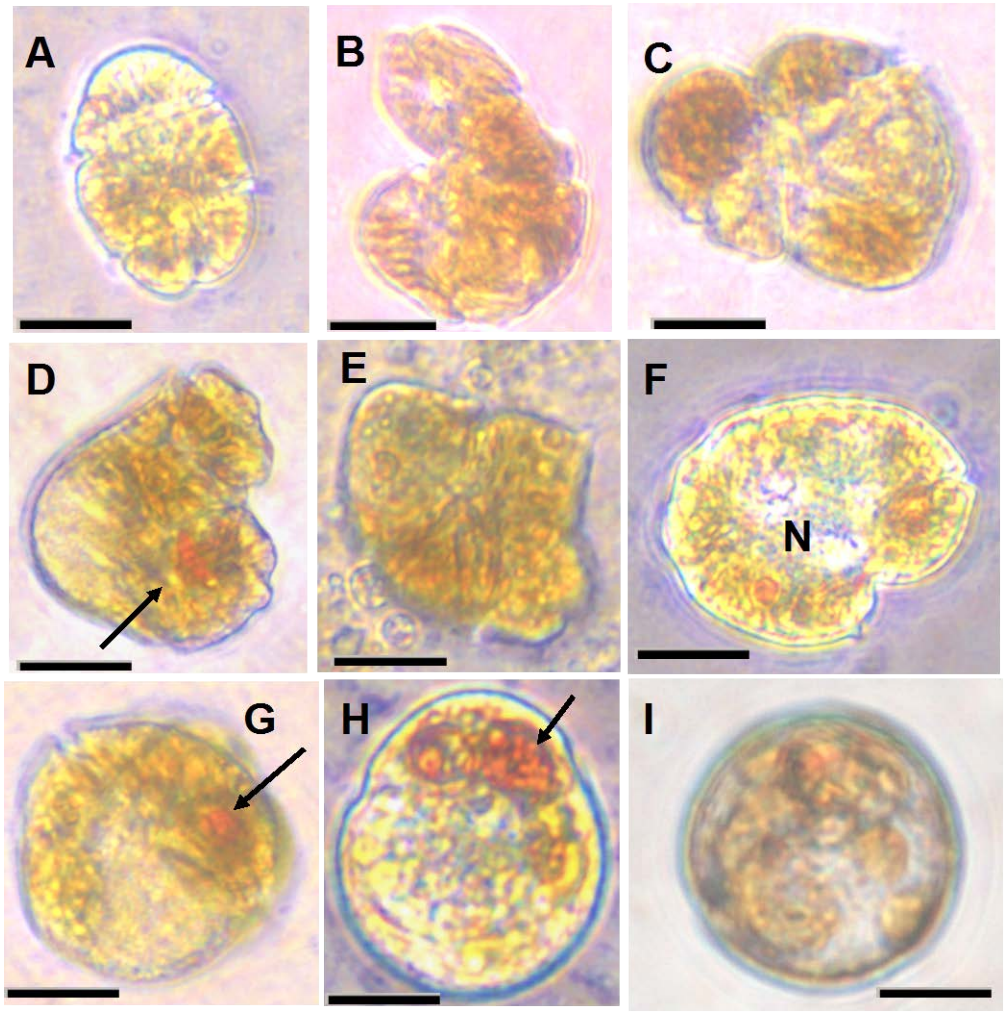


Fig. 1.

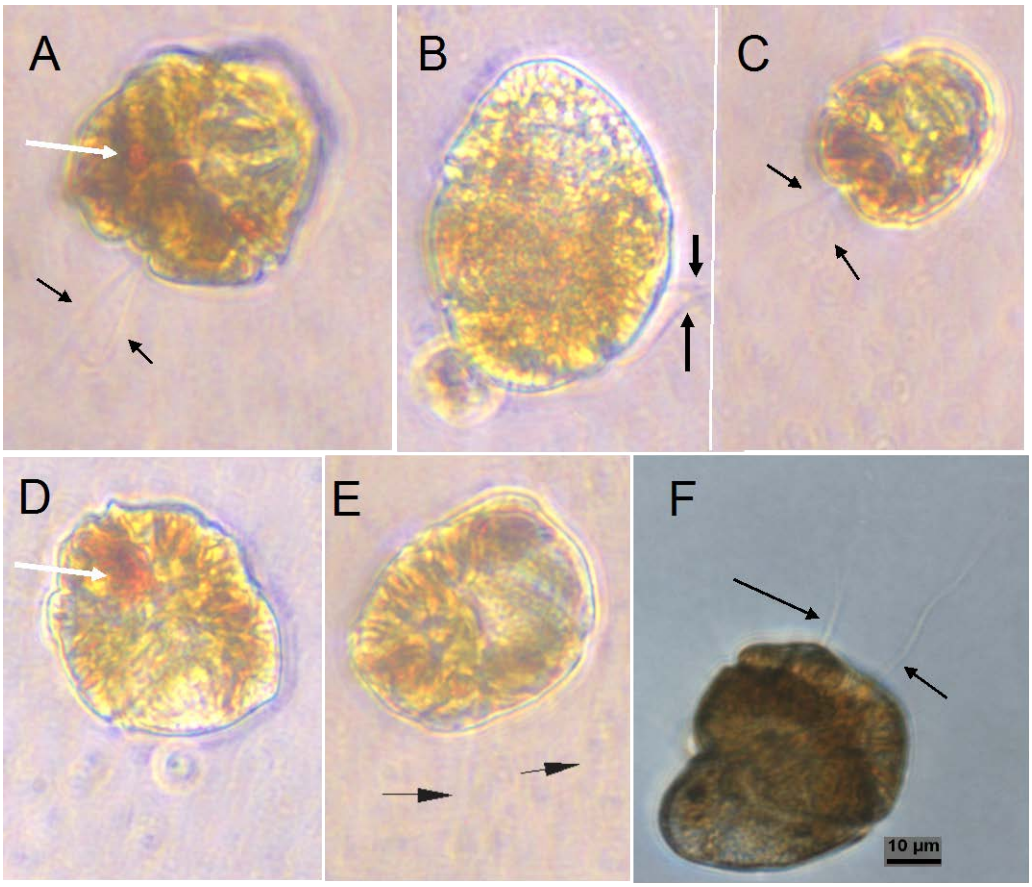


Fig. 2.

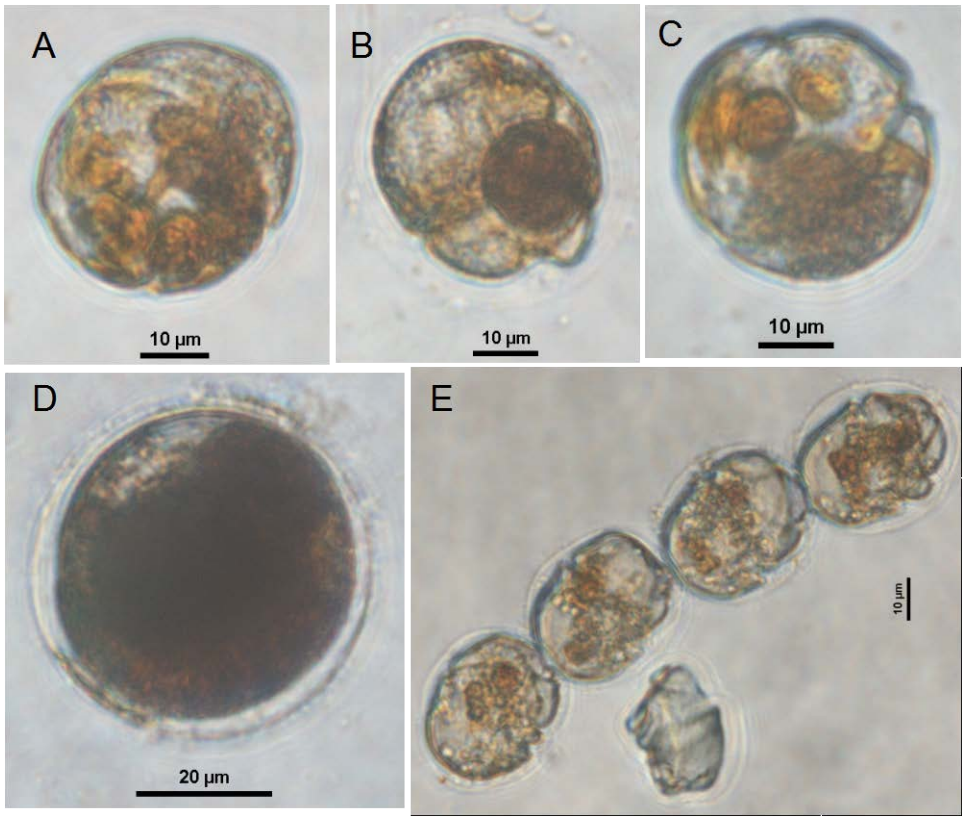
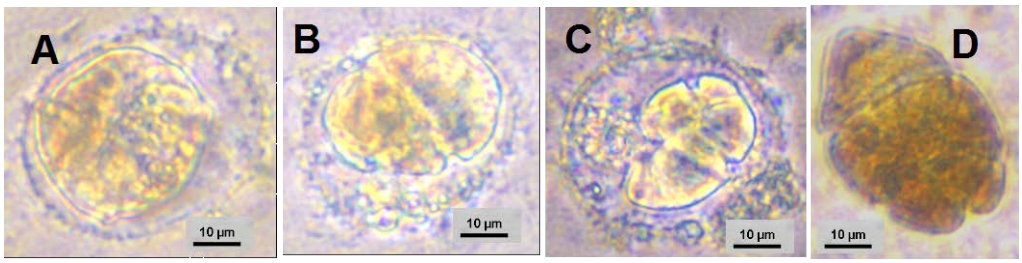


Fig. 3.



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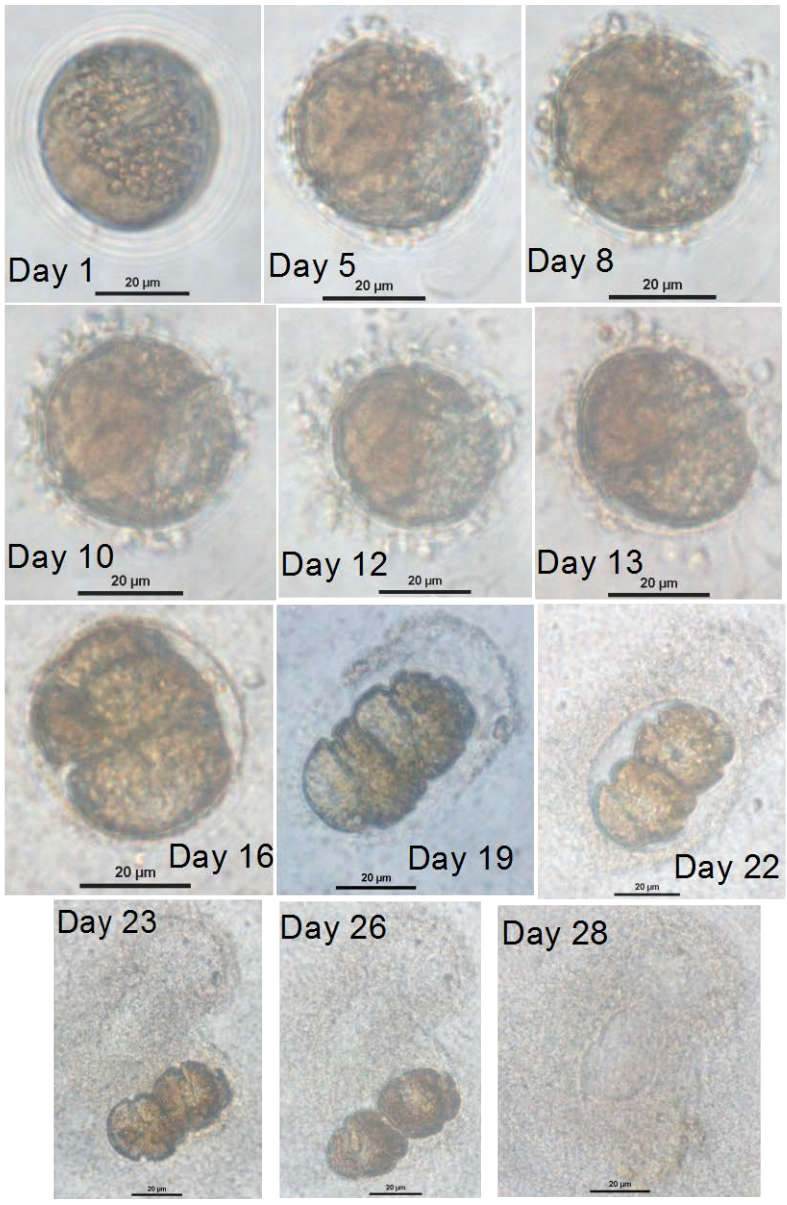
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Fig. 5.

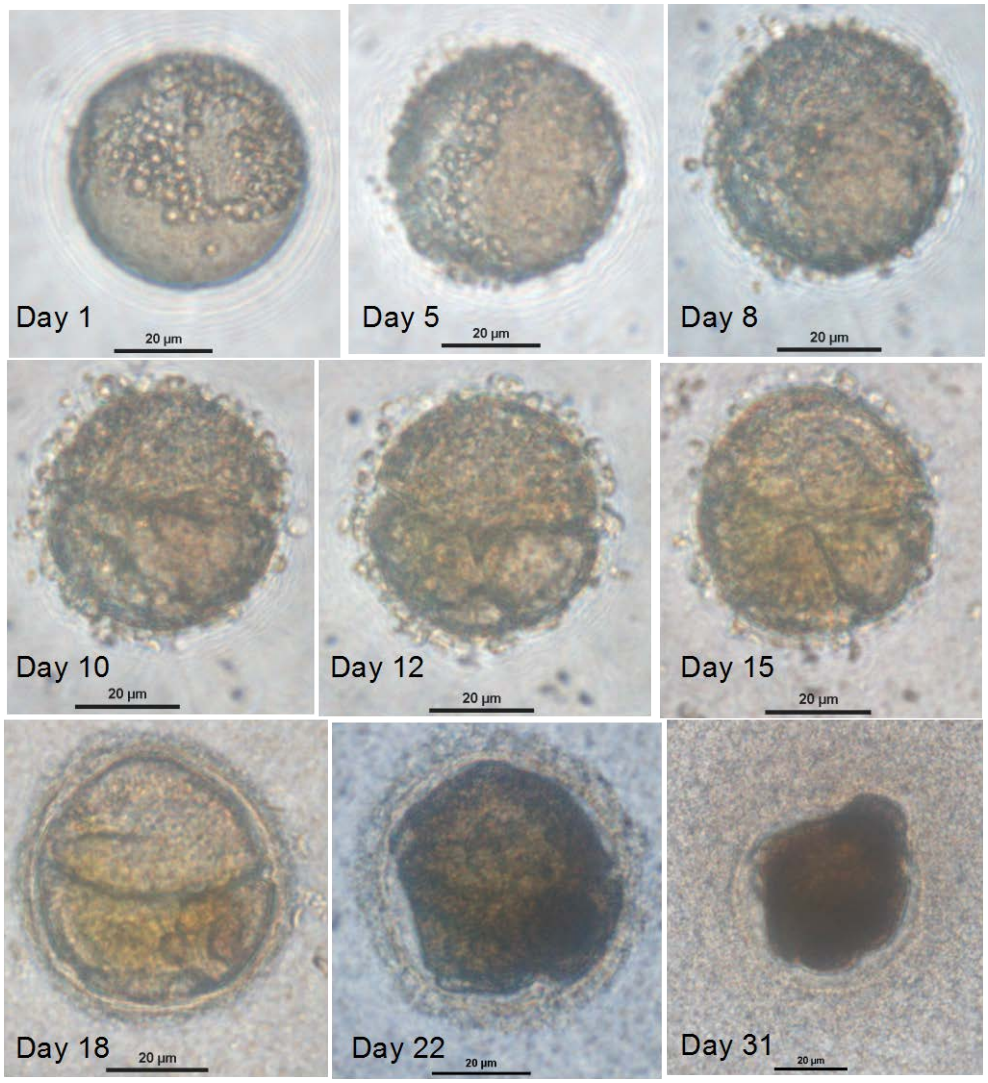
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Fig. 6.

TASK 7. ASSESS THE TEMPERATURE TOLERANCE OF *COCHLODINIUM POLYKRIKOIDES.*

Introduction

Blooms of *C. polykrikoides* have been annually recurred in the estuaries of Long Island highly predictably in late summer from 2004 to 2013 (Gobler et al. 2008; Tang and Gobler, pers. obs.). The species also bloom in other temperate countries at the similar seasons (e.g. Lee & Lee 2006; Tomas & Smayda 2008; Matsuoka and Iwataki 2004). We have recently proved that *C. polykrikoides* can produce resting cysts (Tang and Gobler 2012). However, this species, unlike other resting cyst-producing species which also forms blooms in LI annually such as *Aleandrium tamarense* (Hattenrath et al. 2010), *Scrippsiella trochoidea* (Tang and Gobler, pers. obs.), and *Gymnodinium aureolum* (Tang et al. 2008), does not bloom in spring or early summer. Therefore, we hypothesized that this species may prefer a niche of higher temperature. In order to test it, we conducted a series of grow-out experiments for two cultures of *C. polykrikoides* at different temperatures. The results proved that the species had the highest growth rate at a temperature (25°C) which has been, or close to, the temperature when we observed its blooms in Long Island estuaries.

Materials and Methods

Experiments for temperature tolerance of C. polykrikoides

Two cultures of *C. polykrikoides* were used in the experiments: the highly toxic monoclone CP1 and the less toxic non-clonal CPSB-2A. The stock cultures were incubated in sterile GSe medium with a salinity of 32.5, made with autoclaved and 0.2 μm-filtered seawater, in an incubator with a 12:12h light:dark cycle, illuminated by a

bank of fluorescent lights that provided a light intensity of ~80 µmol quanta m^{-2} s^{-1} to cultures at 21° C. Therefore, the temperature tolerance experiments were conducted stepwise from 21 to 30° C and from 21 to 14° C, respectively, with each step lasted for seven days. Accordingly, the experiments at temperatures 21, 25, and 30° C started from the same stock cultures maintained at 21° C, while the experiments at temperatures 17 and 14° C started with another batch of stock culture also maintained at 21° C. Since all experiments were conducted in the same incubator, all conditions were the same as abovementioned except temperature. An antibiotic solution (a mixture of 10, 000 I.U. penicillin and10, 000 µg mL^{-1} streptomycin, Mediatech. Inc., Hemdon, VA) was added into the medium immediately before inoculation, with a final concentration of 3% to discourage the growth of bacteria in cultures. The stock cultures maintained at 21° C were sampled for enumeration of cell density and diluted with antibiotic-containing GSe medium to 600 cells mL^{-1} for CP1 or 60-400 cells mL^{-1} for CPSB-2A, aliquoted to three 500ml-flasks with each containing 300 mL of culture. The flasks were placed in the incubator with temperature adjusted to the desired level and incubated for seven days. An aliquot of 10 mL was taken on day 3 (72h) and day 7 (168h) and fixed with Lugol's solution (2% final concentration) for enumeration. On day 7, the same cultures were used for the next temperature level after enumeration and dilution with fresh GSe medium.

Results

Within the range of temperature tested in the study (13-30°C), the growth rate of *C. polykrikoides* cultures CP1 and CPSB-2A increased monotonically from 13 to 25°C ($p < 0.001$, one-way ANOVA) and then decreased slightly but significantly ($p < 0.001$, post

hoc comparison of one-way ANOVA) from 25 to 30°C (Table 1; Fig 1). In general, the growth rates at higher temperatures (21 to 30°C) were significantly higher than that at lower temperatures (13 and 17°C) and the growth at 13°C was minimal. Although the growth at 13°C was minimal, cells of *C. polykrikoides* could survive (observation under inverted microscope). In addition, *C. polykrikoides* exhibited an acclimation period during the first 3 days at 17°C and then restored its growth. Although CP1 and CPSB-2A differed in growth rate at the same temperature (CPSB-2A displayed higher growth rates at temperatures 21, 25, and 30°C), both exhibited similar pattern in response to temperature change. These findings suggest that the early onset blooms in County waters when temperatures are warmest may be the most toxic to fisheries.

Table 1. Growth rates (μ) of *Cochlodinium polykrikoides* cultures CP1 and CPSB-2A at different temperatures, which were the higher values calculated from day 1 to day 4 and that from day 4 to day 8.

Temperature (°C)	**14**	**17**	**21**	**25**	**30**
μ of CP1 (d^{-1})	-0.03±0.01	0.083±0.020	0.112±0.008	0.240±0.029	0.166±0.030
μ of CPSB-2A (d^{-1})	0.02±0.01	0.036±0.017	0.167±0.037	0.319±0.019	0.183±0.021

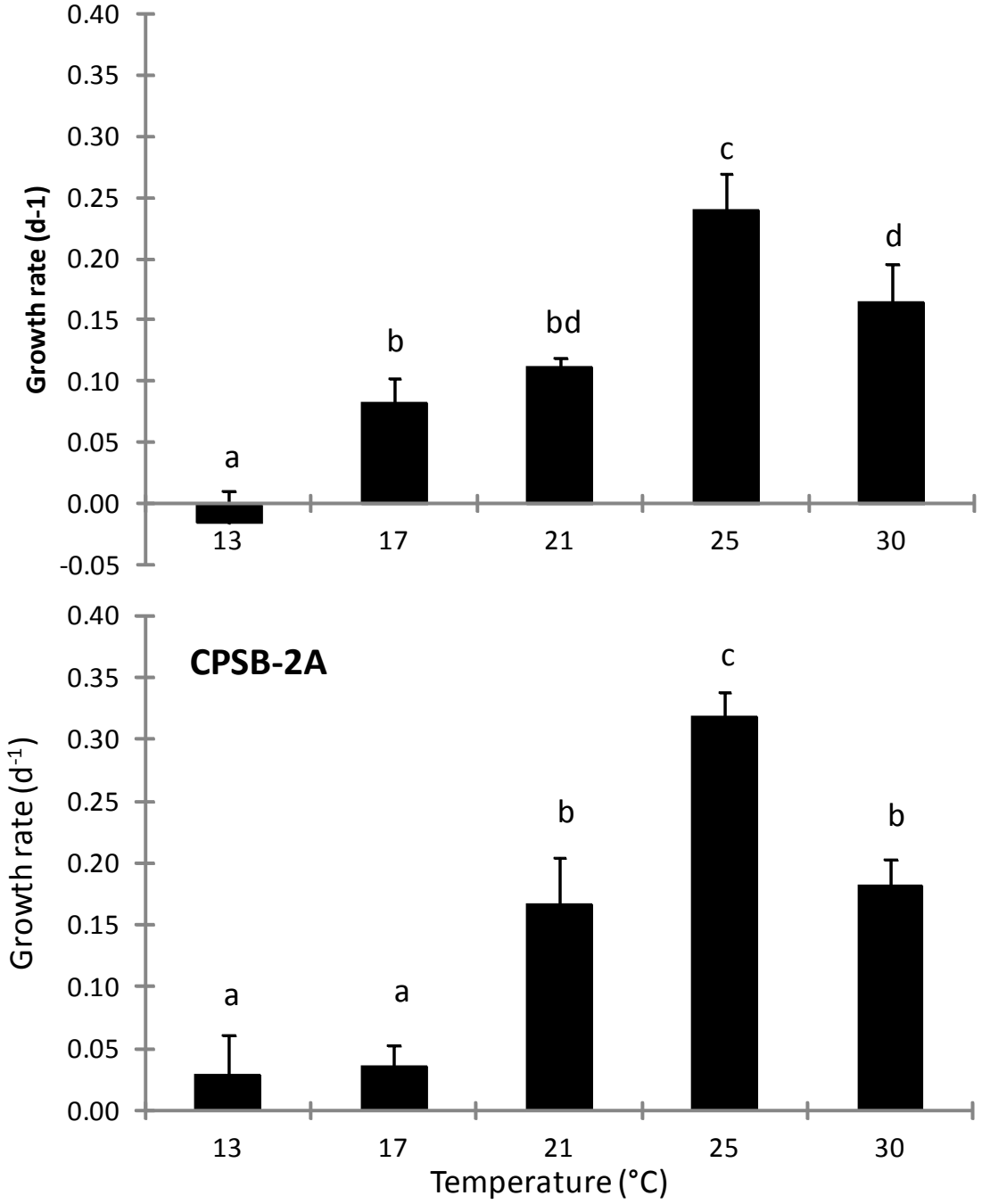

Fig. 1. Dependence of growth rate on temperature for *Cochlodinium polykrikoides* strains CP1 and CPSB-2A. The error bars indicate ± 1 standard deviation, and lowercase letters indicate significant differences.

TASK 8. ASSESS THE MECHANISM OF TOXICITY OF *COCHLODINIUM POLYKRIKOIDES.*

Although the fish and shellfish killing activity of *Cochlodinium* species have been well documented, the associated toxic mechanism or chemical nature of toxicity has been a controversial issue (Onoue et al. 1985; Onoue and Nozawa, 1989a, b; Kim et al. 1999; Whyte et al. 2001; Kim et al. 2002). Landsberg (2002) categorized *Cochlodinium* species as taxa with multiple toxins. Two early studies reported three toxic fractions (i.e. neurotoxic, hemolytic, and hemagglutinative; Onoue and Nozawa 1989a) and zinc-bound paralytic shellfish poisoning (PSP) toxins (Onoue and Nozawa 1989b) from the red tide waters of *Cochlodinium* type' 78 Yatsushiro, which was considered to be conspecific with *C. polykrikoides* (Matsuoka et al. 2008). However, the PSP toxins and two of the non-PSP toxic fractions have not been identified further or described since their initial report (Onoue and Nozawa 1989a,b), while the third fraction, hemolytic agents, has been documented to be associated with fatty acids (Lee 1996). Because these toxic fractions were originally extracted from a mixed biomass sample concentrated from a large volume of field bloom water (1000 L; Onoue and Nozawa 1989b), it is possible that these toxins or toxic fractions came from other sources. Others have demonstrated that reactive oxygen species (ROS; i.e. superoxide anions and hydrogen peroxide) are produced by *C. polykrikoides* cells and may be one of the factors inducing fish kills (Kim et al. 1999). Kim et al. (2002) found, however, that O^{2-} and H_2O_2 production by *C. polykrikoides* was much lower than that by *Chattonella marina*, a species well-known for ROS production and that cell-free aqueous extract of *C. polykrikoides* cultures showed toxic effect on cervical cancer cells (cell line HeLa). Based on the observation of a gradual accumulation

of polysaccharides in the culture medium of *C. polykrikoides*, Kim et al (2002) suggested that biologically active multiple metabolites secreted by *C. polykrikoides* such as cytotoxic agents and mucus substances may contribute to the fish kill mechanism of *C. polykrikoides*. This hypothesis is consistent with the previously proposed suffocation of fish caused by secretion of mucus-like materials from algal cells as a possible mechanismof fish kills by *C. polykrikoides* (Hallegraeff 1992; Lee 1996).

However, our recently published results implicated that ROS or ROS-like agents may be responsible for the toxicity of *C. polykrikoides* to fish, adult and larval shellfish, and other co-occurring phytoplankton (Tang and Gobler 2009a and b, 2010). First, we found that addition of horseradish peroxidase (HRP) and catalase, both are well-known ROS scavengers (i.e. use ROS as their substrates of reactions), could significantly or almost completely remove the toxicity of *C. polykrikoides* to fish, larval shellfish, and phytoplankton. Second, we found *C. polykrikoides* would lose its toxicity if the cells were dead (by heating, freezing, or centrifugation) and physiologically active cells were more toxic than non-active cells (Tang and Gobler 2009a and b, 2010). These findings were consistent or supportive to the ROS hypothesis. Based on these results, we used a number of molecular probes to detect the levels of ROS and other possible candidates of toxins in multiple clones of *C. polykrikoides* while used a number of other phytoplankton as controls. Our results indicated that none of ROS and those detected candidates seemed to be responsible for the toxicity of C. polykrikoides, which, although they are 'negative' results, we believe has deepened our understanding of the toxic mechanisms of this cosmopolitan HAB species.

Materials and Methods

Cultures and conditions of culture maintenance

Cultures of *Cochlodinium polykrikoides* strains CP1, CPSB-1B, and CPPB-12 and other cultures used as controls were obtained by pipetting single cells to 24-well polystyrene cell culture plates containing sterile GSe culture medium under an inverted microscope. Cultures were established from bloom water collected between 2005 and 2010 from the estuaries of Long Island, NY, estuaries of Chesapeake Bay, VA, USA and coastal waters of Singapore (**Table 1**). Molecular (large subunit, LSU, rDNA sequencing) and microscopic identification of strain CP1 was reported previously (Gobler et al., 2008; Iwataki et al., 2008). The *Cochlodinium polykrikoides* culture CPCB-10 was isolated from Cotuit Bay, Massachusetts, USA by DM Anderson Lab and provided by G. Doucette.

All cultures were cultivated in sterile GSe medium with a salinity of 32.5 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. An antibiotic solution (a mixture of 10, 000 I.U. penicillin and10, 000 μg mL^{-1} streptomycin, Mediatech. Inc., Hemdon, VA) was added into the medium immediately before inoculation, with a final concentration of 2% to discourage the growth of bacteria in cultures.

Table 1. Microalgal cultures used in the experiments for detection of reactive oxygen species and fish bioassays

Phylum	**Class**	**Species and strain**	**Clone number**	**Origins**

Dinophyta	Dinophyceae	*Cochlodinium polykrikoides*	CP1	Flanders Bay, NY
	Dinophyceae	*C. polykrikoides*	CPCB-10	Cotuit Bay, MA
	Dinophyceae	*C. polykrikoides*	CPSB-1B	Shinnecock Bay, NY
	Dinophyceae	*C. polykrikoides*	CPPB-12	Pecolic Bay, NY
	Dinophyceae	*Akashiwo sanguinea*	AS2	Elizabeth River, VA,
	Dinophyceae	*Gymnodinium instriatum*	L6	Lafayette River, VA
	Dinophyceae	*Kryptoperidinium foliaceum*	DINO11	Shinnecock Bay, NY
	Dinophyceae	*Karenia brevis*	CCMP2228	Florida, USA
	Dinophyceae	*Karenia mikimotoi*	ISO6	Singapore
	Dinophyceae	*Prorocentrum minimum*	CCMP696	NY, USA
	Dinophyceae	*Prorocentrum donghaiense*	P. donghaiense	East China Sea
Haptophyta	Haptophyceae	*Isochrysis galbana* (Tahiti strain)	T-Iso	-
Ochrophyta	Raphidophyceae	*Chattonella marina*	Chatt1	Singapore

Chemicals and detection of ROS with molecular probes

Molecular probes for detection of reactive oxygen species (ROS) were horseradish peroxidase-Avidin (HRP), catalase, superoxide dismutase (SOD), 3'-(p-aminophenyl) fluorescein (APF), dichlorodihydrofluorescein diacetate (H_2DCFDA), singlet oxygen sensor Green (SOSG), dihydrorhodamine 123 (DHR 123), 1,3-Bis(diphenylphosphino)propane (DPPP), glutathione (GSH), Vitamin E acetate (Vit.E), hydroxocobalamin (HCL) were purchased from Molecular Probes, Inc. or Fisher Scientific, Inc. All molecular probes and their target reactive oxygen species are listed in

Table 2. All experimental procedures of detecting ROS with these probes followed the manufacturers' guidelines and were briefd in legends of figures.

Detection of other possible toxin with fish bioassay

The purposes of fish bioassays were to test the effects of additions of hydrogen peroxide (H_2O_2, 100μM in medium and 20μM in culture), glutathione (GSH, 20μM), hrdroxocobalamin (HCL, 50μM), vitamin E (50μM), and horseradish peroxidase (HRP, 2.5 μg mL^{-1}) on the toxicity of *C. polrkrikoides* strain CP1 (3700 cells mL^{-1}) to the 2 wks-old sheepshead minnows (*Cyprinodon variegatus*). Experiments were conducted with 6-well culture plates, with 10-mL culture or medium and one fish in each well (*n* =6). Experiments lasted for 7 days and when fish did not die in 7 days, their death time was labeled as 168h for the convenience of statistics.

Results

From Fig. 1 to Fig. 6, it can be seen clearly that the ROS levels targeted by probes H_2DCFDA (probe for •OH, $ONOO^-$, ^-OCl, H_2O_2, NO, and ROO•), APF (probe for •OH, $ONOO^-$, ^-OCl), singlet oxygen sensor green (probe for $^1O_2$), dihydrorhodamine 123 (DHR 123; probe for peroxynitrite $ONOO^-$), DPPP (probe for lipid peroxyl), and fluorescien (probes for ROS in general) in the cultures of *C. polykrikoides* (CP1, CPCB10, CPPB12, and CPSB1B) were not significantly higher, or even lower (e.g. *Chattonella marina* Chatt1), than that observed in the medium controls and other comparative cultures. Interestingly but importantly, the measured ROS levels in *C. polykrikoides* cultures were not only significantly lower than that of the H_2O_2 control, and

H_2O_2+$FeSO_4$ (Fenton reaction) control, but also lower than that of the medium control (Figs. 1-6). Except for *Chattonella marina* (Figs. 1 and 3B), and *Gymnodinium instriatum* L6 (Figs. 3B and 5A), all other comparative cultures exhibited ROS levels lower than the medium control but similar to *C. polykrikoides*. Based on these results, ROS are not likely responsible for the toxicity of *C. polykrikoides*.

Based on the results of ROS detections with molecular probes and our previous results showing the significant mitigation effect of the enzymes horseradish peroxidase (HRP) and catalase (Tang and Gobler 2009a and b, 2010), we hypothesized that some of common substrates of these two enzymes may be the culprits. Therefore, a number of molecular probes that can be antidotes of those candidates were applied to live *C. polykrikoides* cultures in a fish bioassay using two weeks-old juvenile sheepshead minnows (*Cyprinodon variegatus*). From the death time of fish exposed to the culture of *C. polykrikoides* CP1 (3700 cells mL^{-1}; Fig. 7), it can be seen that, compared to the CP1 control, additions of hydrogen peroxide (H_2O_2, 100 μM in medium and 20 μM in culture), glutathione (GSH, 20 μM), hrdroxocobalamin (HCL, 50 μM), vitamin E (50 μM) to CP1 did not cause significant mitigation effect to the toxicity of *C. polykrikoides*, while additions of horseradish peroxidase (HRP; 2.5 μg mL^{-1}) and HRP+H_2O_2 (20 μM) significantly reduced or removed the toxicity, consistent with our previous results (Fig. 7). Additions of H_2O_2 (100 μM), GSH, HCL, vitamin E to medium did not cause fish mortality (Fig. 7), demonstrating that these chemicals were not toxic to fish at the levels applied. These results indicated that toxin(s) of *C. polykrikoides* can be detoxified by reactions catalyzed by horseradish peroxidase and this mitigating effect can be enhanced by addition of H_2O_2, but H_2O_2 alone could not mitigate the toxicity at the levels applied

(20 μM). The results for glutathione and vitamin E (both were added as ROS scavengers) further supported that ROS are not responsible for the toxicity of *C. polykrikoides*. The non-mitigating effect of hydroxocobalamin (HCL), as an antidote of cyanide, seemed to indicate cyanide is not responsible either. However, addition of HCL with the same concentration (50 μM) caused significant mitigation effect when lower cell densities of *C. polykrikoides* were used (<1250 cells mL-1; Fig. 8). Considering that one mole CN^- roughly needs 50 moles of HCL, 10μM of CN^- in culture will need 500 μM of HCL to be removed, which is a concentration that may lead to toxicity to *C. polykrikoides* cells. Therefore, using a more specific probe such as the CN^--specific enzymes β-mercaptopyruvate cyanide transulferase may be worthy of a further exploration.

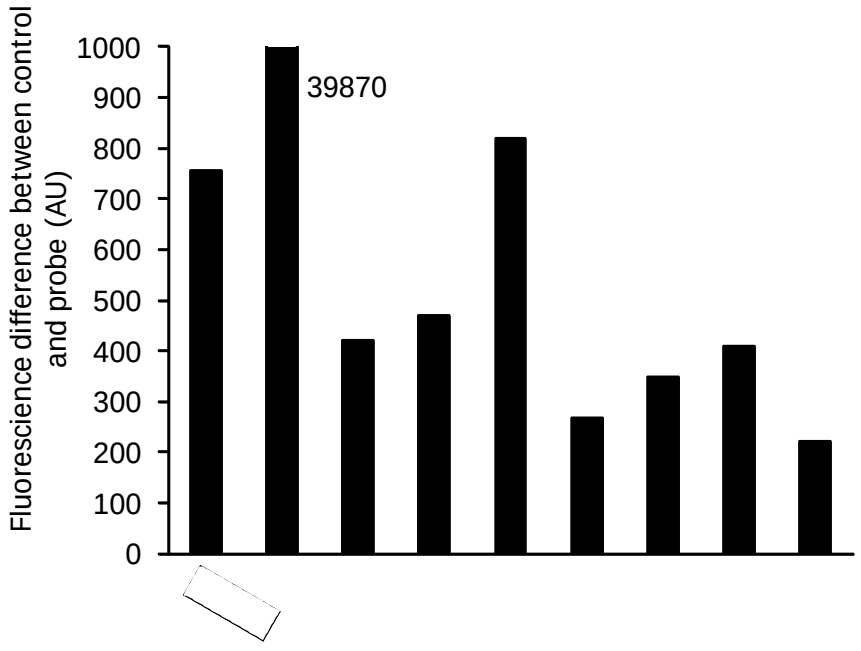

Fig. 1. Fluorescence difference (AU: arbitrary unit) between control (medium or culture before addition of probes) and that with addition of **H_2DCFDA** (probe for •OH, $ONOO^-$,

$^{-}$OCl, H_2O_2, NO, and ROO•) for *C. polykrikoides* and other microalgae. The samples were incubated at 37˚C for 30 min after addition of H_2DCFDA (final concentration 0.01uM) and then fluorescence was measured with a POLARstar Omega Plate Fluoremeter, BMG, LABTECH. The concentration of H_2O_2 was 20 uM. The cell densities for CP1, CPPB12, Chatt1, CCMP2228, L6, DINO11, and *P. donghaiense* were 5.06 x 10^3, 1.89 x 10^3, 1.62 x10^4, 1.77 x 10^4, 1.0 x 10^4, 2.17 x 10^4, and 1.33 x 10^6 cells mL^{-1}, respectively. All displayed numbers were averages of triplicates.

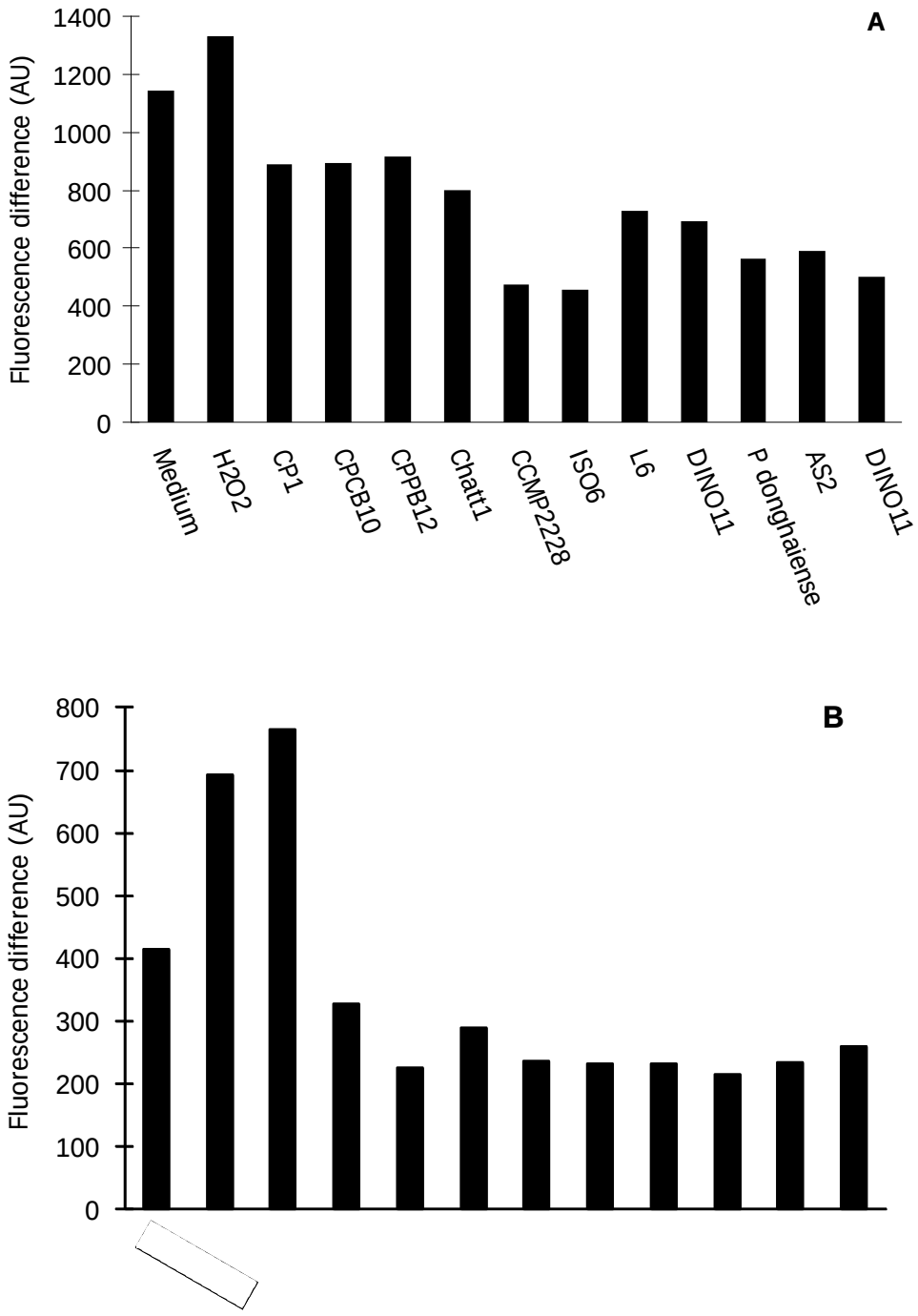

Fig. 2. Fluorescence difference (AU: arbitrary unit) between control (medium or culture before addition of **APF**) and that with addition of APF (probe for •OH, $ONOO^-$, ^-OCl) for *C. polykrikoides* and other microalgae. The samples were incubated at 37˚C after

addition of APF (final concentration 5uM) for (**A**) 40 min or (**B**) 60 min before fluorescence was measured with a POLARstar Omega Plate Fluoremeter, BMG, LABTECH. Note that Fenton reaction's products ($OH^{\bullet}$, $HOO^{\bullet}$, and others) were used as a control (the concentrations of H_2O_2 and $FeSO_4$ were both 20 μM). The cell densities for CP1, CPPB12, Chatt1, CCMP2228, L6, DINO11, and *P. donghaiense* in A were the same as Fig.1 and that for CPCB10, ISO-6, and AS2 were 2.54×10^3, 1.43×10^4, and 6.0×10^3, cells mL^{-1}, respectively, while the cell densities of CP1, CPCB10, CPPB12, Chatt1, L6, CCMP2228, CCMP696, and T-Iso in B were 7.85×10^3, 2.29×10^3, 2.38×10^3, 2.29×10^3, 2.40×10^4, 3.89×10^3, 3.11×10^4, 1.53×10^5, and 2.29×10^5, cells mL^{-1}, respectively. All displayed numbers were averages of triplicates.

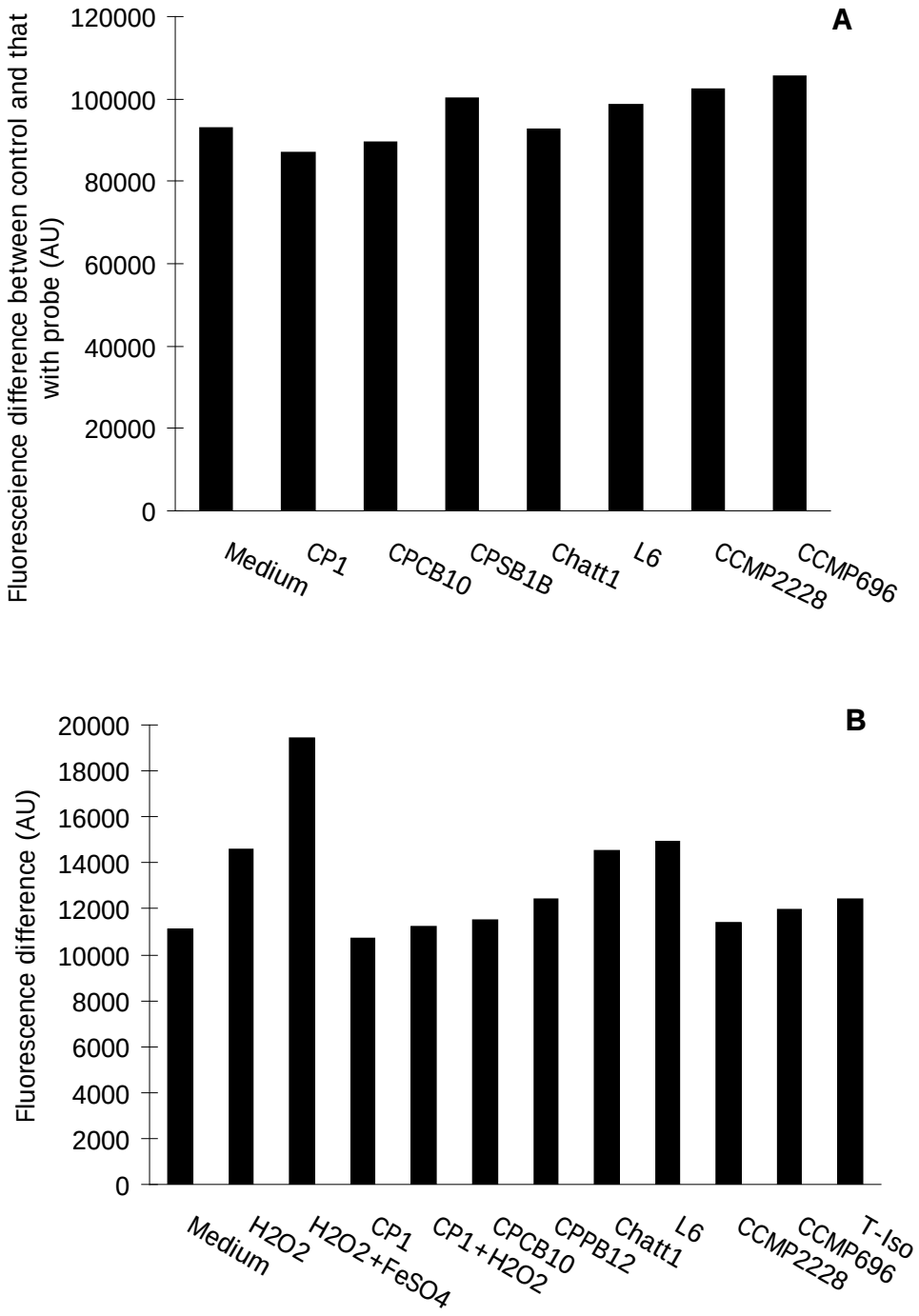

Fig. 3. Fluorescence difference (AU: arbitrary unit) between control (medium or culture before addition of probe) and that with addition of **singlet oxygen sensor green** (probe for $^1O_2$; final concentration 5uM) for *C. polykrikoides* and other microalgae. Samples were incubated in the dark at 22°C for 5 min before fluorescence was measured with a POLARstar Omega Plate Fluoremeter, BMG, LABTECH (excitation

485nm and emission 520nm). (**A**) Experiment 1 and (**B**).Experiment 2. Note that Fenton reaction's products ($OH^{\bullet}$, $HOO^{\bullet}$, and others) were used as a control (the concentrations of H_2O_2 and $FeSO_4$ were both 20 μM) in (B). The cell densities for CP1, CPCB10, CPSB-1B, Chatt1, L6, CCMP2228, and CCMP696 in A were 4.37×10^3, 1.0×10^3, 0.7×10^3, 1.63×10^4, 0.96×10^3, 4.47×10^4, 8.83×10^4, cells mL^{-1}, respectively, while the cell densities in B were the same as Fig. 2B. All displayed numbers were averages of triplicates.

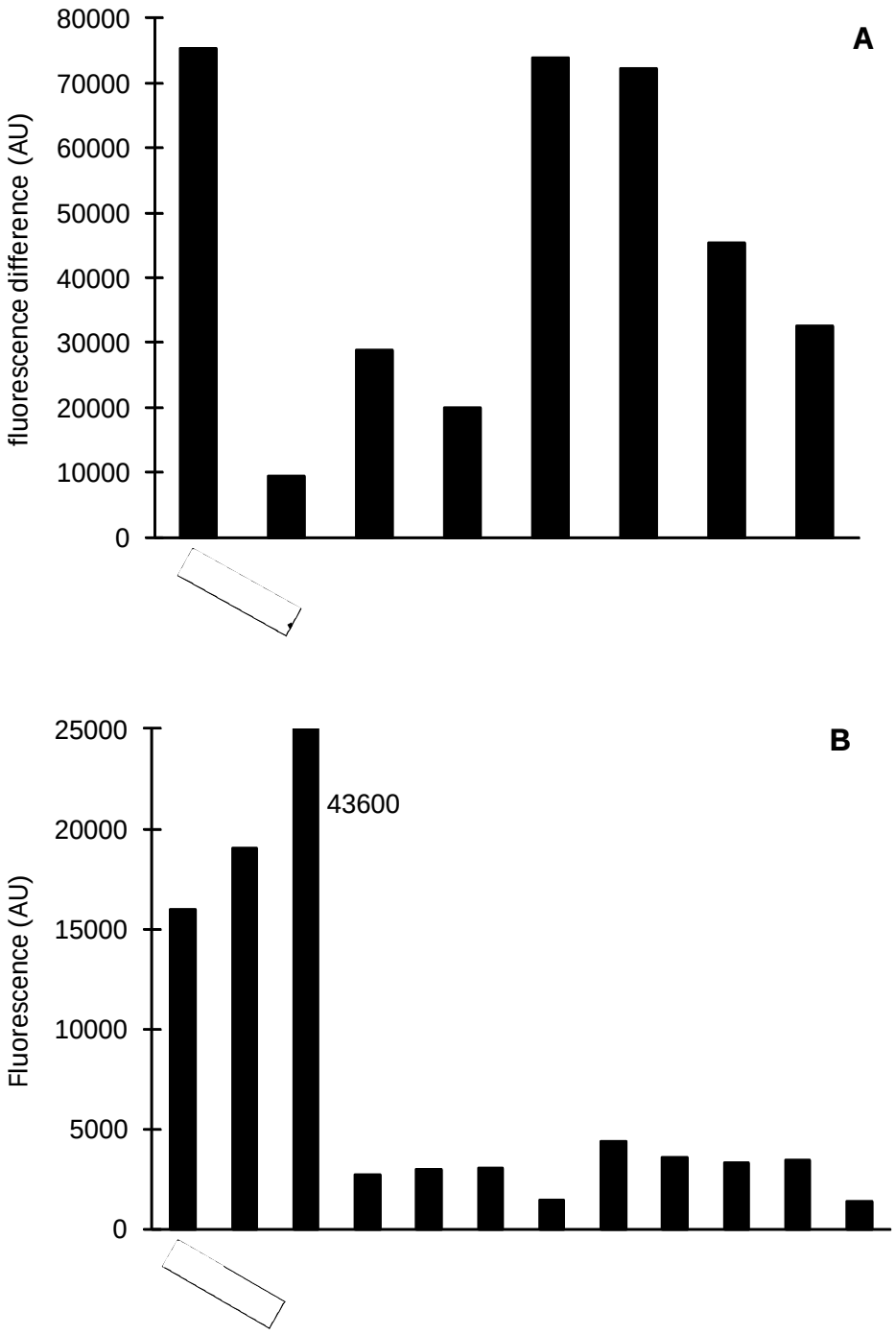

Fig. 4. Fluorescence difference (AU: arbitrary unit) between control (medium or culture without probes) and that with addition of dihydrorhodamine 123 (**DHR 123**, probe for peroxynitrite ONOO) for *C. polykrikoides* and other microalgae. Samples were incubated in the dark at 22°C for 60 min after probe addition (final 5uM) and then fluorescence was measured with a plate

reader (excitation/emission 485/525 nm). (**A**) Experiment 1 and (**B**) Experiment 2. Note that H_2O_2 and Fenton reaction's products ($OH^{\bullet}$, $HOO^{\bullet}$, and others) were used as controls in Experiment 2 (concentrations of H_2O_2 and $FeSO_4$ were both 20 μM). The cell densities were the same as Fig. 3. All displayed numbers were averages of triplicates.

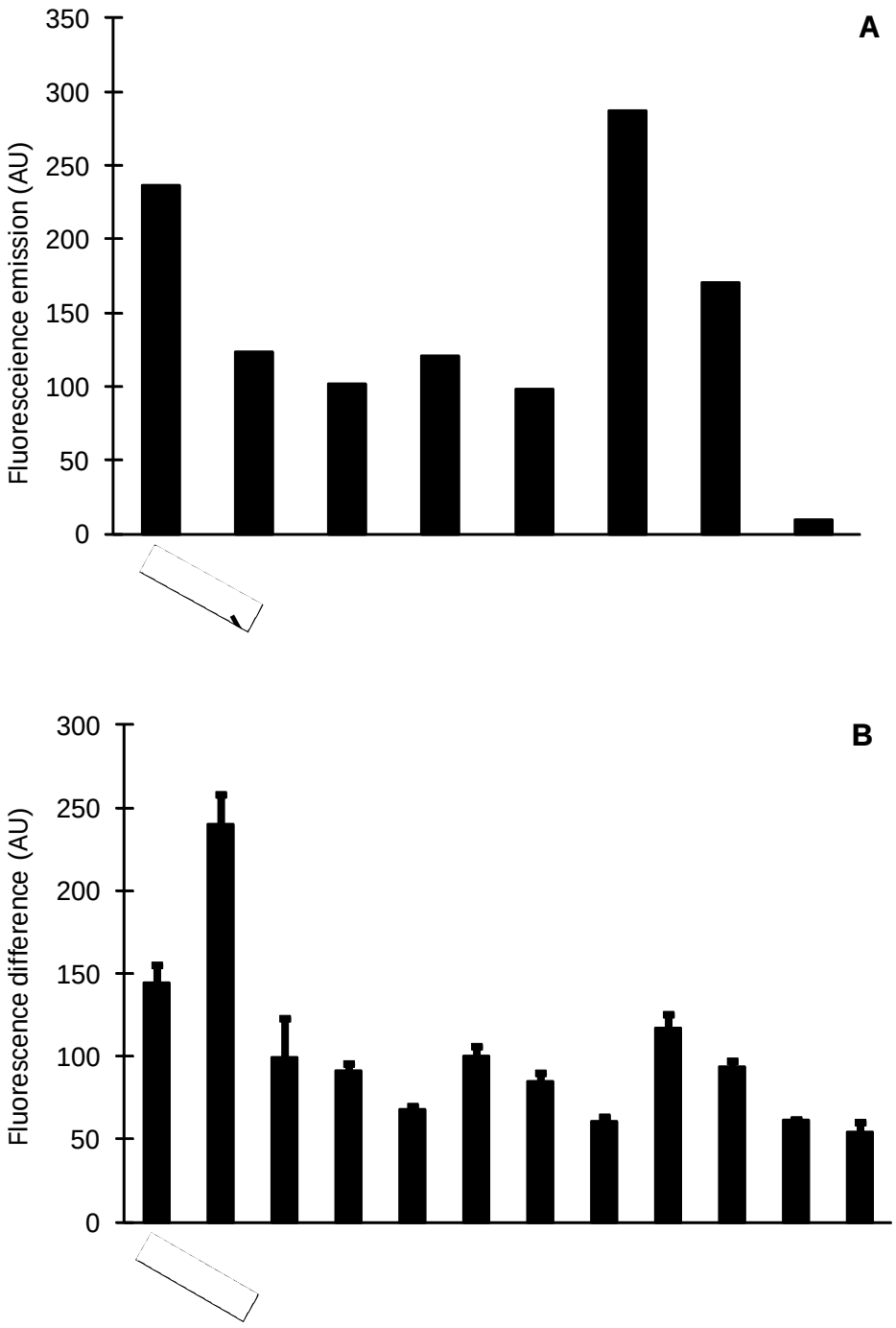

Fig. 5.. Fluoresceience difference (AU: arbitrary unit) between control (medium or culture without probes) and culture with addition of **DPPP** (**lipid peroxyl** probe) for *C. polykrikoides* and other comparative cultures. After addition of DPPP (2uM final for **A** or

5uM for **B**), samples were incubated at 37˚C for 60 min before measured with a F-4500 Fluorescence spectrophotometer, Hitachi, Japan at excitation /emission 351/382 nm). Note that H_2O_2 and Fenton reaction's products ($OH^{\bullet}$, $HOO^{\bullet}$, and others) were used as controls in B (concentrations of H_2O_2 and $FeSO_4$ were both 20 μM). The cell densities were the same as Fig. 3. Error bars indicate +1 standard deviation of $n = 3$.

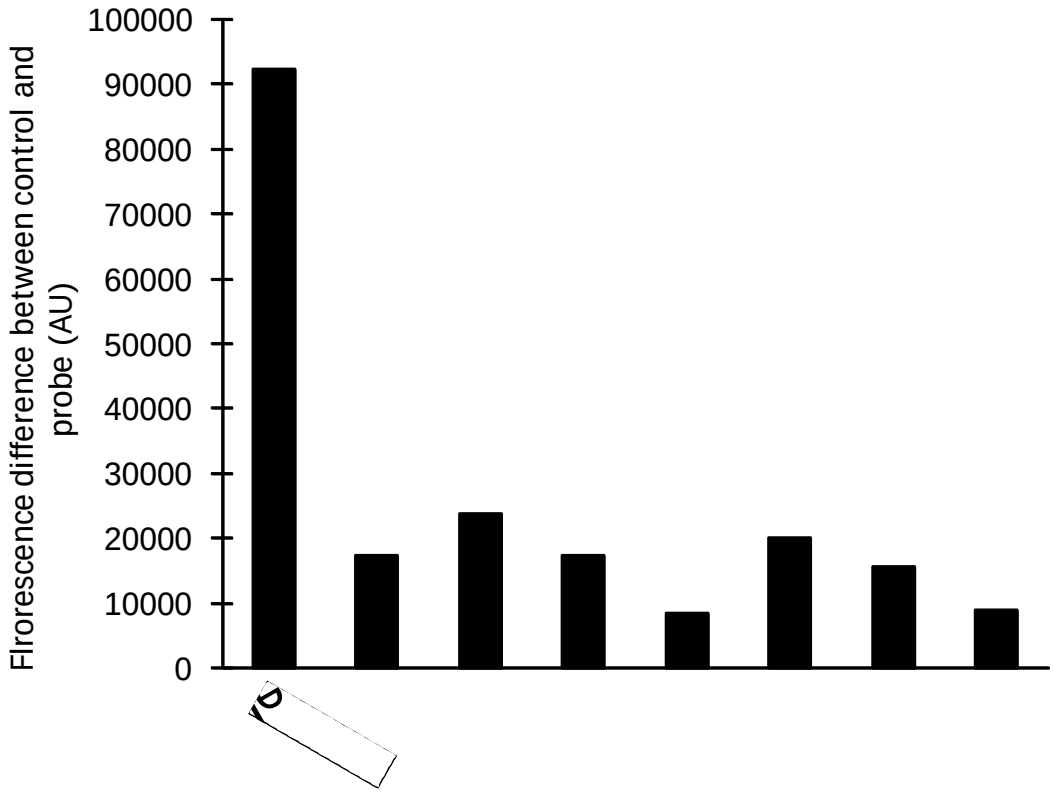

Fig. 6. Quenching effects of microalgal cultures on the fluorescence of fluorescien. Fluorescence was measured with plate reader (excitation/emission 485/520 nm) 10 min after addition of fluorescien in cultures and incubated at room temperature. The cell densities were the same as Fig. 3A.

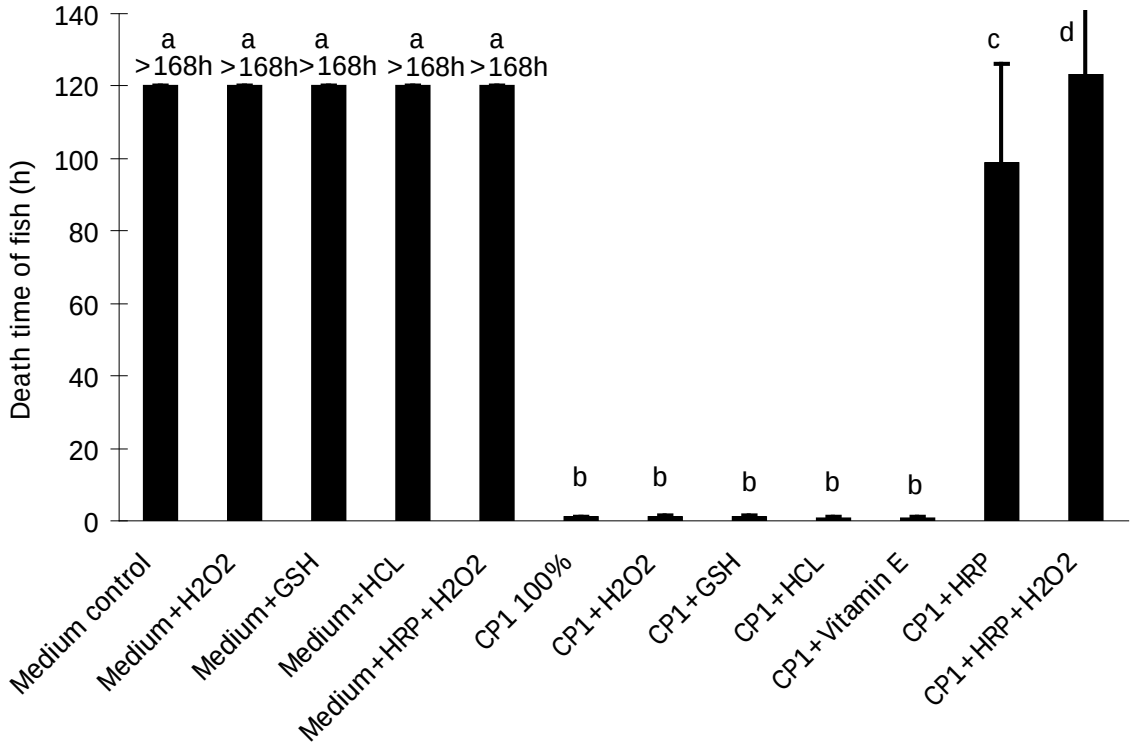

Fig. 7. Fish bioassays for testing the effects of additions of hydrogen peroxide (H_2O_2, 100μM in medium and 20μM in culture), glutathione (GSH, 20μM), hrdroxocobalamin (HCL, 50μM), vitamin E (50μM), and horseradish peroxidase (HRP, 2.5 μg mL^{-1}) on the toxicity of *C. polrkrikoides* strain CP1 (3700 cells mL^{-1}) to the 2 wks-old sheepshead minnows (*Cyprinodon variegatus*). Experiments were conducted with the 6-well culture plates, with 10-mL culture or medium and one fish in each well (*n* =6). Error bars indicate +1 standard deviation of *n* = 6; >168h indicates fish were still alive when experiments were terminated on day 7; different lower case letters indicate significant differences ($p < 0.05$) among treatments according to one-way ANOVA. Note that higher concentration of H_2O_2 was used in medium (100 μM) than in the culture (20 μM) to demonstrate non-killing effect of H_2O_2 at concentration as high as 100 μM and to avoid negative effect on the viability of *C. polykrikoides* cells, as determined with preliminary experiments. Also note that no negative effects in the viability of *C. polykrikoides* cells were observed with additions of 100μM H_2O_2, 20μM glutathione (GSH), 50μM hrdroxocobalamin (HCL), 50μM vitamin E, and 2.5 μg mL^{-1} horseradish peroxidase (HRP). H_2O_2 was added alone to CP1 culture to supposedly oxidize the toxin of reductant nature, GSH and vitamin E were added as ROS scavengers, hyrdroxocobalamin (HCL) was added as antidote of cynide, H_2O_2 was added along with HRP to produce free radicals and oxidize the cryptic reductant.

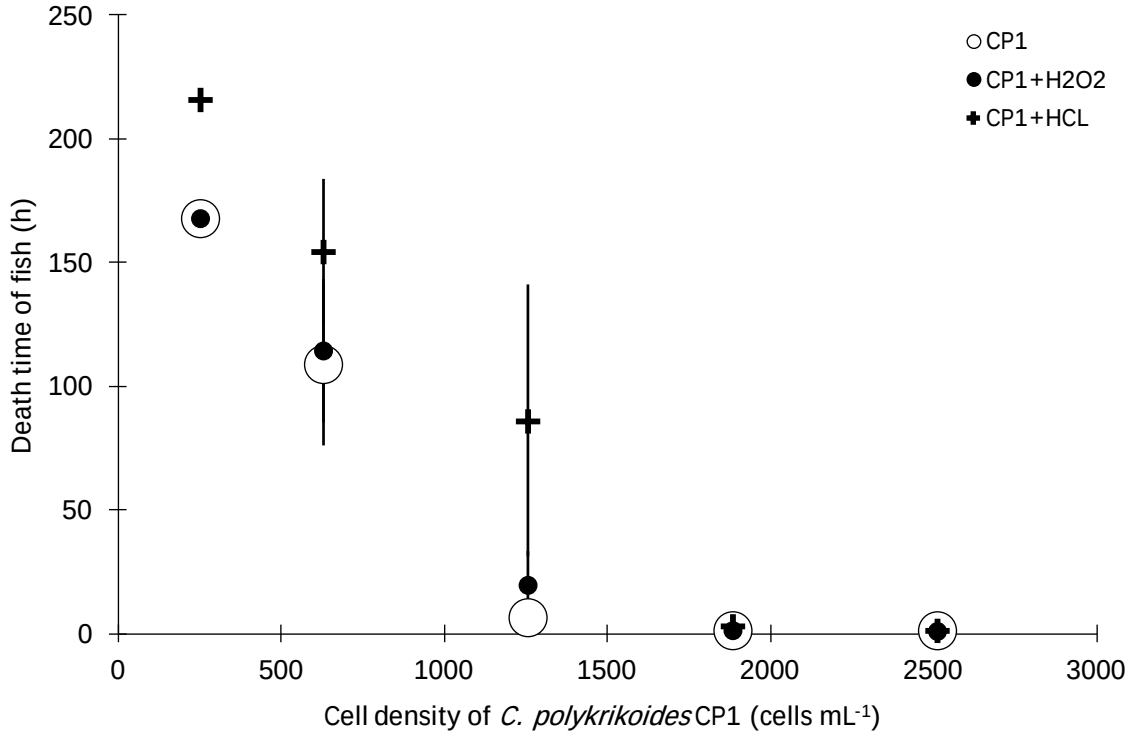

Fig. 8. Dependence of death time of 2 wks-old sheepshead minnows (*Cyprinodon variegatus*) on cell density of *C. polrkrikoides* strain CP1 in cultures with and without addition of H_2O_2 (20μM) or hrdroxocobalamin (HCL; 50μM). Experiments were conducted with 6-well culture plates that contain 10-mL culture or medium and one fish in each well (*n*=6). Error bars indicate +1 standard deviation of *n* = 6. Note: This graph seems showing an effect of HCL on the toxicity. Since one mole CN^- roughly needs 50 moles of HCL, 500 μM of HCL will be needed if the concentration of CN^- is 10μM, which may lead to toxicity to CP cells.

Table 2. Probes for reactive oxygen species (ROS) and reactive nitrogen species (RNS) and their targeted species.

	Superoxide	Singlet oxygen	Hydroxyl radical	Hydroperoxyl radical	Alkyl peroxyl	Phenoxyl	Hydrogen peroxide	Alkoxyl	Hypochlorite	Hypochlorous acid	Nitric oxide	Nitrogen dioxide	Peroxynitrite (peroxonitite)	Carbonate radical
	$O^{\bullet}_2$	$^1O2$	$OH^{\bullet}$	$HOO^{\bullet}$	$ROO^{\bullet}$	PhO•	H_2O_2	$RO^{\bullet}$	^-OCl	HOCl	$^{\bullet}NO$	$^{\bullet}NO2$	$ONOO^-$	$^{\bullet}CO_3^-$
H2DCFDA			Y		Y?		Y?				Y?		Y	
APF			Y						Y				Y	
HPF			Y										Y	
Singlet Oxygen Sensor Green		Y												
Dihydrorhodamine 123 (DHR 123)*							Y						Y	
DPPP				Y	Y									
Fluorescien							Reductants							

$ROO^{\bullet}$ Alkylperoxyl (or Lipid hydroperoxide, or peroxyl radical)

Peroxyl radical: alkylperoxyl ($ROO^{\bullet}$), hydroperoxyl ($HOO^{\bullet}$)

* DHR seemed highly reactive with products of Fenton reaction of H_2O_2 as seen in Fig. 4B.

Table 3. Scavengers or antidotes and their targets (substrates)

	Specific?	Targets	Remarks	H_2O_2 needed?
Catalase		H_2O_2; also function as peroxidase (see note below)	Use the heated as control	H_2O_2
Horseradish	No	Many as listed	Use the heated	H_2O_2

peroxidase		below;	as control	
Vitamin E Acetate, 97%	Y	Peroxyl radicals ROO•;	In 10 mM level; Fisher (100g, $77)	
Glutathione (GSH)	No	PhO•; OH•, HOCl, $ONOO^-$, RO•, ROO•, carbon-centered radicals; •NO2; $•CO_3$ and $^1O2$ to generate superoxide,	In 10 mM level;	No
Hydroxycobalamin (HCL)		**Antidote for cyanide**		
H_2O_2		**Supposedly oxidize cyanide, formic acid or any reductant**		

Appendix: Supplementary information about ROS scavengers and ROS probes

1. SOD: The specificity of SOD for reaction with $O_2^{•-}$ has frequently been used to probe for the involvement of this radical in biological systems. Although SOD^-

appears specific for catalytic removal of $O_2^{\bullet -}$, the SOD proteins (like any other protein) can react directly with certain ROS/RNS. Thus SODs react with $OH^{\bullet}$, peroxyl/alkoxyl radicals and singlet oxygen because they contain histidine and other side chains that react with these species. CuZnSOD interacts with peroxynitrite, decomposing it to a nitrating species. For bovine CuZnSOD, one SOD molecule catalyses nitration of a second molecule (or of any other nitratable protein molecules added to the reaction mixture). MnSOD also catalyses nitration and is slowly inactivated on addition of $ONOO^-$. Thus if a large quantity of SOD (or any other protein) is added to a system producing ROS/RNS, an artefactual inhibition of damage due to direct scavenging might result. Controls with heat-denatured SOD, other proteins (e.g. albumin) in equimolar amounts or SOD apoenzymes (the protein with the metals removed from the active site) can usually be performed to address this issue. A similar point applies to the use of other enzymes, such as catalase to implicate H2O2 involvement in an observed reaction: any protein at high concentrations can act as a 'general' ROS/RNS scavenger (Halliwell and Gutteridge 2007).

2. Catalase: The reaction of catalase is, like that of SOD, essentially a dismutation (disproportionation); One H2O2 is reduced to H2O and the other oxidized to O2: Catalase-Fe(III) + H2O2 $\rightarrow$ Compound I + H2O; Compound I + H2O2 $\rightarrow$ catalase-Fe(III) + O2. Catalase can be inhibited by azide, cyanide, and HOCl, but these are non-specific as they inhibit many other enzymes. Mammalian catalase can also catalyse certain peroxidase-type reactions. Compound I in the above reaction will oxidize methanol and ethanol to their corresponding aldehydes, but

propanols or butanols are much poorer substrates. Formic acid (HCOOH) can be oxidized to CO2 by the peroxidase action of compound I. Catalase can also oxidize nitrite (NOO-) into nitrate (NO3-) in vitro. The presence of peroxidatic substrates for catalase in vivo will decrease the concentration of Compound I, causing more free catalase to be formed, and this is another variable that must be considered in assessing how quickly H_2O_2 can be removed in vivo. .. The separated catalase subunits show little catalase activity, but have peroxidase activity on a much wider range of substrates, including NADH, because the active site haem is now more accessible. Dissociation of catalase into its subunits, which easily occurs on storage, freeze-drying or exposure of the enzyme to acid or alkali, causes loss of catalase activity. It has been suggested that catalase is responsible for oxidizing cyanamide (H2N-C≡N) to the product that inhibits aldehyde dehydrogenase: during the oxidation, the catalase itself loses activity (Halliwell and Gutteridge 2007). **Hydroperoxidase I** (HPI) shows both catalase and a wide range of peroxidase activities, i.e. it is a bifunctional catalase-peroxidase. Note that, when catalase and peroxidase coexist, catalase becomes more important (more efficient) in removing H2O2 than does peroxidase because of the high Km of catalase.

3. Horseradish peroxidase (HRP): Perhaps the most studied non-specific peroxidase. Several different forms of HRPs exist, each containing bound carbohydrate and calcium ions, but they all have broad substrate specificity. For example, HRPs will oxide guaiacol, pyrogallol, CN- ion, NADH, thiol compounds, phenols, and the plant hormone, indoleacetic acid (auxin). Oxidations by HRP, and probably by

most other non-specific peroxidases, usually occur by the following series of reactions, in which SH2 is the substrate: Peroxidase + H_2O_2 → Compound I; Compound I + SH2 → SH• + Compound II; Compound II + SH2 → SH• + peroxidase. The two electrons are replaced in two one-electron steps, in each of which a substrate molecule forms a radical, SH• (e.g. phenols (ROH) give phenoxyl, R-O•, radicals). Hence, peroxidase/H_2O_2 mixtures have been used to generate free radicals in the lab from almost every compound under the sun. The substrate-derived radicals (SH•) can then undergo a disproportionation reaction, one reducing the other to SH2 and simultaneously being itself oxidized to SH: SH• + SH• → S + SH2. Phenoxyl radicals can alternatively link together to give biphenols, e.g. tyrosine phenoxyl radicals can produce bityrosine. H_2O_2 is not always needed to be added, because trace amount of H_2O_2 may be present in the live system. HRP and other non-specific peroxidases can oxidize thiols into thiyl radicals in the presence of H_2O_2, e.g. H_2O_2 + 2GSH (HRP) → 2H_2O + 2GS•. These radicals can then participate in several reactions that result in O_2 uptake, e.g. for GS•: GS• + GS- (ionized form of GSH) → GSSG•-; GSSG•- +O_2 → GSSG + O_2•-; GS• +O_2 → GSO_2• (Halliwell and Gutteridge 2007). HRP: The enzyme exhibits a high specificity. Activity is observed with H_2O_2, MeOOH, and EtOOH (Maehly and Chance 1954). See also Chmielnicka et al. (1971) and Morrison and Bayse (1973). And also ascorbate, ferrocyanide, cytochrome C and the leuco form of many dyes.

4. Vitamin E: a scavenger of peroxyl radicals, is probably the most important (but not the only), inhibitor of the free-radical chain reaction of lipid peroxidation in animals (Halliwell and Gutteridge 2007).
5. Glutathione (GSH): In vitro, GSH can react with $OH^{\bullet}$, HOCl, $ONOO^{-}$, $RO^{\bullet}$, $ROO^{\bullet}$, carbon-centered radicals and $^{1}O2$ (Halliwell and Gutteridge 2007). its reaction with free radicals will generate thiyl ($GS^{\bullet}$) radicals. $GS^{\bullet}$ radicals can generate $O^{\bullet-}_{2}$ by the reaction: $GS^{\bullet} \rightarrow (GS^{-})\ GSSG^{\bullet-} \rightarrow (O_2)\ GSSG + O^{\bullet-}_{2}$.. since GSH is present at millimolar intracellular concentrations, scavenging of the above species is feasible in vivo (Halliwell and Gutteridge 2007; Winterbourn 2008).
6. Dihydrorhodamine 123 (DHR) can be used as an indicator of peroxynitrite formation.1 It is oxidized by peroxynitrite to the highly fluorescent product rhodamine in vitro. Neither NO, superoxide, nor hydrogen peroxide alone appear to oxidize DHR.1 Formation of rhodamine can be monitored by fluorescence spectroscopy using excitation and emission wavelengths of 500 and 536 nm, respectively, or by absorbance spectroscopy at 500 nm (ε = 78,800 M−1cm−1).2,3,1

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TASK 9. EXPLORE THE VULNERABILITY OF SUFFOLK COUNTY FISH POPULATIONS TO *COCHLODINIUM POLYKRIKOIDES.*

This research was performed by Stony Brook University doctoral candidate, Konstantine J. Rountos as part of his PhD dissertation. Ying-Zhong Tang, Robert M. Cerrato, and Ellen K. Pikitch also contributed significantly to this work.

Introduction

Harmful algal blooms (HABs) caused by the dinoflagellate, *Cochlodinium polykrikoides*, have increased in geographic extent, frequency, and duration in many coastal areas worldwide. These blooms have negatively impacted many coastal fisheries, causing mass mortalities of both wild and farmed fish. Forage fish species may be particularly susceptible to HABs as they feed on plankton and are usually highly abundant in coastal ecosystems where these blooms occur. While mortalities associated with HABs have been well documented for juvenile and adult fish, the potential impacts to early life stages (i.e. embryos and eleutheroembryos) have not been explored. We conducted a series of toxicity experiments using a clonal laboratory culture of *C. polykrikoides* and three forage fish species (Atlantic silverside: *Menidia menidia*, inland silverside: *M. beryllina*, and sheepshead minnow: *Cyprinodon variegatus*) all common on the U.S. East Coast and Suffolk County estuaries.

Embryo toxicity experiments

Time to embryo death. Embryo survival for *Menidia menidia* across all treatments (Experiment 1) was high (>90%; Fig. 1A) and time to embryo death was not significantly different between control and *Cochlodinium polykrikoides* treatments (Table 2). Survival of *M. beryllina* embryos (Experiment 2) was >81% for controls but was only 4% when embryos were exposed to 5×10^3 *C. polykrikoides* cells ml^{-1} (Fig. 1A). Survival of

embryos exposed to lower doses of *C. polykrikoides* did not differ from controls (Fig. 1A) and time to death of *M. beryllina* embryos was not significantly different between controls and *C. polykrikoides* treatments (Table 2). Survival of *Cyprinodon variegatus* embryos (Experiment 3) in control and lower *C. polykrikoides* cell densities ranged from 80% to 100%, while exposure to high densities of *C. polykrikoides* (6.4×10^3 cell ml^{-1}) reduced survival to 46% (Fig. 1A). Time to embryo death, however, was not significantly different between controls and *C. polykrikoides* treatments (Table 2).

Time to hatch. Hatching success across all treatments in Experiment 1 (*Menidia menidia*) was ≥91% (Fig. 1B). Embryos in control treatments hatched significantly later than embryos in *Cochlodinium polykrikoides* treatments ($p < 0.0001$, Table 2). Hatching success of *M. beryllina* (Experiment 2) was 4% at 5×10^3 *C. polykrikoides* cells ml^{-1} and >81% in all other treatments (Fig. 1B). Time to hatch in *C. polykrikoides* treatments also occurred significantly later for *M. beryllina* embryos compared to controls ($p = 0.0007$, Table 2). No statistically significant differences in time to hatch or hatching success were found for *Cyprinodon variegatus* embryos in Experiment 3 (Table 2, Fig. 1B).

Time to eleutheroembryo death. Time to death of eleutheroembryos in *Cochlodinium polykrikoides* treatments occurred significantly earlier than in control treatments for *M. beryllina* and *M. menidia* eleutheroembryos ($p = 0$ and $p < 0.0001$ respectively, Table 2). Survival of *Menidia menidia* eleutheroembryos (Experiment 1) was >85% for all *C. polykrikoides* treatments ≤7.6×10^2 cells ml^{-1}, but was ≤5% for treatments with >7.6×10^2 *C. polykrikoides* cells ml^{-1} (Fig. 1C). Survival of *M. beryllina* eleutheroembryos ranged from 41% to 90% in *C. polykrikoides* treatments ≤4.9×10^2 cells ml^{-1} and the control (Experiment 2; Fig. 1C). No *M. beryllina* eleutheroembryos survived in *C.*

polykrikoides treatments ≥1.2 x 10^3 cells ml^{-1}. Survival of *Cyprinodon variegatus* eleutheroembryos in experiment 3 was ≥89% in all treatments (Fig. 1C).

Eleutheroembryo acute toxicity experiments. Eleutheroembryos of all fish species exposed to high densities of *Cochlodinium polykrikoides* experienced complete mortality during the first day of post-hatch exposure (Fig. 2). Survival in lower *C. polykrikoides* cell density treatments varied by fish species and *C. polykrikoides* cell density. All *Cyprinodon variegatus* in experiment 7 survived *C. polykrikoides* treatments with densities ≤1.6 x 10^3 cells ml^{-1}, while *Menidia menidia* (Experiment 5) and *M. beryllina* (Experiment 6) had high survival, >75 and 100% respectively, in *C. polykrikoides* treatments ≤4.0-5.0 x 10^2 cells ml^{-1} (Fig. 2). Survival analyses revealed that time to eleutheroembryo death decreased significantly with increasing *C. polykrikoides* cell densities in all these experiments ($p = 0$ for experiments 5-7, Table 2).

Eleutheroembryo exposure/recovery experiments

***Menidia menidia*.** Time to eleutheroembryo death occurred significantly earlier ($p < 0.0001$) in full exposure (i.e. 3.7 x 10^3 cells ml^{-1} : 3.7 x 10^3 cells ml^{-1}, 1.9 x 10^3 cells ml^{-1} : 1.9 x 10^3 cells ml^{-1}) and the high cell density (3.7 x 10^3 cells ml^{-1} : 0 cells ml^{-1}) partial exposure treatments compared to the control (0 cells ml^{-1} : 0 cells ml^{-1}) and the lower cell density (1.9 x 10^3 cells ml^{-1} : 0 cells ml^{-1}) partial exposure treatments at both exposure times tested (i.e. 0.5 and 0.75 h) (Table 3, Fig. 3). Eleutheroembryos in lower cell density partial exposure (1.9 x 10^3 cells ml^{-1} : 0 cells ml^{-1}) treatments had greater survival than full exposure (1.9 x 10^3 cells ml^{-1} : 1.9 x 10^3 cells ml^{-1}) treatments. In contrast, there was no significant differences in time to eleutheroembryo death were found between partial (1.9 x 10^3 cells ml^{-1} : 0 cells ml^{-1}) and control (0 cells ml^{-1} : 0 cells ml^{-1}) treatments (Table

3). Finally, a 0.5 h exposure to 3.7 x 10^3 *C. polykrikoides* cells ml^{-1} was enough to cause 83% mortality after just 1 h and complete mortality after 2.7 h in the high cell density partial exposure (3.7 x 10^3 cells ml^{-1} : 0 cells ml^{-1}) treatment (Fig. 3A).

***Menidia beryllina* and *Cyprinodon variegatus*.** There were differences in the survival of *Menidia beryllina* and *Cyprinodon variegatus* eleutheroembryos after a 0.25 h exposure to similar *Cochlodinium polykrikoides* cell densities (Fig. 4). Survival analyses across all treatments for *M. beryllina* and *Cyprinodon variegatus* revealed that time to eleutheroembryo death only occurred significantly earlier in the high cell density full exposure (6.0 x 10^3 cells ml^{-1} : 6.0 x 10^3 cells ml^{-1}) *C. polykrikoides* treatments compared to the controls ($p = 0.005$ and $p < 0.0001$ respectively, Table 3). Survival of *M. beryllina* in the lower cell density (3.0 x 10^3 cells ml^{-1} : 0 cells ml^{-1}) partial exposure treatment was high (82%), and nearly identical to the control (0 cells ml^{-1} : 0 cells ml^{-1}), while survival in the full exposure treatment (3.0 x 10^3 cells ml^{-1} : 3.0 x 10^3 cells ml^{-1}) was only 10% (Fig. 4A). Similarly, survival in the high cell density (6.0 x 10^3 cells ml^{-1} : 0 cells ml^{-1}) partial exposure treatments was higher (30%) than the high cell density full exposure (6.0 x 10^3 cells ml^{-1} : 6.0 x 10^3 cells ml^{-1}) treatment (0%) for *M. beryllina*. Complete survival of *Cyprinodon variegatus* eleutheroembryos was found in all treatments except for the high cell density *C. polykrikoides* full exposure (6.0 x 10^3 cells ml^{-1} : 6.0 x 10^3 cells ml^{-1}) treatment (10%; Fig. 4B).

***Cyprinodon variegatus*.** Survival in the partial exposure (6.2 x 10^3 cells ml^{-1} : 0 cells ml^{-1}) treatments was >82% at exposure times ≤2.5 h and 55% in the 3.5 h exposure treatment, while no eleutheroembryos survived in any of the high cell density full exposure treatments. Time to death occurred significantly earlier, in about 2.73 to 3.75

days, in full exposure (6.2 x 10^3 cells ml^{-1} : 6.2 x 10^3 cells ml^{-1}) *C. polykrikoides* treatments compared to control (0 cells ml^{-1} : 0 cells ml^{-1}) and partial exposure (6.2 x 10^3 cells ml^{-1} : 0 cells ml^{-1}) treatments ($p < 0.0001$ for all exposure times; Table 3). Time to eleutheroembryo death in partial (6.2 x 10^3 cells ml^{-1} : 0 cells ml^{-1}) exposure treatments only occurred significantly earlier (~2.42 d) than control (0 cells ml^{-1} : 0 cells ml^{-1}) treatments following a 3.5 h exposure to *C. polykrikoides* ($p < 0.05$, Table 3).

Exposures to *Cochlodinium polykrikoides* caused swimming to be inhibited within 3.5 h for all eleutheroembryos in full (6.2 x 10^3 cells ml^{-1} : 6.2 x 10^3 cells ml^{-1}) and 96% of fish in partial (6.2 x 10^3 cells ml^{-1} : 0 cells ml^{-1}) exposure treatments. Time to swimming inhibition occurred significantly earlier in these exposure treatments compared to controls (0 cells ml^{-1} : 0 cells ml^{-1}) (Table 4), where swimming was inhibited in only 7% of eleutheroembryos. Immobilized control individuals however began to swim again in less than 1 h and remained swimming for the remainder of the experiment. All eleutheroembryos in full exposure treatments died by the end of the experiment while only 18% of eleutheroembryos perished in partial exposure *C. polykrikoides* treatments (Table 4). As such, motility data was analyzed by excluding dead individuals from all analyses. Individuals in the full exposure *C. polykrikoides* treatments experienced significantly shorter time to swimming inhibition as well as significantly longer total time immobilized and time to swimming recovery compared to the control ($p <0.0001$). Eleutheroembryo motility in partial exposure treatments varied with exposure times, with longer *C. polykrikoides* exposure times generally leading to increased immobilization and swimming recovery times (Fig. 6). Total immobilization times in partial exposure treatments were significantly greater than controls at 1.75 h ($p< 0.005$), 2.5 h ($p <$

0.0001) and 3.5 h ($p < 0.0001$) exposure times (Table 4). A similarly significant pattern emerged for swimming recovery times between controls and partial exposure treatments (Table 4). Eleutheroembryos in the 3.5 h partial exposure treatment had significantly longer recovery times (median: 36.75 h) than fish exposed to high levels of *C. polykrikoides* for 1 h (median: 6 h, $p < 0.0001$), 1.75 h (median: 6 h, $p < 0.0001$) but not 2.5 h (median: 30.5 h (Table 4, Fig. 6).

CONCLUSIONS

Our experiments demonstrated that *C. polykrikoides* caused mortalities in both embryos and eleutheroembryos, but that sensitivity to toxicity differed among fish species (*M. beryllina* > *M. menidia* > *C. variegatus*) and among life stages (eleutheroembryos > embryos). Althoughembryos were somewhat resistant to the *C. polykrikoides* biotoxins until they hatched, once they hatched, they experienced rapid mortality and impaired swimming ability. By testing ecologically relevant exposure times to *C. polykrikoides*, we found that eleutheroembryos can become incapacitated relatively quickly (i.e. hours), although surviving fish could recover swimming ability when removed from *C. polykrikoides* exposure. This is the first evidence of sublethal impacts from *C. polykrikoides* exposures to fish, advancing our understanding of the potential ecosystem impacts of this harmful alga. Our understanding of the toxicity, toxinology, and ecosystem effects of *Cochlodinium polykrikoides* blooms are still in their infancy (Gobler et al. 2008, Kim et al. 2009, Tang & Gobler 2009). We present the first results of lethal and sublethal effects of *C. polykrikoides* on early life stage fish. Our results specifically demonstrate that embryos of three common estuarine forage fish species are relatively resistant to *C. polykrikoides* biotoxins until they hatch. However, once they hatch, they can experience rapid mortality and incapacitation. By testing ecologically relevant exposure times, we found that eleutheroembryos exposed to *C. polykrikoides* became incapacitated relatively quickly, but could still completely recover if provided enough time in control conditions. Given the heterogeneous and episodic nature of *C. polykrikoides* blooms, these results can provide coastal managers and scientists with a better understanding of the potential impacts posed to coastal fish populations. As forage

fish species are some of the most ecologically and economically important components of many coastal marine ecosystems (Pikitch et al. 2012a, Pikitch et al. 2012b) these impacts could be significant.

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Tables:

Experiment #	Species	Control survival (%)	Maximum *C. polykrikoides* cell density tested (cells ml^{-1})	Treatments (% of maximum *C. polykrikoides* cell density)	Replicates per treatment (n)	Total fish used (n)	Sampling frequency(d)	Duration (d)
Embryo toxicity experiments								
1	MM	100	3×10^4	(0,1.5,10,25,50,100)	18,21,20,20,19,22,18	138	0,1,7,8,10	10
2	MB	82	5×10^4	(0,1.5,10,25,50,100)	21,22,22,22,22,22,23	154	0,2,4,7,8,9	9
3	CV	80	8×10^4	(0,1.25,50,75,100)	15,13,13,9,9,13	72	0,2,3,4,5,6,7,8	8
Embryo protocol experiments								
4	MB	98	4×10^4	Antibiotic additions (0,100)	23,23	46	0,1,2,3,4,5,6,7	7
Eleutheroembryo acute toxicity experiments								
5	MM	100	5×10^4	(0,1.5,10,50,100)	24,24,24,24,24,24	144	0,1,2	2
6	MB	75	8×10^4	(0,1.5,10,25,50,100)	24,24,24,24,24,24,24	168	0,1,2	2
7	CV	100	8×10^4	(0,1.25,50,75,100)	18,18,18,18,18,18	108	0,1,2	2
Eleutheroembryo exposure/recovery experiments								
8	MM	75	4×10^4	0.5h(0:0), 0.5h(50:0), 0.5h(50:50), 0.5h(100:0), 0.5h(100:100), 0.75h(0:0), 0.75h(50:0), 0.75h(50:50), 0.75h(100:0), 0.75h(100:100)	12,12,12,12,12, 12,12,12,12,12	120	0,0.02,0.04,0.07,0.11, 0.18,0.2,0.24,0.29,1.18,2.03	2.03
9	MB	78	8×10^4	0.25h(0:0), 0.25h(50:0), 0.25h(50:50),0.25h(100:0),0.25h(100:100)	10,10,10,10,10	50	0,0.04,0.08,0.13,0.17,	2.75
	CV	100	8×10^4	0.25h(0:0), 0.25h(50:0),0.25h(50:50),0.25h(100:0),0.25h(100:100)	10,10,10,10,10	50	0.21,0.25,0.5,0.75,1.75,2.75	
10	CV	100	8×10^4	1h(0:0), 1h(100:0),1h(100:100), 1.75h(0:0), 1.75h(100:0), 1.75h(100:100), 2.5h(0:0), 2.5h(100:0), 2.5h(100:100), 3.5h(0:0), 3.5h(100:0), 3.5h(100:100)	12,12,12,12,12,12, 12,12,12,12,12,12	144	0,0.17,0.1,0.15,0.17,0.21,0.25, 1.25,1.75,2.0,3.0,4.0	4

Table 1. Inventory of *Cochlodinium polykrikoides* toxicity experiments with early life stages of *Menidia menidia* (MM), *M. beryllina* (MB) and *Cyprinodon variegatus* (CV) species.

Experiment #	Species	Test	Factor tested	χ^2	*df*	p value	N
Embryo toxicity experiments							
1	MM	Time to death (embryo)	*C. polykrikoides* dilutions	-13890	1	ns	138
		Time to hatch	*C. polykrikoides* dilutions	15.87	1	< 0.0001	133
		Time to death (eleutheroembryo)	*C. polykrikoides* dilutions	89.67	1	0	133
2	MB	Time to death (embryo)	*C. polykrikoides* dilutions	-6351.4	1	ns	154
		Time to hatch	*C. polykrikoides* dilutions	11.58	1	0.0007	116
		Time to death (eleutheroembryo)	*C. polykrikoides* dilutions	57.9	1	< 0.0001	116
3	CV	Time to death (embryo)	*C. polykrikoides* dilutions	0.98	1	ns	72
		Time to hatch	*C. polykrikoides* dilutions	0.02	1	ns	48
		[a]Time to death (eleutheroembryo)	*C. polykrikoides* dilutions	-	-	-	48
Embryo protocol experiments							
4	MB	Time to death (embryo)	Antibiotic addition (0 cells ml^{-1}) vs Antibiotic addition (4 x 10^4 *C. polykrikoides* cells ml^{-1})	1.97	1	ns	46
		Time to hatch		2.29	1	ns	42
		Time to death (eleutheroembryo)		33.19	1	< 0.0001	42
Eleutheroembryo acute toxicity experiments							
5	MM	Time to death (eleutheroembryo)	*C. polykrikoides* dilutions	184.25	1	0	144
6	MB	Time to death (eleutheroembryo)	*C. polykrikoides* dilutions	131.23	1	0	144
7	CV	Time to death (eleutheroembryo)	*C. polykrikoides* dilutions	131.46	1	0	108
a = Insufficient number of days post-hatch to conduct statistical test							

Table 2. Summary of survival analyses in *Cochlodinium polykrikoides* toxicity experiments (Experiments 1-7) with *Menidia menidia* (MM), *M. beryllina* (MB) and *Cyprinodon variegatus* (CV) early life stage fish. Chi-square (χ^2), degrees of freedom (*df*), statistical significance (p value) and number of fish in each analysis (N) are given. Values that were not significant (ns) had a p value >0.05 and "-" indicates values that could not be calculated.

Experiment #	Species	Test	Exposure time (hr)	Exposure type											
				50:0			50:50			100:0			100:100		
				χ^2	df	p value	χ^2	df	p value	χ^2	df	p value	χ^2	df	p value
Eleutheroembryo exposure/recovery experiments															
8	MM	Time to death (eleutheroembryo)	0.5	0.86	1	ns	26.72	1	<0.0001	71.24	1	<0.0001	73.21	1	<0.0001
			0.75	0.12	1	ns	32.09	1	<0.0001	25	1	<0.0001	93.25	1	<0.0001
9	MB	Time to death (eleutheroembryo)	0.25	0.32	1	ns	3.72	1	ns	2.95	1	ns	15.99	1	0.005
	CV		0.25	-	-	ns	-	-	ns	-	-	ns	23.25	1	<0.0001
10	CV	Time to death (eleutheroembryo)	1	na	na	na	na	na	na	3.01	1	ns	38.55	1	<0.0001
			1.75	na	na	na	na	na	na	-	-	ns	32.83	1	<0.0001
			2.5	na	na	na	na	na	na	1.45	1	ns	36.91	1	<0.0001
			3.5	na	na	na	na	na	na	8.42	1	0.04	34.27	1	<0.0001

Table 3. Statistical significance of time to eleutheroembryo death in exposure/recovery experiments using pairwise t-tests with Bonferroni adjusted error rates for *Menidia menidia* (MM), *Menidia beryllina* (MB) and *Cyprinodon variegatus* (CV) species. Full (i.e. 50:50 and 100:100) and partial (i.e. 50:0 and 100:0) exposure treatments were compared to their respective control (0:0) treatment. 0, 50 and 100 represent the percentage of the maximum *C. polykrikoides* cell density used for each respective experiment, found in Table 1.

Time to swimming inhibition			Exposure time (h)											
			1			1.75			2.5			3.5		
			0:0	100:0	100:100	0:0	100:0	100:100	0:0	100:0	100:100	0:0	100:0	100:100
Exposure time (h)	1	0:0												
		100:0	0.0474											
		100:100	na	na										
	1.75	0:0	ns	0.0145	na									
		100:0	ns	ns	na	0.0197								
		100:100	na	na	na	na	na							
	2.5	0:0	ns	0.0322	na	ns	0.0451	na						
		100:0	0.0047	ns)	na	0.0451	ns	na	0.003					
		100:100	na	na	na	na	na	na	na	na				
	3.5	0:0	ns	0.0017	na	ns	0.0021	na	ns	0.0001	na			
		100:0	ns	ns	na	0.0329	ns	na	ns	ns	na	0.0053		
		100:100	na	na	na	na	na	na	na	na	na	na	na	
Time immobilized			0:0	100:0	100:100	0:0	100:0	100:100	0:0	100:0	100:100	0:0	100:0	100:100
Exposure time (h)	1	0:0												
		100:0	ns											
		100:100	na	na										
	1.75	0:0	ns	ns	na									
		100:0	0.0043	ns	na	0.0043								
		100:100	na	na	na	na	na							
	2.5	0:0	ns	ns	na	ns	0.0041	na						
		100:0	<0.0001	0.0013	na	<0.0001	0.0109	na	<0.0001					
		100:100	na	na	na	na	na	na	na	na				
	3.5	0:0	na	ns	na	ns	0.0039	na	ns	<0.0001	na			
		100:0	<0.0001	<0.0001	na	<0.0001	<0.0001	na	<0.0001	ns	na	<0.0001		
		100:100	na	na	na	na	na	na	na	na	na	na	na	
Time to recovery			0:0	100:0	100:100	0:0	100:0	100:100	0:0	100:0	100:100	0:0	100:0	100:100
Exposure time (h)	1	0:0												
		100:0	ns											
		100:100	na	na										
	1.75	0:0	ns	0.0475	na									
		100:0	0.002	ns	na	0.0017								
		100:100	na	na	na	na	na							
	2.5	0:0	ns	0.0492	na	ns	0.0018	na						
		100:0	<0.0001	0.0014	na	<0.0001	0.0103	na	<0.0001					
		100:100	na	na	na	na	na	na	na	na				
	3.5	0:0	ns	0.037	na	ns	0.0013	na	ns	<0.0001	na			
		100:0	<0.0001	<0.0001	na	<0.0001	<0.0001	na	<0.0001	ns	na	<0.0001		
		100:100	na	na	na	na	na	na	na	na	na	na	na	

Table 4. Statistical significance of sublethal effects of *Cochlodinium polykrikoides* to *Cyprinodon variegatus* eleutheroembryos across all exposure types (i.e. 0:0, 100:0 and 100:100) and exposure times (i.e. 1 h, 1,75 h, 2.5 h and 3.5 h) using pairwise t-tests with Bonferroni adjusted error rates. ns denotes p values that were not significant ($p > 0.05$) and na indicates that comparisons were not applicable because of dead individuals. Exposure type values (i.e. 0 and 100) refer to the percentage of the maximum *C. polykrikoides* cell density used for experiment 10 (Table 1).

Figures:

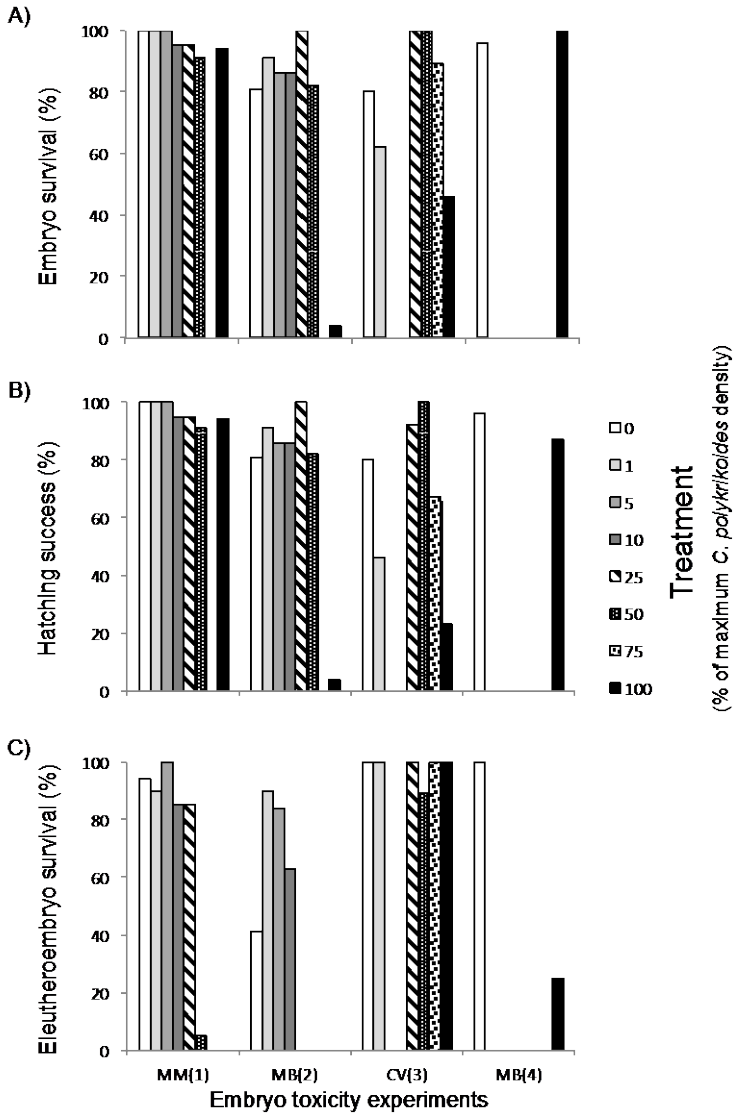

Fig. 1. (A) Embryo survival, (B) hatching success and (C) survival of newly hatched eleutheroembryos in embryo experiments with *Menidia menidia* (MM), *Menidia beryllina* (MB) and *Cyprinodon variegatus* (CV). Numbers in parentheses correspond to the experiment number in Table 1.

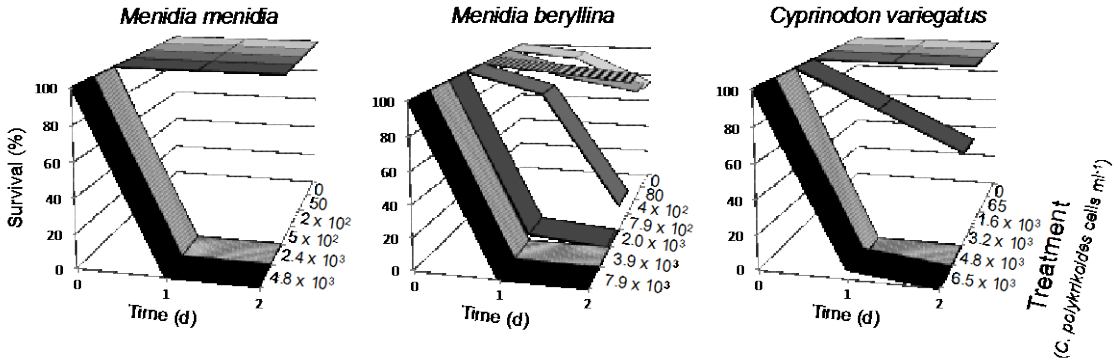

Fig. 2. Survival of *Menidia menidia*, *M. beryllina* and *Cyprinodon variegatus* eleutheroembryos in static *Cochlodinium polykrikoides* acute toxicity experiments.

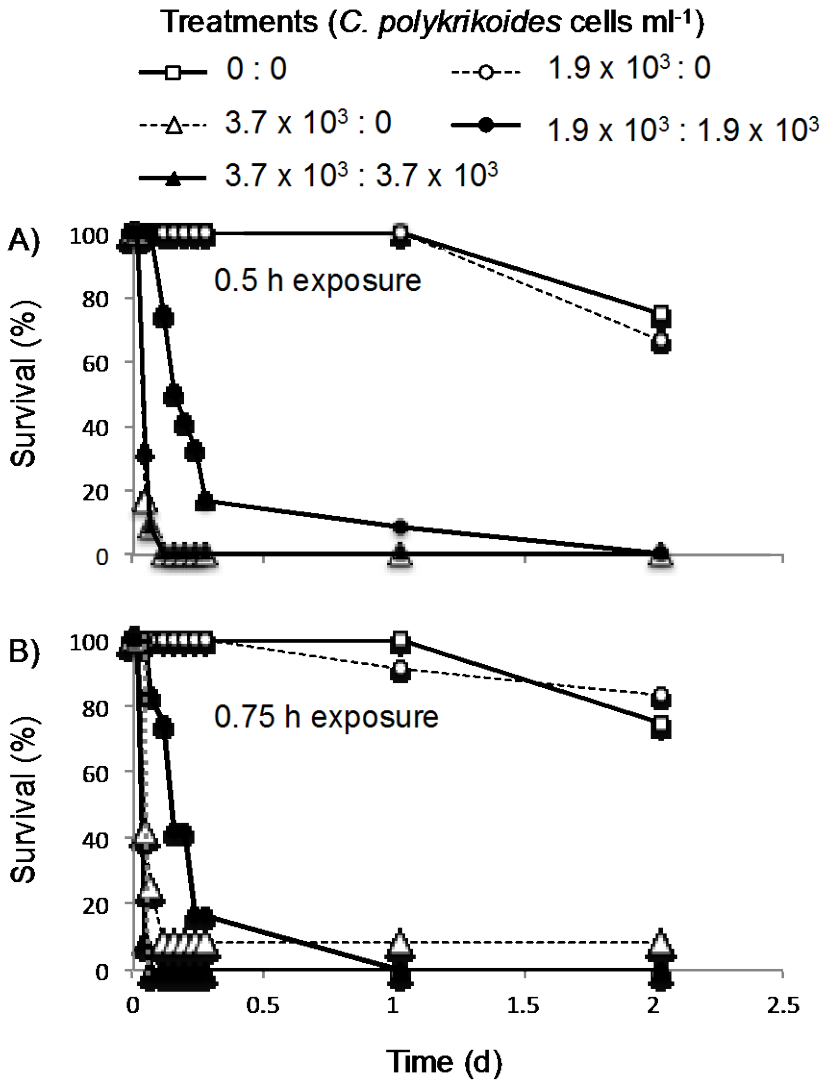

Fig. 3. Survival of *Menidia menidia* eleutheroembryos after (A) 0.5 h and (B) 0.75 h exposures to *Cochlodinium polykrikoides* culture treatments. Treatments indicate the *C. polykrikoides* cell densities before and after (before : after) eleutheroembryos were transferred following the designated exposure time.

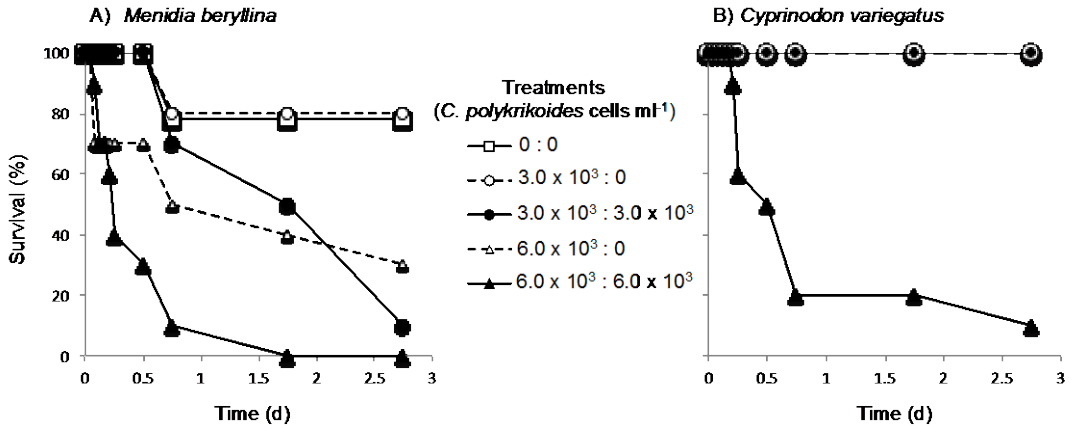

Fig. 4. Survival of A) *Menidia beryllina* and B) *Cyprinodon variegatus* eleutheroembryos after a 0.25 h exposure to *Cochlodinium polykrikoides* culture. Treatments indicate the *C. polykrikoides* cell densities before and after (before : after) eleutheroembryos were transferred at 0.25 h.

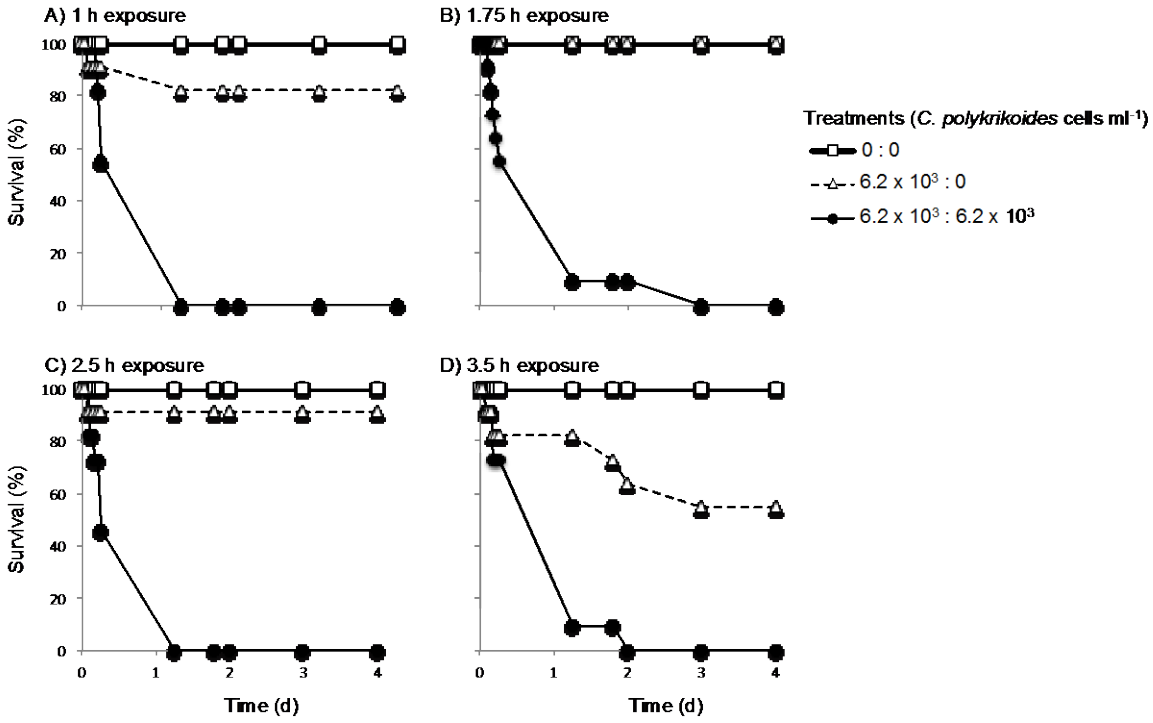

Fig. 5. Survival of *Cyprinodon variegatus* eleutheroembryos after (A) 1 h, (B) 1.75 h, (C) 2.5 h, and (D) 3.5 h exposures to *Cochlodinium polykrikoides*. Treatments indicate the *C. polykrikoides* cell densities before and after (before : after) eleutheroembryos were transferred following the designated exposure times.

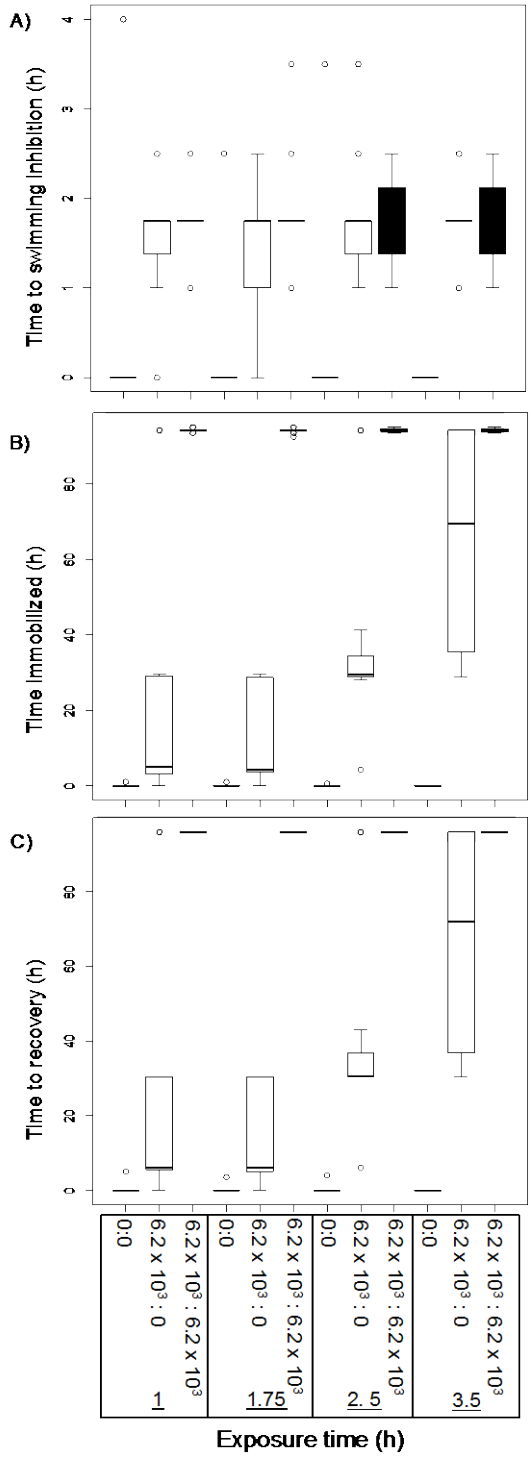

Fig. 6. Sublethal effects of *Cochlodinium polykrikoides* culture to (A) time to swimming inhibition, (B) total time immobilized and (C) time to swimming recovery for

Cyprinodon variegatus eleutheroembryos. Lines inside box plots represent the median values, while the upper and lower segments represent the 0.75 and 0.25 quartiles respectively. Treatments indicate the *C. polykrikoides* cell densities before and after (before : after) eleutheroembryos were transferred following the designated exposure times. Open circles represent data outliers identified by the R statistical software.

TASK 10. PREPARE A FINAL REPORT REGARDING *COCHLODINIUM POLYKRIKOIDES* RESULTS.

This report fulfills this task.