Vol. 505: 81–94, 2014
doi: 10.3354/meps10793

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published May 28

Toxicity of the harmful dinoflagellate *Cochlodinium polykrikoides* to early life stages of three estuarine forage fish

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ABSTRACT: Harmful algal blooms (HABs) caused by the dinoflagellate *Cochlodinium polykrikoides* have increased in geographic extent, frequency, and duration in 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 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 3 forage fish species (Atlantic silverside *Menidia menidia*, inland silverside *M. beryllina*, and sheepshead minnow *Cyprinodon variegatus*) all common on the US East Coast. Our experiments demonstrated that *C. polykrikoides* caused mortalities in both embryos and eleutheroembryos, but that sensitivity to acute toxicity differed among fish species (*M. beryllina* > *M. menidia* > *C. variegatus*) and among life stages (eleutheroembryos > embryos). Although embryos were somewhat resistant to *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. within hours), and that surviving fish could recover swimming ability following removal from *C. polykrikoides* exposure. This research provides the first evidence of sublethal impacts on fish exposed to *C. polykrikoides*, and advances understanding of the potential ecosystem impacts of this harmful alga.

KEY WORDS: *Cochlodinium polykrikoides* · *Cyprinodon variegatus* · Early life history · Forage fish · Harmful algal bloom · *Menidia beryllina* · *Menidia menidia* · Toxicity

INTRODUCTION

Harmful algal blooms (HABs) are caused by a variety of marine phytoplankton species, many of which produce potent biotoxins (Landsberg 2002). HABs can be ecologically and economically destructive, causing mass mortalities in both wild and farmed fish and shellfish species globally (Shumway 1990, Whyte et al. 2001, Lee et al. 2002, Imai et al. 2006, Anton et al. 2008, Gobler et al. 2008, Richlen et al. 2010). While these impacts are substantial, little is known about the broader ecological impacts of many HABs (Smayda 1991, Landsberg 2002, Kudela & Gobler 2012). Forage fish play a crucial role in marine ecosystems by feeding on algae and other plankton and transferring energy to upper trophic levels (Pikitch et

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al. 2012), and may be particularly susceptible to HABs. Thus impacts to this group of fishes may have cascading effects within aquatic food webs, as well as important economic consequences (Pikitch et al. 2014)

Blooms of the toxic dinoflagellate *Cochlodinium polykrikoides* have increased in geographic extent, frequency, and duration in many coastal ecosystems worldwide (Kudela & Gobler 2012). In many regions of Asia, Europe, and North America, these blooms are now annual events (Lee et al. 2002, Gobler et al. 2008, Tomas & Smayda 2008, Richlen et al. 2010, Kudela & Gobler 2012), presenting significant challenges to the management and conservation of coastal living resources (Landsberg 2002, Anderson 2009, Kim 2010).

Much of our current understanding of the ichthyotoxicity of *C. polykrikoides* and other HABs is based on traditional laboratory controlled experiments or field observations using mostly larval, juvenile, or adult fish (Onoue et al. 1985, Kim et al. 1999, Landsberg 2002, Gobler et al. 2008, Tang & Gobler 2009). While studies on these life stages have been useful in determining the toxicity of *C. polykrikoides*, more research is needed to identify the causative agents of *C. polykrikoides* toxicity (Kim et al. 1999, Tang & Gobler 2009, Kudela & Gobler 2012). In addition, no study to date has evaluated the toxicity of *C. polykrikoides* to the early life stages (ELSs) of fish (i.e. embryos and eleutheroembryos). Toxicity to ELS fish could be ecologically significant, but may go unnoticed in the wild because of their relatively small size (Tang & Gobler 2009). Impacts to ELS fish may affect the recruitment of fish populations (Houde 1989, Rothschild 2000, Houde 2008), the productivity of fisheries, upper trophic level predators, and food web dynamics (Rothschild 2000, Pikitch et al. 2004, 2012, 2014).

The use of ELS fish bioassays for HAB toxicology research has been very limited, both in terms of the algae and the model organisms investigated. Only 3 fish species, the Japanese medaka *Oryzias latipes* (Kimm-Brinson & Ramsdell 2001), the zebrafish *Danio rerio* (Lefebvre et al. 2004, Berry et al. 2007), and the red drum *Sciaenops ocellatus* (Riley et al. 1989) have been used in studies conducted to date. Of these, red drum was the only species that was gathered from the wild and not produced from a hatchery (Riley et al. 1989).

This study assessed the toxicity of *C. polykrikoides* to the ELSs of 3 species of forage fish common to US East Coast estuaries. This research furthers understanding of the potential ecosystem effects of *C. polykrikoides* blooms, and underscores the importance and utility of incorporating ELS fish in future toxicology studies with HABs.

MATERIALS AND METHODS

Cochlodinium polykrikoides clonal culture

The dinoflagellate *C. polykrikoides* (strain CP1) was isolated from bloom water collected in Flanders Bay, Peconic Estuary, New York, USA in 2006 (Tang & Gobler 2009). Clonal culture of *C. polykrikoides* was maintained in sterile GSe culture medium prepared according to Tang & Gobler (2009). Briefly, the culture medium was prepared with autoclaved and filtered (0.22 µm) coastal Atlantic Ocean seawater (salinity: 30) supplemented with stock nutrients and an antibiotics solution (a mixture of 10 000 IU penicillin and 10 000 µg ml^{-1} streptomycin; Mediatech) with a final concentration of 2% (Tang & Gobler 2009). Cultures of *C. polykrikoides* were incubated at 21°C following a 12 h light:12 h dark photoperiod with a light intensity of ~100 µmol quanta m^{-2} s^{-1} (Tang & Gobler 2009). Batches of *C. polykrikoides* clonal cultures were generally within early exponential growth phases when they were used for experiments, as cells in this phase are most physiologically active (Tang & Gobler 2009).

Fish collection and selection

We followed procedures approved by the Institutional Animal Care and Use Committee at Stony Brook University (Stony Brook, New York, USA) for fish collection, handling, experimental design, and disposal, using organisms approved for toxicity research (EPA 2002). Atlantic silverside (*Menidia menidia*) embryos were obtained from either strip spawning adult fish collected by beach seine from Shinnecock Bay, New York, USA (temperature: 21 to 23°C; salinity: 28 to 30) using methods described in Costello et al. (1957) or by natural photoperiod-induced spawning of a separate F1 population of fish maintained at Flax Pond Laboratory, Stony Brook, New York, USA (temperature: 24°C; salinity: 25). Inland silverside (*Menidia beryllina*) embryos were obtained from hatchery stocks maintained by Aquatic Resource Organisms (Hampton, New Hampshire, USA) (temperature: 25°C; salinity: 25 to 30), while sheepshead minnow (*Cyprinodon variegatus*) embryos were obtained from Aquatic Bio Systems (Fort

Collins, Colorado, USA) (temperature: 20 to 25°C; salinity 25 to 30).

Embryos used in experiments were <24 h old post-fertilization (hpf), which allowed for differentiation between successfully fertilized embryos with healthy appearance (i.e. translucent yellow color) from those that were unfertilized or dead (i.e. opaque white color) (EPA 1996). Prior to all experiments, embryos were selected using flexible forceps, counted, and placed into sterilized nutrient-ameliorated culture media for at least 2 h. This was done to wash any residues from the embryos and allow them to acclimate. Culture medium served as a control treatment in our experiments (Tang & Gobler 2009). Rinsed embryos were then visually inspected under an inverted light microscope (Nikon Eclipse TS100, Nikon) at 40× to confirm that sufficient numbers of healthy, fertilized embryos were available for experiments.

Eleutheroembryos, defined as newly hatched fish that are still feeding on their yolk sac (Belanger et al. 2010, Embry et al. 2010), for all 3 species were obtained by hatching embryos. Embryos used for hatching were placed into 1 l beakers filled with filtered (0.2 µm) and UV sterilized seawater (salinity: 30). Beakers received constant bubble aeration and followed an approximate photoperiod of 12 h light: 12 h dark at ~25°C. Beakers were periodically checked for hatching over several days, depending on the species, and water exchanges (0.5 l) occurred every 2 to 3 d. Eleutheroembryos used in experiments were <24 h old post-hatch (hph) for all species. Once hatched, eleutheroembryos were counted and transferred into sterilized nutrient-ameliorated culture media (salinity: 30) using a modified transfer pipette (Samco Scientific). Eleutheroembryos were acclimated for at least 2 h in fresh sterile medium before experiments.

Embryo toxicity experiments (Expts 1 to 3)

Embryo experiments were conducted using 24-well polystyrene cell culture plates. All experiments included a culture medium control (0 cells ml^{-1}) and an undiluted *C. polykrikoides* clonal culture treatment (3×10^3 to 6×10^3 cells ml^{-1}) (Table 1). Cell densities of undiluted *C. polykrikoides* clonal cultures were similar to those documented in natural *C. polykrikoides* blooms (Gobler et al. 2008). Depending on the experiment, several intermediary *C. polykrikoides* treatments were prepared by diluting *C. polykrikoides* clonal culture with culture medium (Table 1). All dilution treatments in each experiment were prepared in the same flask, starting with the control treatment and increasing in *C. polykrikoides* cell density. Dilution treatments were dispensed into replicate wells (3 ml in each well). Acclimated embryos were then haphazardly selected and allocated into plate wells in a sequential manner starting with the first replicate (well) on each treatment plate, before proceeding to other wells in a treatment plate. This process was repeated until all replicates in plates had an embryo to reduce any potential selection bias across treatment plates. After allocation of embryos, an inverted light microscope was used to confirm that each well had an embryo and a digital image was captured (Nikon Insight camera, Nikon) to assess the size of embryos. Dilution treatments, fish per treatment, and total sample sizes for all ELS experiments are shown in Table 1. All embryos were exposed to *C. polykrikoides* treatments continuously throughout their embryonic development (i.e. 5 to 8 d depending on the fish species) and the remaining duration of the experiments (Table 1). Embryos were microscopically examined periodically (Table 1) throughout the duration of experiments and mortality and hatching was assessed. Post-hatch mortality was recorded and analyzed in Expts 1 and 2, but this was not possible for Expt 3.

Eleutheroembryo experiments

To determine the toxicity of *C. polykrikoides* to eleutheroembryos, 2 types of experiments were conducted: (1) 2 d static exposure experiments to assess the acute toxicity of *C. polykrikoides* clonal culture, and (2) exposure and recovery experiments to evaluate lethal and sublethal toxicity of short-term exposure of eleutheroembryos to *C. polykrikoides* culture. Both experiment types were conducted using multiple 24-well polystyrene plates and utilized eleutheroembryos (<24 hph) for each fish species. Calibrated digital images of eleutheroembryos were taken at either time of death or experimental termination for length measurements using NIS-Elements BRTM Imaging (Build 728) software 3.22.11 (LO, Nikon).

Eleutheroembryo acute toxicity experiments (Expts 4 to 6). For the 2 d static acute toxicity experiments, dilutions of *C. polykrikoides* culture were prepared in the same manner as the embryo experiments and at similar cell densities (0 to 8×10^3 cells ml^{-1}) (Table 1). Mortality was assessed every 24 h by visual inspection under an inverted light microscope. An eleutheroembryo was considered dead if there

Table 1. Inventory of *Cochlodinium polykrikoides* toxicity experiments with early life stages of *Menidia menidia* (MM), *M. beryllina* (MB), and *Cyprinodon variegatus* (CV) species. In the exposure and recovery experiments, full (i.e. 50:50 and 100:100) and partial (i.e. 50:0 and 100:0) exposure treatments were compared with their respective control (0:0) treatment

Expt	Species	Max. *C. polykrikoides* (cells ml^{-1})	Treatments (% of max. *C. polykrikoides*)	Fish per treatment (n)	Total no. of fish (n)	Sampling times (d)	Duration (d)
Embryo toxicity experiments							
1	MM	3×10^3	(0, 1, 5, 10, 25, 50, 100)	18, 21, 20, 20, 19, 22, 18	138	0, 1, 7, 8, 10	10
2	MB	5×10^3	(0, 1, 5, 10, 25, 50, 100)	21, 22, 22, 22, 22, 22, 23	154	0, 2, 4, 7, 8, 9	9
3	CV	6×10^3	(0, 1, 25, 50, 75, 100)	15, 13, 13, 9, 9, 13	72	0, 2, 3, 4, 5, 6, 7, 8	8
Eleutheroembryo acute toxicity experiments							
4	MM	5×10^3	(0, 1, 5, 10, 50, 100)	24, 24, 24, 24, 24, 24	144	0, 1, 2	2
5	MB	8×10^3	(0, 1, 5, 10, 25, 50, 100)	24, 24, 24, 24, 24, 24, 24	168	0, 1, 2	2
6	CV	6×10^3	(0, 1, 25, 50, 75, 100)	18, 18, 18, 18, 18, 18	108	0, 1, 2	2
Eleutheroembryo exposure and recovery experiments							
7	MM	4×10^3	0.5 h (0:0), 0.5 h (50:0), 0.5 h (50:50), 0.5 h (100:0), 0.5 h (100:100), 0.75 h (0:0), 0.75 h (50:0), 0.75 h (50:50), 0.75 h (100:0), 0.75 h (100:100)	12, 12, 12, 12, 12, 12, 12, 12, 12, 12	120	0, 0.02, 0.04, 0.07, 0.11, 0.16, 0.2, 0.24, 0.28, 1.03, 2.03	2.03
8	MB	6×10^3	0.25 h (0:0), 0.25 h (50:0), 0.25 h (50:50), 0.25 h (100:0), 0.25 h (100:100)	10, 10, 10, 10, 10	50	0, 0.04, 0.08, 0.13, 0.17, 0.21, 0.25, 0.5, 0.75, 1.75, 2.75	2.75
	CV	6×10^3	0.25 h (0:0), 0.25 h (50:0), 0.25 h (50:50), 0.25 h (100:0), 0.25 h (100:100)	10, 10, 10, 10, 10	50		
9	CV	6×10^3	1 h (0:0), 1 h (100:0), 1 h (100:100), 1.75 h (0:0), 1.75 h (100:0), 1.75 h (100:100) 2.5 h (0:0), 2.5 h (100:0), 2.5 h (100:100), 3.5 h (0:0), 3.5 h (100:0), 3.5 h (100:100)	11, 11, 11, 11, 11, 11, 11, 11, 11, 11, 11, 11	132	0, 0.07, 0.1, 0.15, 0.17, 0.21, 0.25, 1.25 1.79, 2.0, 3.0, 4.0	4

was no sign of a heartbeat after 20 s of inspection (EPA 1996).

Exposure and recovery experiments (Expts 7 to 9). Given the heterogeneous nature of *C. polykrikoides* blooms (Kudela & Gobler 2012), exposure and recovery experiments were designed to assess how short-term exposures (i.e. swimming in) to a bloom 'patch' may impact fish. Exposure and recovery experiments followed a randomized block design using 24-well plates, in which each plate contained one replicate of each treatment. Experiments varied in both *C. polykrikoides* cell densities (i.e. undiluted *C. polykrikoides*, 50% diluted *C. polykrikoides* and control) and exposure time (Table 1). In these experiments, a treatment replicate consisted of 2 wells on a given plate, an exposure well and a recovery well. Following the exposure period, all eleutheroembryos were transferred to an adjacent recovery well, which contained either *C. polykrikoides* at the same cell density or culture medium (control). Eleutheroembryos whose exposure well contained culture medium (0 cells ml^{-1}) were transferred to recovery wells containing culture medium (0 cells ml^{-1}) to account for the potential effects of pipetting and transferring on fish. Treatments in which eleutheroembryos were transferred to control recovery wells after *C. polykrikoides* exposure will be referred to as 'partial' exposure treatments, while those transferred to the same *C. polykrikoides* treatments will be referred to as 'full' exposure treatments henceforth. Eleutheroembryos remained in recovery wells for the duration of these experiments and were periodically checked for viability (Expts 7, 8, and 9) and motility (Expt 9) (Table 1).

***Expt 7* (*Menidia menidia*):** Eleutheroembryos in partial exposure treatments were exposed to undiluted *C. polykrikoides* (4×10^3 cells ml^{-1}) or 50% diluted *C. polykrikoides* (2×10^3 cells ml^{-1}) for either 0.5 or 0.75 h before being transferred into recovery wells containing control culture medium (0 cells ml^{-1}). Full exposure treatments were transferred to recovery wells that contained identical *C. polykrikoides* cell densities following the same exposure times. Eleutheroembryos in all treatments were monitored for 2 d.

***Expt 8* (*Menidia beryllina and Cyprinodon variegatus*):** Eleutheroembryos of 2 fish species (*M. beryllina* and *C. variegatus*) were exposed to 3 concentra-

tions of *C. polykrikoides*; undiluted (6×10^3 cells ml^{-1}), 50% diluted (3×10^3 cells ml^{-1}) and control (0 cells ml^{-1}). Eleutheroembryos were transferred into recovery wells after 0.25 h in exposure wells. Eleutheroembryo mortality was monitored for 2.75 d.

***Expt 9* (*Cyprinodon variegatus*):** Because *C. variegatus* eleutheroembryos exposed to *C. polykrikoides* displayed the highest survival among the 3 species investigated, the effects of this alga on the swimming abilities of this fish species were investigated. Eleutheroembryos in partial exposure treatments were exposed to undiluted *C. polykrikoides* cultures (6×10^3 cells ml^{-1}) for 1.0, 1.75, 2.5, or 3.5 h before being transferred to control recovery wells. Full exposure treatments were transferred to recovery wells of similar *C. polykrikoides* densities following these same exposure times. Eleutheroembryo mortality and motility were monitored for 4 d periodically throughout the experiment (Table 1). Eleutheroembryo motility was classified as 'swimming' or 'not swimming', depending on whether the fish propelled itself in any direction after 20 s of observation at each check point throughout the experiment (Table 1). Time to swimming inhibition, the maximum time immobilized, and time to swimming recovery were calculated for each eleutheroembryo. Time to swimming inhibition was defined as the time period from the beginning of the experiment until the eleutheroembryo was first classified as 'not swimming'. Maximum time immobilized was defined as the longest time period that an eleutheroembryo was consecutively classified as 'not swimming', and time to swimming recovery was the time period required for a previously 'not swimming' eleutheroembryo to be consistently classified as 'swimming'.

Statistical analyses

Data collected from all experiments were analyzed using the R statistical software (CRAN R, version 2.15.0, www.R-project.org) with a time to event package (package survival). Specifically, time to death of embryos or eleutheroembryos and time to hatch of embryos were analyzed by survival analysis with censoring. Censoring allowed for individuals surviving or hatching beyond the experiment end to be included in our analyses. Several parametric hazard distributions were evaluated and ultimately the Weibull distribution was used because it generally provided the best fit to experimental data. Embryo experiments evaluated time to death and time to hatch of embryos primarily, but time to death for hatched eleutheroembryos was also analyzed when possible. Because of heterogeneity of variance in Expts 7, 8, and 9, likely due to a large number of treatments with 0 or 100% survival, treatments were compared using pairwise *t*-tests with Bonferroni-adjusted error rates. This adjustment procedure is considered conservative and reduces Type I errors (Sokal & Rohlf 1995, McDonald 2009). Eleutheroembryo experiments evaluated time to death as well as sublethal effects to motility (Expt 9). Time to swimming inhibition, total time immobilized, and time to swimming recovery, were also analyzed using a pairwise *t*-test with Bonferroni-adjusted error rates. To account for the influence of eleutheroembryos dying during the experiment, motility data were analyzed by excluding dead individuals entirely from the analysis. All experiments with multiple *C. polykrikoides* dilution treatments were analyzed as a continuous factor, since they covered a wide range of cell densities, from 0 cells ml^{-1} to bloom concentrations (Table 1).

RESULTS

Embryo toxicity experiments (Expts 1 to 3)

Time to embryo death. Embryo survival for *Menidia menidia* across all treatments (Expt 1) was high (>90%) and time to embryo death was not significantly different between control and *Cochlodinium polykrikoides* treatments (Table 2). Survival of *Menidia beryllina* embryos (Expt 2) was >81% for the control and lower dilutions of *C. polykrikoides* and only 4% (1 out of 23 embryos) when embryos were exposed to 5×10^3 *C. polykrikoides* cells ml^{-1}. Survival of embryos exposed to lower doses of *C. polykrikoides* did not differ from controls 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 (Expt 3) in control and lower *C. polykrikoides* cell densities ranged from 80 to 100%, while exposure to the high densities of *C. polykrikoides* (6.4×10^3 cell ml^{-1}) reduced survival to 46% (6 out of 13 embryos). 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 Expt 1 (*M. menidia*) was ≥91%. Embryos in control treatments hatched significantly later than embryos in *C. polykrikoides* treatments ($p < 0.0001$,

Table 2). Hatching success of *M. beryllina* (Expt 2) was 4% at 5 × 10^3 *C. polykrikoides* cells ml^{-1} and >81% in all other treatments. Time to hatch in *C. polykrikoides* treatments also occurred significantly later for *M. beryllina* embryos compared with controls ($p = 0.0007$, Table 2). No statistically significant differences in time to hatch or hatching success were found for *C. variegatus* embryos in Expt 3 (Table 2).

Time to eleutheroembryo death. Time to death of eleutheroembryos in *C. polykrikoides* treatments occurred significantly earlier than in control treatments for *M. menidia* and *M. beryllina* eleutheroembryos ($p = 0$ and $p < 0.0001$ respectively, Table 2). Survival of *M. menidia* eleutheroembryos (Expt 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}. Survival of *M. beryllina* eleutheroembryos (Expt 2) ranged from 41 to 90% in *C. polykrikoides* treatments ≤4.9 × 10^2 cells ml^{-1} and the control. No *M. beryllina* eleutheroembryos survived in *C. polykrikoides* treatments ≥1.2 × 10^3 cells ml^{-1}. Survival of *C. variegatus* eleutheroembryos (Expt 3) was ≥89% in all treatments.

Table 2. Summary of survival analyses in *Cochlodinium polykrikoides* toxicity experiments (Expts 1 to 6) 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

Expt	Species	Test	χ^2	df	p-value	N
Embryo toxicity experiments						
1	MM	Time to death (embryo)	−13889.51	1	ns	138
		Time to hatch	15.87	1	<0.0001	133
		Time to death (eleutheroembryo)	89.67	1	0	133
2	MB	Time to death (embryo)	−6351.36	1	ns	154
		Time to hatch	11.58	1	0.0007	116
		Time to death (eleutheroembryo)	57.90	1	<0.0001	116
3	CV	Time to death (embryo)	0.98	1	ns	72
		Time to hatch	0.02	1	ns	48
		Time to death (eleutheroembryo)[a]	–	–	–	48
Eleutheroembryo acute toxicity experiments						
4	MM	Time to death (eleutheroembryo)	184.25	1	0	144
5	MB	Time to death (eleutheroembryo)	131.23	1	0	144
6	CV	Time to death (eleutheroembryo)	131.46	1	0	108

[a]Insufficient number of days post-hatch to conduct statistical test

Eleutheroembryo experiments

Eleutheroembryo acute toxicity experiments (Expts 4 to 6). Eleutheroembryos of all fish species exposed to high densities of *C. polykrikoides* experienced complete mortality during the first day of post-hatch exposure (Fig. 1). Survival in lower *C. polykrikoides* cell density treatments varied by fish species and *C. polykrikoides* cell density. All *C. variegatus* in Expt 6 survived *C. polykrikoides* treatments with densities ≤1.6 × 10^3 cells ml^{-1}, while *M. menidia* (Expt 4) and *M. beryllina* (Expt 5) had high survival, >75 and 100% respectively, in *C. polykrikoides* treatments ≤4.0 × 10^2 to 5.0 × 10^2 cells ml^{-1} (Fig. 1). Survival analyses revealed that time

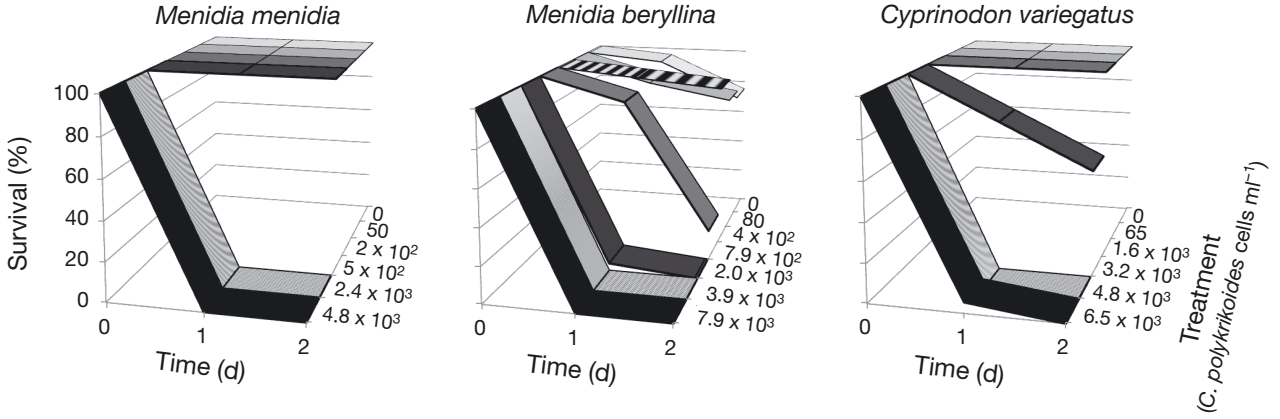

Fig. 1. Survival of *Menidia menidia*, *M. beryllina* and *Cyprinodon variegatus* eleutheroembryos in static *Cochlodinium polykrikoides* acute toxicity experiments

Table 3. Statistical significance of time to eleutheroembryo death in exposure and recovery experiments using pairwise *t*-tests with Bonferroni-adjusted error rates for *Menidia menidia* (MM), *M. 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 with their respective control (0:0) treatment. 0, 50 and 100 represent the percentage of the maximum *Cochlodinium polykrikoides* cell density used for each respective experiment, found in Table 1. ns denotes p-values that were not significant ($p > 0.05$) and na indicates that these treatments were not used

Expt	Species	Test	Exposure time (h)	Exposure type: 50:0 χ^2	50:0 df	50:0 p-value	50:50 χ^2	50:50 df	50:50 p-value	100:0 χ^2	100:0 df	100:0 p-value	100:100 χ^2	100:100 df	100:100 p-value
Eleutheroembryo exposure and recovery experiments															
7	MM	Time to death	0.5	–0.86	1	ns	26.72	1	<0.0001	71.24	1	<0.0001	73.21	1	<0.0001
		(eleutheroembryo)	0.75	0.12	1	ns	32.09	1	<0.0001	25.00	1	<0.0001	93.25	1	<0.0001
8	MB	Time to death	0.25	0.32	1	ns	3.72	1	ns	2.95	1	ns	15.99	1	0.005
	CV	(eleutheroembryo)	0.25	–	–	ns	–	–	ns	–	–	ns	23.25	1	<0.0001
9	CV	Time to death	1.0	na	na	na	na	na	na	3.01	1	ns	38.55	1	<0.0001
		(eleutheroembryo)	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

to eleutheroembryo death decreased significantly with increasing *C. polykrikoides* cell densities in all these experiments ($p = 0$ for Expts 4 to 6, Table 2).

Eleutheroembryo exposure and recovery experiments (Expts 7 to 9). ***Expt 7 (Menidia menidia):*** Time to eleutheroembryo death occurred significantly earlier ($p < 0.0001$) in full exposure (i.e. 3.7×10^3 cells ml^{-1}:3.7×10^3 cells ml^{-1}, 1.9×10^3 cells ml^{-1}:1.9×10^3 cells ml^{-1}) and the high cell density (3.7×10^3 cells ml^{-1}:0 cells ml^{-1}) partial exposure treatments compared with the control (0 cells ml^{-1}:0 cells ml^{-1}) and the lower cell density (1.9×10^3 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. 2). Eleutheroembryos in the lower cell density partial exposure (1.9×10^3 cells ml^{-1}:0 cells ml^{-1}) treatments had greater survival (i.e. 0.5 h = 67%, 0.75 h = 83%) than full exposure (1.9×10^3 cells ml^{-1}:1.9×10^3 cells ml^{-1}) treatments (i.e. 0% for both exposure times). In contrast, no significant differences in time to eleutheroembryo death were found between partial (1.9×10^3 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 3.7×10^3 *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 (3.7×10^3 cells ml^{-1}:0 cells ml^{-1}) treatment (Fig. 2A).

Expt 8 (Menidia beryllina and Cyprinodon variegatus): There were differences in the survival of *M. beryllina* and *C. variegatus* eleutheroembryos after a 0.25 h exposure to similar *C. polykrikoides* cell densities (Fig. 3). Survival analyses across all treatments for *M. beryllina* and *C. variegatus* revealed that time to eleutheroembryo death only occurred significantly earlier in the high cell density full exposure (6.0×10^3 cells ml^{-1}:6.0×10^3 cells ml^{-1})

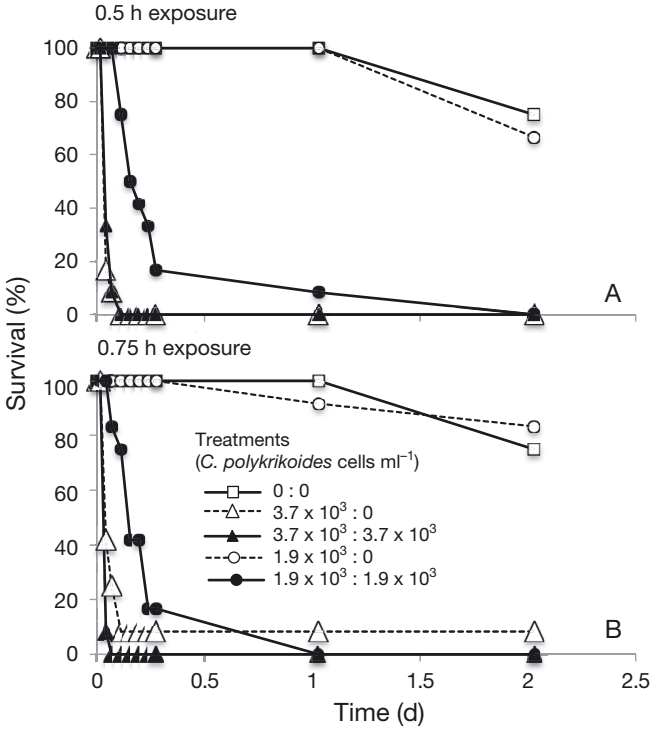

Fig. 2. Survival of *Menidia menidia* eleutheroembryos after (A) 0.5 h and (B) 0.75 h exposures to *Cochlodinium polykrikoides* culture treatments. Treatments indicate the *C. polykrikoides* cell densities before and after (before:after) eleutheroembryos were transferred following the designated exposure time

C. polykrikoides treatments compared with the controls ($p = 0.005$ and $p < 0.0001$ respectively, Table 3). Survival of *M. beryllina* in the lower cell density (3.0×10^3 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 (3.0×10^3 cells ml^{-1}:3.0×10^3 cells ml^{-1}) was only 10% (Fig. 3A). Similarly, survival in the high cell density (6.0×10^3 cells ml^{-1}: 0 cells ml^{-1}) partial exposure treatments was higher (30%) than the high cell density full exposure (6.0×10^3 cells ml^{-1}:6.0×10^3 cells ml^{-1}) treatment (0%) for *M. beryllina*. Complete survival of *C. variegatus* eleutheroembryos was found in all treatments except for the high cell density *C. polykrikoides* full exposure (6.0×10^3 cells ml^{-1}:6.0×10^3 cells ml^{-1}) treatment, where it was 10% (Fig. 3B).

Expt 9 (Cyprinodon variegatus): Survival in the partial exposure (6.2×10^3 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 (Fig. 4). Time to death occurred significantly earlier, in about 2.73 to 3.75 d, in full exposure (6.2×10^3 cells ml^{-1}:6.2×10^3 cells ml^{-1}) *C. polykrikoides* treatments compared with control (0 cells ml^{-1}:0 cells ml^{-1}) and partial exposure (6.2×10^3 cells ml^{-1}:0 cells ml^{-1}) treatments ($p < 0.0001$ for all exposure times; Table 3). Time to eleutheroembryo death in partial (6.2×10^3 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 *C. polykrikoides* caused swimming to be inhibited within 3.5 h for all eleutheroembryos in full (6.2×10^3 cells ml^{-1}:6.2×10^3 cells ml^{-1}) and 96% of fish in partial (6.2×10^3 cells ml^{-1}:0 cells ml^{-1}) exposure treatments. Time to swimming inhibition occurred significantly earlier in these exposure treatments compared with controls (0 cells ml^{-1}:0 cells ml^{-1}) (Table 4), where swimming was inhibited in only 7% of eleutheroembryos. Immobilized individuals in controls, however, began to swim again in less than 1.0 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. As such, motility data was analyzed by excluding dead individuals from all analyses. Eleutheroembryo motility in partial exposure treatments varied with exposure times, with longer *C. polykrikoides* exposure times

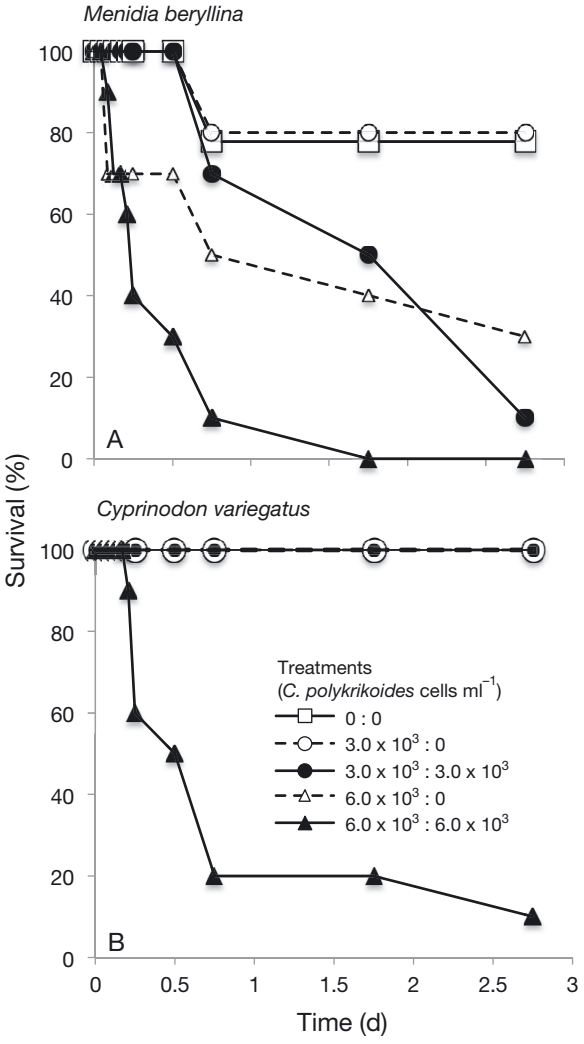

Fig. 3. Survival of (A) *Menidia beryllina* and (B) *Cyprinodon variegatus* eleutheroembryos after a 0.25 h exposure to *Cochlodinium polykrikoides* culture. Treatments indicate the *C. polykrikoides* cell densities before and after (before: after) eleutheroembryos were transferred at 0.25 h

generally leading to increased immobilization and swimming recovery times (Fig. 5). 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 when dead individuals were excluded from the analysis (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 1.0 h (median: 6.0 h, $p < 0.0001$), 1.75 h (median: 6.0 h, $p < 0.0001$), but not 2.5 h (median: 30.5 h) (Table 4, Fig. 5).

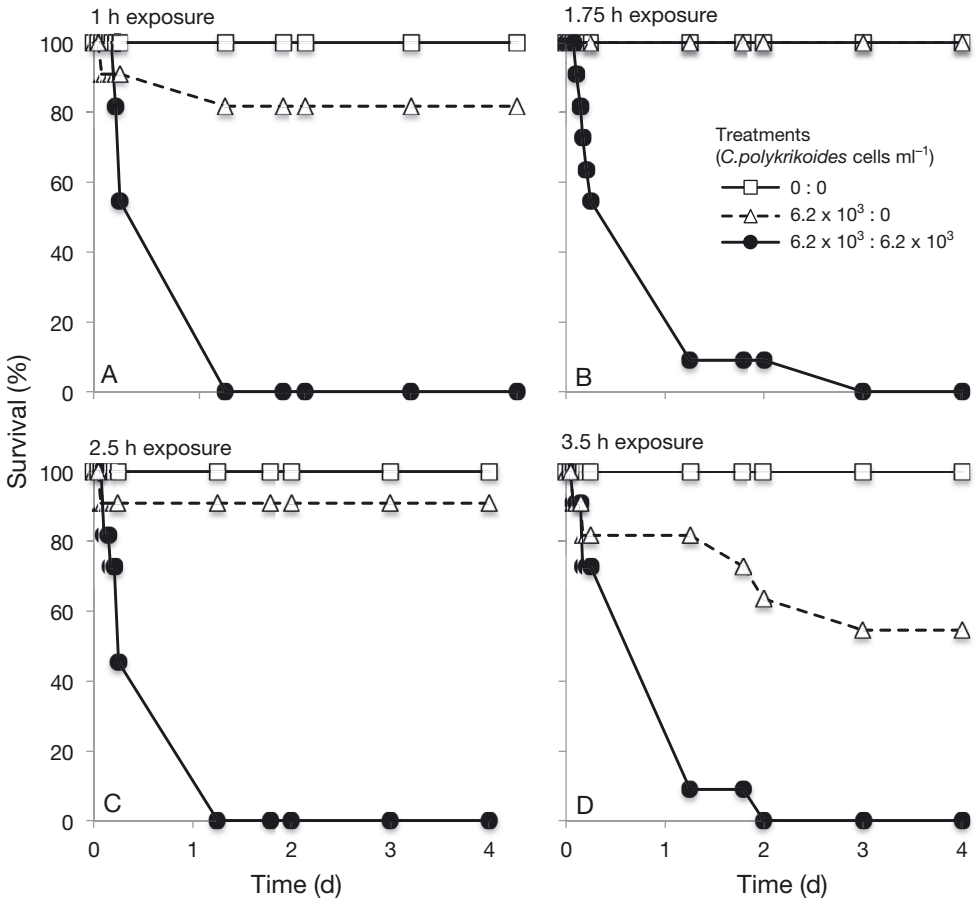

Fig. 4. Survival of *Cyprinodon variegatus* eleutheroembryos after (A) 1.0, (B) 1.75, (C) 2.5 and (D) 3.5 h exposures to *Cochlodinium polykrikoides*. Treatments indicate the *C. polykrikoides* cell densities before and after (before:after) eleutheroembryos were transferred following the designated exposure times

DISCUSSION

Effects of *Cochlodinium polykrikoides* on early life stage fish in an ecosystem context

Blooms of *C. polykrikoides* often occur in high-density (10^3 to 10^5 cells ml^{-1}) patches which can persist for many months and cover large coastal areas (>1 km^2) throughout the world (Onoue et al. 1985, Anton et al. 2008, Gobler et al. 2008, Richlen et al. 2010). It is thus likely that these blooms overlap with multiple life history stages of coastal fish populations, especially when blooms last for periods >8 mo (Richlen et al. 2010) or occur during seasons of high coastal fish activity in temperate latitudes (Gobler et al. 2008). Tang & Gobler (2009) hypothesized that *C. polykrikoides* blooms may cause cryptic mortality of smaller life stages of fish. We confirm that newly hatched eleutheroembryos of several forage fish species common to the US East Coast can experience rapid mortality and sublethal effects to motility when exposed to *C. polykrikoides*. Although *C. polykrikoides* cultures have been found to be more toxic than bloom water at equivalent cell densities (Tang & Gobler 2009), similar toxicological responses are observed at higher bloom water concentrations (≥10^4), which are commonly found in natural bloom patches (Gobler et al. 2008, Tang & Gobler 2009).

Menidia menidia and *M. beryllina* species were more sensitive to *C. polykrikoides* than *Cyprinodon variegatus*, and eleutheroembryos were more sensitive than the embryo life stage. In general, survival was relatively high in embryos exposed to *C. polykrikoides* treatments until they hatched, suggesting that the toxic agents in *C. polykrikoides* do not easily penetrate the chorionic membrane of these species, similar to a variety of other toxicants (Riley et al. 1989, Lammer et al. 2009, Embry et al. 2010). However, in the *M. beryllina* embryo experiment (Expt 2), survival of embryos was greatly reduced when exposed to an elevated cell density of *C. polykrikoides* (5×10^3 cells ml^{-1}), suggesting that mortality of embryos does occur at higher cell densities and/or longer exposures. The effects of *C. polykrikoides* exposure on embryo hatch times were less evident and contrasting. Although statistically significant differences were found between treatments for *M. menidia* and *M. beryllina*, they are likely not eco-

Table 4. Statistical significance of sublethal effects of *Cochlodinium polykrikoides* to *Cyprinodon variegatus* eleutheroembryos across all exposure types (i.e. control (0:0), partial (100:0), and full (100:100)) and exposure times (i.e. 1.0, 1,75, 2.5 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 Expt 9 (Table 1)

			Exposure time (h)											
			1.0			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
Time to swimming inhibition														
Exposure time (h)	1.0	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														
Exposure time (h)	1.0	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														
Exposure time (h)	1.0	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	

logically significant, because fertilization times could have naturally ranged by as much as ±5 to 7 h in our experiments. In addition, no significant differences in time to hatch were found in the *C. variegatus* embryo experiment. Based on these results and the heterogeneous and episodic nature of *C. polykrikoides* blooms, it is unlikely that they pose an acute threat to wild fish embryos, at least until they hatch. However, there is increasing evidence that embryonic exposures to some toxicants can have delayed consequences that only become manifested in older life stages of fish (Weis & Weis 1995a,b, Timme-Laragy et al. 2006, Levin et al. 2011). These types of effects should be evaluated in future embryo toxicity experiments with this HAB.

Static acute toxicity experiments revealed rapid (<24 h) mortality of eleutheroembryos in all 3 fish species when exposed to the high *C. polykrikoides* cell density treatments (2×10^3 to 7.9×10^3 cells ml^{-1}), which are typical of dense blooms (Kudela & Gobler

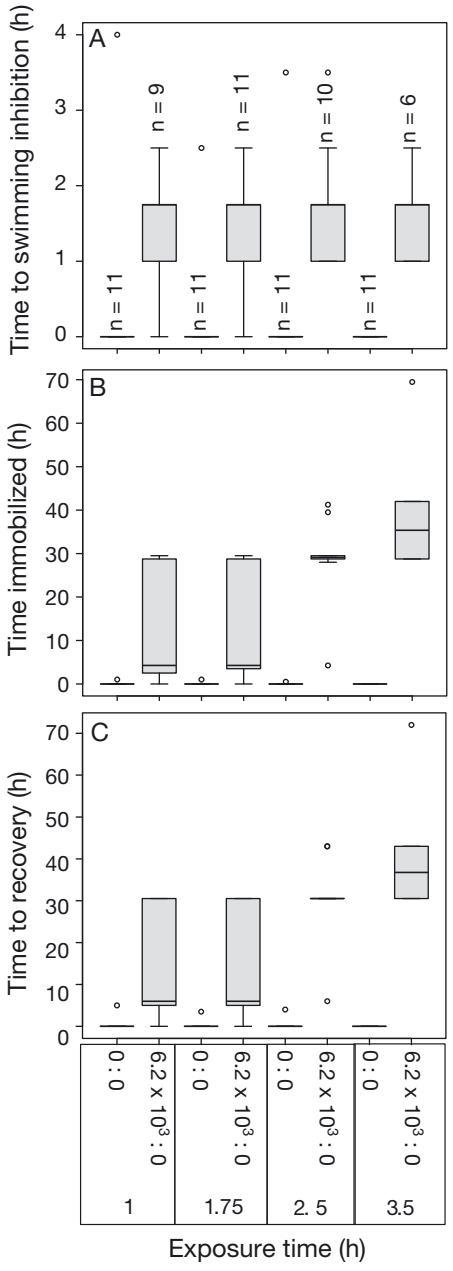

Fig. 5. Sublethal effects of *Cochlodinium polykrikoides* culture to (A) time to swimming inhibition, (B) total time immobilized, and (C) time to swimming recovery for surviving *Cyprinodon variegatus* eleutheroembryos. Lines inside box plots represent the median values, while the upper and lower segments 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

2012). The *C. polykrikoides* cell densities at which the greatest mortality occurred varied by fish species (Fig. 1). Complete mortality occurred after exposure to 2.4×10^3 cells ml^{-1} for *M. menidia*, while similar depressed survival occurred at lower (7.9×10^2 cells ml^{-1}) and higher (4.8×10^3 cells ml^{-1}) *C. polykrikoides* cell densities for *M. beryllina* and *C. variegatus*, respectively (Fig. 1). These results demonstrate that individual fish species may have different sensitivities to *C. polykrikoides*.

While static acute toxicity experiments are valuable for determining baseline toxicology data, they are often difficult to interpret in an ecological context (Kimball & Levin 1985, de Vlaming & Norberg-King 1999). Exposures of marine organisms to HABs are likely not static in the natural environment, as exposure is influenced by a variety of factors, including water circulation and the potential motility of both target and HAB species (Landsberg 2002). *C. polykrikoides* blooms in particular can form large heterogeneous blooms that can persist for many months (Gobler et al. 2008, Richlen et al. 2010, Kudela & Gobler 2012). As such, the effects of relatively short-term (i.e. minutes to hours) *C. polykrikoides* exposures to eleutheroembryos were examined using critical exposure times and cell densities found in this and previous static acute toxicity studies that are ecologically relevant (Gobler et al. 2008, Tang & Gobler 2009). In general, exposure and recovery experiments demonstrated that both exposure to higher *C. polykrikoides* cell densities and longer exposure times significantly influenced the survival and time to death of eleutheroembryos for all species examined. Short-term (i.e. 0.5 and 0.75 h) exposures to *C. polykrikoides* (3.7×10^3 cells ml^{-1}) treatments caused >50% mortality to *M. menidia* eleutheroembryos within 1.0 h of exposure and >90% mortality at 2 d, regardless of whether fish were transferred to control (0 cells ml^{-1}) conditions after exposure. At lower *C. polykrikoides* cell densities in partial exposure (1.9×10^3 cells ml^{-1}: 0 cells ml^{-1}) treatments, eleutheroembryos exhibited high survival similar to controls (0 cells ml^{-1}:0 cells ml^{-1}) (Fig. 2). A similar result in the survival of *M. beryllina* eleutheroembryos was found when examining a shorter exposure time (0.25 h) to higher *C. polykrikoides* cell densities (Fig. 3A). These results demonstrate that in an ecosystem setting, fish exposed to high levels of *C. polykrikoides* (>10^3 cells ml^{-1}) for a brief period of time and subsequently exposed to very low levels — a potentially common occurrence given the heterogeneous nature of these blooms — are more likely to survive than individuals chronically exposed to high levels. As such, the impacts of *C. polykrikoides* to fish may be less severe than previously predicted by chronic exposure experiments (e.g. Tang & Gobler 2009).

Even though short-term exposures to *C. polykrikoides* may not necessarily be lethal, important sublethal effects were found in this study. The loss of swimming ability in *C. variegatus* eleutheroembryos is the first evidence of behavioral impacts from *C. polykrikoides* toxicity to fish. Behavioral information is especially valuable in understanding the broader ecological impacts of many toxicants (Little & Finger 1990), particularly in ELS fish (Sloman & McNeil 2012). Negative impacts on swimming ability can have potentially large consequences for the survival of eleutheroembryos in coastal ecosystems, including the inability to escape predators or capture prey items (Weis & Weis 1995a, Zhou & Weis 1998, Samson et al. 2008, Sloman & McNeil 2012). Our results suggest that short-term exposures of eleutheroembryos to *C. polykrikoides* blooms could render exposed fish vulnerable, as they are incapacitated well before death actually occurs and do not recover their swimming ability for hours after exposure. In this regard, *C. polykrikoides* blooms may significantly impact the recruitment of coastal fish species, their distributions, and even food web dynamics, particularly when these bloom events coincide with reproductive events of fish. Further research, however, is still clearly needed to fully understand the ecosystem effects of this HAB species.

Use of ELS fish bioassay in future HAB toxicology research

ELS fish bioassays are now commonly used across many disciplines of toxicology (Berry et al. 2007, Lammer et al. 2009, Embry et al. 2010). Although ELS fish tests are regarded as comparable and effective alternatives to traditional acute toxicological experiments (Dave 1993, Braunbeck et al. 2005, Wedekind et al. 2007, Lammer et al. 2009, Embry et al. 2010), their use in HAB toxicology research has been relatively limited (Riley et al. 1989, Kimm-Brinson & Ramsdell 2001, Lefebvre et al. 2004, Berry et al. 2007). We demonstrated that ELS fish can be effectively incorporated in toxicology research with *C. polykrikoides* and likely other HABs. The results of acute toxicity experiments using eleutheroembryos were similar to those using older life stage fishes, including larvae, juveniles, and adults (Kim et al. 1999, Gobler et al. 2008, Tang & Gobler 2009). Our findings suggest that future toxicology research with *C. polykrikoides* could utilize ELS *M. menidia*, *M. beryllina*, and *C. variegatus* instead of older conspecifics to achieve similar objectives. Using ELS fish also provides benefits compared with traditional bioassays with older life stages, including reduced research time and expenses, and may allow for a broader range of potential species to be examined (Dave 1993). For instance, it may be impractical or impossible to evaluate juvenile or adult life stages of certain fish species using traditional fish bioassays because of their size (Dave 1993). ELS fish are much smaller, can equilibrate to external experimental conditions more quickly, and generally have lower dissolved oxygen requirements than older conspecifics (Braunbeck et al. 2005). In addition, embryos and eleutheroembryos do not have exogenous dietary considerations, as experiments on older life stage fish would require. This allows for the relative dietary condition of fishes used in experiments to be standardized, as they are all feeding on their yolk sacs and not exogenously (Belanger et al. 2010). Using ELS fish bioassays may also aid in examining the effects of HABs on fish growth, development, and population recruitment (Lefebvre et al. 2004, Berry et al. 2007). As >95% of fish species fertilize embryos externally, researchers could assess the effects of HABs on a variety of marine fish, including those that are known to be ecologically or economically important.

In this study, a few preliminary experiments exhibited poor embryo survival in controls. We suspect that this was likely due to unforeseen interactions (i.e. the stimulation of bacterial or mycotic growth in control culture media) rather than infirm organisms, as survival was acceptable in other respective treatments. Bacterial growth in culture media of *C. polykrikoides* has been demonstrated before (Tang & Gobler 2012) and although embryos were rinsed in sterile culture media prior to experiments during the present study, it is likely that they still contained microbial contaminants. We found that the application of increased antimicrobial solution every 3 d to replicates greatly improved survival of *M. beryllina* embryos and eleutheroembryos. Future embryo experiments using nutrient-ameliorated culture media should consider using similar antimicrobial applications if control survival of embryos is unexpectedly poor.

CONCLUSIONS

Our understanding of the toxicity, toxinology, and ecosystem effects of *Cochlodinium polykrikoides* blooms is still in its 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 3 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 recover swimming ability 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. 2012, 2014) these impacts could be significant.

Acknowledgements. This research was supported by the Lenfest Ocean Program, the Institute for Ocean Conservation Science, Suffolk County Department of Health Services, the Pew Charitable Trusts, and the Shinnecock Bay Restoration Program. The Shinnecock Bay Restoration Program received major funding from the Laurie Landau and Jim and Marilyn Simons Foundations. Special thanks go to Dr. H. Baumann, Dr. K. Broms, S. Caldarello, S. Cernadas-Martin, E. DePasquale, N. Gownaris, T.K. Hattenrath-Lehmann, Dr. F. Koch, C. Santora, and J. Steve for laboratory assistance and fruitful discussions.

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Editorial responsibility: Stylianos Somarakis, Heraklion, Greece

Submitted: September 9, 2013; Accepted: March 19, 2014
Proofs received from author(s): May 19, 2014