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HARMFUL ALGAE POSE ADDITIONAL CHALLENGES FOR OYSTER RESTORATION: IMPACTS OF THE HARMFUL ALGAE *KARLODINIUM VENEFICUM* AND *PROROCENTRUM MINIMUM* ON EARLY LIFE STAGES OF THE OYSTERS *CRASSOSTREA VIRGINICA* AND *CRASSOSTREA ARIAKENSIS*

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ABSTRACT The eastern oyster, *Crassostrea virginica* (Gmelin, 1791) has been in decline along the eastern seaboard, and especially in Chesapeake Bay, for decades because of over-harvesting, disease and declines in water quality and suitable habitat. Eutrophication has also been increasing over the past half century, leading to increases in hypoxia and harmful algal blooms (HABs). The effects of two common Chesapeake Bay HAB dinoflagellates, *Karlodinium veneficum*, and *Prorocetnrum minimum* were tested on larvae of *C. virginica* and the Asian oyster being considered for introduction to Chesapeake Bay, *C. ariakensis*. When embryos from freshly spawned *C. virginica* and *C. ariakensis* were exposed immediately to *K. veneficum* at 10^4 cells mL^{-1}, virtually all of the developed larvae were deformed within 48 h in one experimental trial, but not in a second trial in which algae were at a different growth stage. No deformities, and mortalities of <45%, were observed in controls to which a standard diet of the haptophyte *Isochrysis* was added. When 2-wk-old larvae of both species were exposed to the same HAB species, the effect was a severe reduction in motility with *K. veneficum*, but with *P. minimum* only *C. ariakensis* was affected and not *C. virginica*. Comparisons were made of the frequency of these HABs in Chesapeake Bay from long-term data analysis and the temporal period of spawning. Whereas both blooms are more common during the summer months, the frequency of blooms of *K. veneficum* and the period of oyster spawning, June to September, coincide more strongly. To compare spatial similarity, results of a larval transport model were compared with observational data for *K. veneficum*. This comparison demonstrated a significant overlap in July, particularly in the northern reaches of the Bay. These eutrophication-related HABs thus have the potential to reduce survival of early life history stages of oysters and hence to reduce oyster recruitment. Any reduction in recruitment either spatially or temporally, combined with an overall reduction in sheer numbers of larvae that survive, will make restoration or establishment of significant, self-sustaining populations of natural or introduced oyster species much more difficult.

KEY WORDS: oysters, larvae, harmful algae, HABs, Chesapeake Bay, oyster restoration, *Karlodinium veneficum*, *Prorocentrum minimum*, *Crassostrea virginica, C. ariakensis*

INTRODUCTION

The eastern oyster, *Crassostrea virginica* (Gmelin, 1791), is ecologically and economically important in estuaries from Maritime Canada to the Gulf of Mexico, but has been negatively impacted by over-harvesting, disease and declines in water quality and suitable habitat (Wesson et al. 1999, Ocean Studies Board 2004). In Chesapeake Bay, for example, abundances have declined nearly 100-fold over the last century (Rothschild et al. 1994, Jordan et al. 2002). Oyster restoration is a goal of state and federal agencies, with the aim to enhance the ecosystem services provided by oysters, including improved water clarity from oyster filtration, increased oyster reef habitat, and sustainable commercial harvest (Chesapeake Bay Program 2000 (http://www.chesapeakebay.net/agreement.htm, USACOE 2004). Introduction of the Asian oyster, *C. ariakensis*, has been proposed as one strategy to restore oysters in Chesapeake Bay, as they are faster growing and thought to be less susceptible to the common diseases than is the native oyster, *C. virginica* (Zhou & Allen 2003).

Whereas disease and habitat loss are thought to be the major threats to oysters in Chesapeake Bay (e.g., Wesson et al. 1999), eutrophication has also been increasing over the past half century, leading to increases in hypoxia and harmful algal blooms (HABs); (Glibert et al. 2001, Deeds et al. 2002, Glibert & Magnien 2004, Hagy et al. 2004, Kemp et al. 2005), which may pose yet another challenge for the successful re-establishment of oysters. The most familiar impact of HABs is their intoxication of shellfish, leading to contaminated seafood (reviewed by Shumway 1990, Landsberg 2002, Backer & McGillicuddy 2006). Other impacts of HABs include fish kills and the production of high biomass, which alter ecosystem function (e.g., Anderson et al. 2002, Glibert et al. 2005). Here we show that HABs also have direct impacts on the early life stages of oysters, leading to significantly increased mortalities at the larval stage. Without successful recruitment, populations face further obstacles for recovery.

Harmful algal blooms (HABs) in Chesapeake Bay are now more frequent, and of substantially higher densities than several decades ago (e.g., Glibert & Magnien 2004). For example, the dinoflagellate *Prorocentrum minimum* (Pavillard) Schiller 1933 (Dinophyceae, formerly named *P. marie-laboriae*, also classified as *P. cordatum* (Ostenfeld) Dodge, Taylor et al. 2003) is now observed in blooms at densities 3-fold higher than were noted in the 1970s, reaching 10^5 cells mL^{-1} (Tyler & Seliger 1978, Fan et al. 2003, Glibert & Magnien 2004, Tango et al. 2005). Another species of HAB that is increasing in frequency and abundance, and is now recognized as a dominant summer species, is *Karlodinium veneficum* Ballentine 1956 (Dinophyceae, formerly named *K. micrum* and *Gyrodinium galatheanum*; Goshorn et al. 2004, Marshall et al. 2005, Bergholtz et al.

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2005). This species has been implicated in fish kills in Chesapeake Bay and South Carolina (Deeds et al. 2002, Kempton et al. 2002). Bloom densities of *K. veneficum* in Chesapeake Bay can reach 10^5 cells mL^{-1} (Goshorn et al. 2004).

It is well known that oyster growth is a function of the quantity and quality of food in their diet, as well as other factors controlling oyster physiology (e.g., Newell & Langdon 1996, Langdon & Newell 1996). HABs have several direct effects on oysters, but their impact differs by the growth state of the oysters at the time of their exposure, the particular HAB species or strain, as well as its stage of growth or toxicity level (Landsberg 2002, Pate et al. 2003). Early life history stages of oysters may be particularly susceptible to the effects of HABs because of their soft, exposed tissues and requirement for ''good food'' within a few days of hatching and developing into mature larvae. Whereas effects of HABs on the early life history stages (embryos and larvae) of most shellfish and fish are largely unknown, recent studies suggest that HABs can affect growth and, in some cases, development and survivorship of larvae (Wikfors & Smolowitz 1995, Kim-Brinson & Ramsdell 2001, Jeong et al. 2004, Leverone et al. 2006, Padilla et al. 2006, Bricelj & MacQuarrie 2007). HABs have the potential to reduce the growth and survival of embryos and larvae, which in turn will decrease recruitment resulting from a spawning event. Here, our objectives are to demonstrate the impacts of the common Chesapeake Bay HAB species, *P. minimum* and *K. veneficum*, on early life stages of *C. virginica* and *C. ariakensis* based on experimental studies, and to provide comparisons of the distribution of *C. virginica* larvae and *K. veneficum* and *P. minimum* in Chesapeake Bay in time and space, based on comparisons of known HAB distribution from long-term monitoring data and *C. virginica* distributions from a newly developed, larval transport model (North et al. in press).

METHODS

Algal cultures and hatchery-produced embryos and larvae were used to determine the effects of exposure to *P. minimum* and *K. veneficum* on survival and behavior of early life stages of the native and Asian oyster species. Oysters were grown and spawned in the Oyster Hatchery of the Horn Point Laboratory; Asian oysters were maintained in quarantine facilities throughout all phases of experimentation. Oysters were spawned in filtered natural seawater of salinity near 10, and temperature of ~25°C. Algal exposures, as detailed below, were at levels designed to mimic blooms.

Two types of experiments were conducted and focused on different stages of larval development. Newly fertilized eggs hatch within a few hours changing into embryos which quickly undergo a series of developmental stages that lead to the development of veligers. After two to four weeks, veligers develop into the pediveliger stage and begin to seek suitable substrate on which to settle. The first experiment focused on the impact of HABs on embryos or larvae less than a few days old; the second experiment focused on the impact of HABs on pediveliger larvae that were ~2 wk old.

In the first experiment, methods followed the guidelines outlines by American Society for Testing and Materials (1998, International standard guide for conducting static acute toxicity tests starting with embryos, Designation E724-98). Two trials of the first type of experiment were conducted, with each treatment in triplicate for each trial. For the first trial, oysters were strip-spawned to obtain gametes; for the second trial, oysters were spawned naturally. Oyster larvae at a final concentration of 20 larvae mL^{-1} (first trial) or 30 larvae mL^{-1} (second trial) were placed in 1 L glass bottles with gentle aeration. The treatments consisted of: (a) *P. minimum* at 10^4 cells mL^{-1}; (b) *K. veneficum* at 10^4 cells mL^{-1}; (c) the haptophyte *Isochrysis* sp. (strain C-ISO) at 10^5 cells mL^{-1} (standard diet); and (d) none (unfed control). Larvae of both species were tested in each trial, but because of experimental problems, results of percent mortality of only the *C. ariankensis* are reported for the first trial. In the first trial, bottles were subsampled in triplicate at 48 h, and embryos/larvae were enumerated in a Sedgwick Rafter counting chamber at $\times 200$ magnification and photographed microscopically. In the second trial, the entire contents of each bottle was drained and enumerated, thereby reducing the variability associated with subsample counting.

The algal strain of *P. minimum* used, Strain PM-1, was originally isolated from the Choptank River, a tributary of the Chesapeake Bay, in spring 1995 by Dr. A. Li. The strains of *K. veneficum* used were CCMP 1974 and CCMP 1975, also originally isolated from Chesapeake Bay by Dr. A. Li. Stain CCMP 1975 was used for the acute toxicity experiments, and CCMP 1974 was used for the second set of experiments described below. These strains have been shown to produce potent hemolytic toxins, karlotoxins KmTx 1, and KmTx 2, that can be found associated with the dinoflagellate cells and also free in the water (Wang et al. 2005, Deeds & Place 2006, Stoecker et al. in review). The haptophyte *Isochrysis* (Strain C-ISO) was used as the control diet and is routinely used as a food in the oyster hatchery. The algal cultures were grown at a salinity of 10 at 20°C on a 12:12 L:D cycle at ~100 $\mu E\ m^{-2}s^{-1}$. All algae were cultured in f/2 medium (Andersen 2005), prepared without addition of silicate. Early stationary phase cultures were used in the experiments.

In the second type of experiment, 2-wk-old larvae of both species were exposed to the same HAB and control species as above. The experiment was conducted in triplicate in 4 mL well plates. To each well, ~50 larvae and the following algae were added: (a) *P. minimum* at 3.1×10^4 cells mL^{-1}; (b) *K. veneficum* at 5.7×10^4 cells mL^{-1}; (c) *Isochrysis* sp. (C-ISO) at 5×10^5 cells mL^{-1}; and (d) none (unfed control). After 72 h of exposure, the wells were video taped to reveal any differences in larval swimming behavior. The larvae were grown in the oyster hatchery of the Horn Point Laboratory, under standard hatchery protocols, with *Isochrysis* provided as food prior to the experiment.

RESULTS

In the first experiment, even though percent mortality could not be calculated for *C. virginica* in the first trial, when freshly spawned oyster larvae of both species were exposed to *K. veneficum*, virtually all (>95%) of the larvae were found to be deformed (Fig. 1). No deformities were observed in controls to which the standard diet of *Isochrysis* was added, or in treatments to which *P. minimum* was added. Although the same strain (CCMP 1975) of *K. veneficum* was used in the second trial, the percent deformities was far less (<5%).

In all cases, mortalities were <45% for the control treatments that had *Isochrysis* as food (Fig. 2). High mortalities

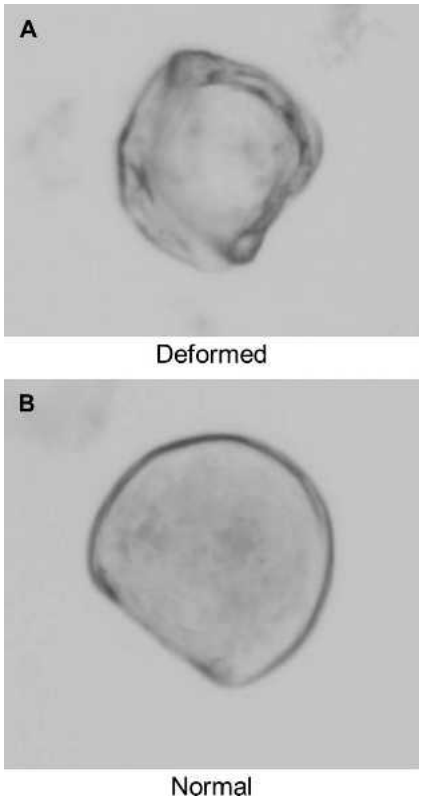

Figure 1. (A) Photomicrograph of larvae of the oyster *Crassostrea virginica* exposed immediately after spawning to the toxic dinoflagellate *Karlodinium veneficum* at 10^4 cells mL^{-1} for 48 h. (B) Normal D-hinge larvae of the same species not exposed to the toxic dinoflagellate.

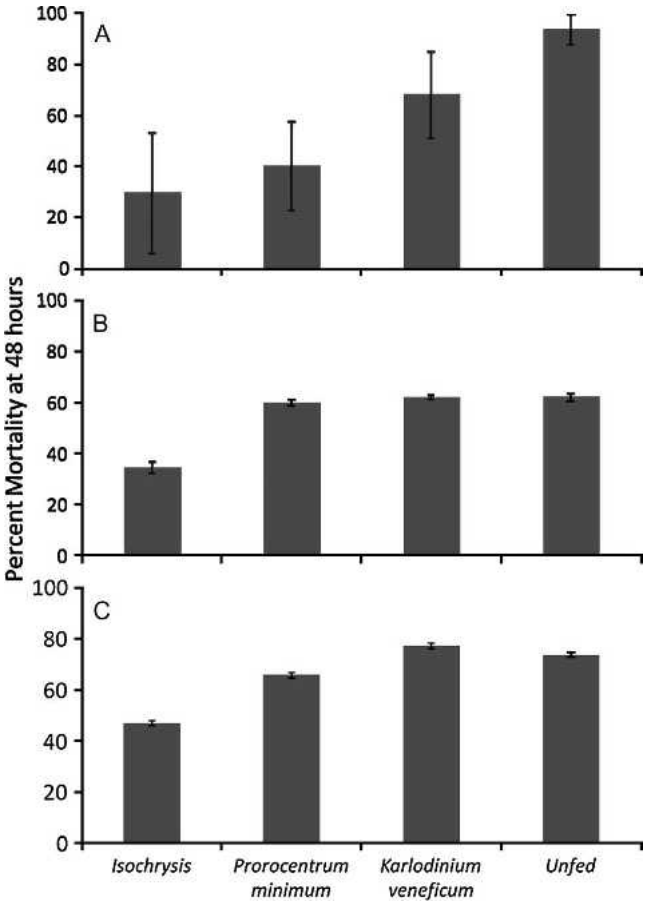

Figure 2. Percent mortality (mean ± 1 standard deviation) of oyster embryos/larvae exposed to varying algal treatments within hours of spawn (Experiment 1). All exposures were 48 h in duration. Panels A and B (trials 1 and 2) represent responses of *Crassostrea ariakensis*, and panel C (trial 2) represents *C. virginica*. Details of the experimental protocols are given in text.

(>60%), however, were observed in all unfed control treatments. The treatments with *P. minimum* had slightly, but not significantly (ANOVA, $P > 0.05$), higher mortality than the treatment with *Isochrysis* in the first trial (Fig. 2A). In the second trial, the mortalities of larvae exposed to *P. minimum* were significantly higher in both oyster species relative to the *Isochrysis* treatment ($P < 0.05$; Figs. 2B,C). Mortality with *K. veneficum* in all cases was always significantly higher than with the standard *Isochrysis* diet ($P < 0.05$), but was only higher than the mortality in the *P. minimum* treatments in trial 1, but not trial 2 (Fig. 2).

In the second experiment, in which 2 wk old larvae were exposed to varying algal treatments to assess effects on motility, differences between the responses of the two oyster species to the HAB species were noted. All larvae of both species exposed to *Isochrysis* sp. and also those that were unfed during the experiment demonstrated no change in percent motility. However, >60% of the *C. ariakensis* larvae exposed to *P. minimum* were nonmotile after 72 h, whereas the percent motility of the *C. virginica* larvae was comparable to the controls, although the swimming speed (not shown) was significantly slower than controls (Table 1). Both larval species were negatively affected by *K. veneficum*, with 100% of the larvae becoming nonmotile (Table 1).

DISCUSSION

The results herein, as well as those of additional related studies (Stoecker et al. in review) demonstrate that the early life stages are very susceptible to impacts of these HABs. These findings suggest that harmful algae, in particular *K. veneficum*, can be lethal to larvae of *C. virginica* and would also be lethal to larvae of *C. ariakensis* if this oyster species were introduced to the Bay. These experiments also demonstrated important behavioral changes in pediveliger larvae when exposed to *K. veneficum*, leading them to stop swimming and to sink. Even if such effects did not immediately result in mortality, any change in behavior would influence larval dispersal and probably would reduce feeding and growth and perhaps increase the susceptibility of larvae to predation. Nonmotile larvae that have not begun to attempt settlement are also likely to suffer from increased mortality sue to sinking and landing on unsuitable substrate. It is only through the ability to study these oysters in hatchery settings that these effects could be documented. Deformed or nonmotile larvae would be less likely to be observed or correctly identified in natural samples.

Spawning by *C. virginica* in Chesapeake Bay overlaps temporally with the period during which *K. veneficum* and *P. minimum* are most common. *Karlodinuum veneficum* occurs over a range of salinity (3-29) and temperature (7°C–28°C), but blooms are most common at salinities of 7-17 and

TABLE 1.

Percent nonmotile larvae of oyster species after 72 h of exposure to varying algal diets in 4 ml well plates as described in text. Values indicated are mean of triplicates, and ±1 SD is given in parentheses.

Oyster Species	*Isochrysis* sp.	Unfed Control	*Prorocentrum minimum*	*Karlodinium veneficum*
Crassostrea ariakensis	0 (0)	0 (0)	65.4 (12.8)	100 (0)
Crassostrea virginica	0 (0)	0 (0)	0 (0)	100 (0)

when surface water temperature is >13°C (Li et al. 2000). Bloom densities are commonly between 10^2 and 10^3 cells mL^{-1} during summer, but can reach densities of 10^4–10^6 cells mL^{-1} (Goshorn et al. 2004). Indeed, an assessment of long-term monitoring data ($n = 1311$ samples) from Chesapeake Bay from 1981 to 2001 revealed that highest average monthly densities were observed from April through September, >200 cells mL^{-1}, and were most common in the northern Bay (Goshorn et al. 2004). With natural spawning of *C. virginica* occurring mostly from mid-June through mid-September, depending on water temperature, these blooms and planktonic larvae overlap temporally in the Bay (Fig. 3).

Prorocentrum minimum grows over a very wide temperature (<5°C to 30°C) and salinity gradient, but blooms of >3000 cells mL^{-1} are typically found when temperatures are between 12°C to 22°C and salinity is 5-10 (Tango et al. 2005). In recent years, blooms reaching 1×10^5 cells mL^{-1} have been documented in several Chesapeake Bay tributaries (Fan et al. 2003, Tango et al. 2005). Based on the assessment of long-term monitoring data ($n = 902$ samples) for the Chesapeake Bay from 1985–2001, blooms, defined as $>3 \times 10^3$ cells mL^{-1}, were found to be most frequent during the months of April and May, but also remain fairly common throughout the summer months in the northern and middle reaches of the Bay (Tango et al. 2005).

To further assess the potential for temporal and spatial overlap of *K. veneficum* and oyster larvae, the results of a Bay-wide oyster transport model for *C. virginica* were compared for July, 1995, for which high resolution coverage is available for this alga. The oyster larvae transport model (North et al. in press) couples a high-resolution hydrodynamic model (Li et al. 2005) and a particle tracking model with subgrid scale turbulence routines (North et al. 2006). This model is designed to predict the transport of individual oyster larvae that are spawned from >2,700 simulated oyster reefs under differing physical conditions throughout Chesapeake Bay and its major tributaries.

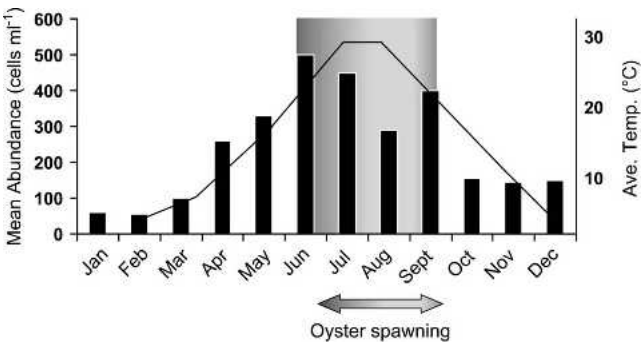

Figure 3. Temporal correspondence of the mean monthly density of *Karlodinium veneficum* in Chesapeake Bay [black bars, based on 1,311 samples collected between 1988 and 2001 (redrawn from Goshorn et al. 2004)], mean annual temperature (black line), and the period during which most oyster larvae are likely found in Chesapeake Bay (grey shading) based on larval stage durations and observed peaks in settlement (Kennedy 1996).

The model includes the best present-day estimate of oyster habitat (Greenhawk 2005) and algorithms that give particles "oyster-larvae-like" behaviors. Larval swimming behavior is an important factor that influences the direction and distance of larval transport (North et al. in press). The behaviors of the larvae in the model were constrained to observed swimming speeds (Mann & Rainer 1990, Kennedy 1996) and cued by salinity gradients that were deduced from laboratory experiments (Hidu & Haskin 1978, R. Newell unpub. data, J. Manuel unpub. data) and inferred from field studies (Andrews 1983, Mann & Rainer 1990, Kennedy 1996, Baker 2003). The current model does not estimate mortality other than lack of encounter with suitable substrate. Nevertheless, the model provides guidance for where and when the larvae could occur over a range of physical conditions (e.g., during periods of high, low and average freshwater flow).

In the model output shown here (Fig. 4A,B), particle release occurred after the day on which mean water temperatures reached 25°C in 1995 (the average mass spawning temperature for *C. virginica*; Shumway 1996). Based on model predictions from this average flow year, it can be seen that oyster-larvae-like particles could have been distributed throughout the Bay, with high concentrations in the northern reaches. Observational studies for this same time period have shown that significant *K. veneficum* concentrations occurred during July 1995, and overlapped in space with oyster-larvae-like particles in the northern Bay (Fig. 4C; Li et al. 2000). Whereas the concentrations from the 1995 field data were less than those used in the laboratory experiments reported here, they may be sufficiently high to cause increased mortality in nature, because toxin concentrations of *K. veneficum in situ* can be one to two orders of magnitude higher than in culture (Brownlee et al. in press, A. Place, unpub. data). Variability in toxin content may also have contributed to the different responses to *K. veneficum* in trials 1 and 2; one culture led to deformed larvae, whereas the other did not. Although the overlap of *K. veneficum* blooms with oyster larvae in time and space suggest that *K. veneficum* may adversely impact oyster larvae survival, the association has not yet been linked to mortality in the field. It is possible that *C. virginica* larvae could avoid high concentrations of *K. veneficum* by swimming vertically, thereby minimizing mortality and developmental disorders (although costs associated with poor feeding and slower growth could still be incurred).

The effects of *P. minimum* were more variable in both experiments than those of *K. veneficum*. *Prorocentrum minimum* is highly variable in its potential toxicity (Heil et al. 2005,

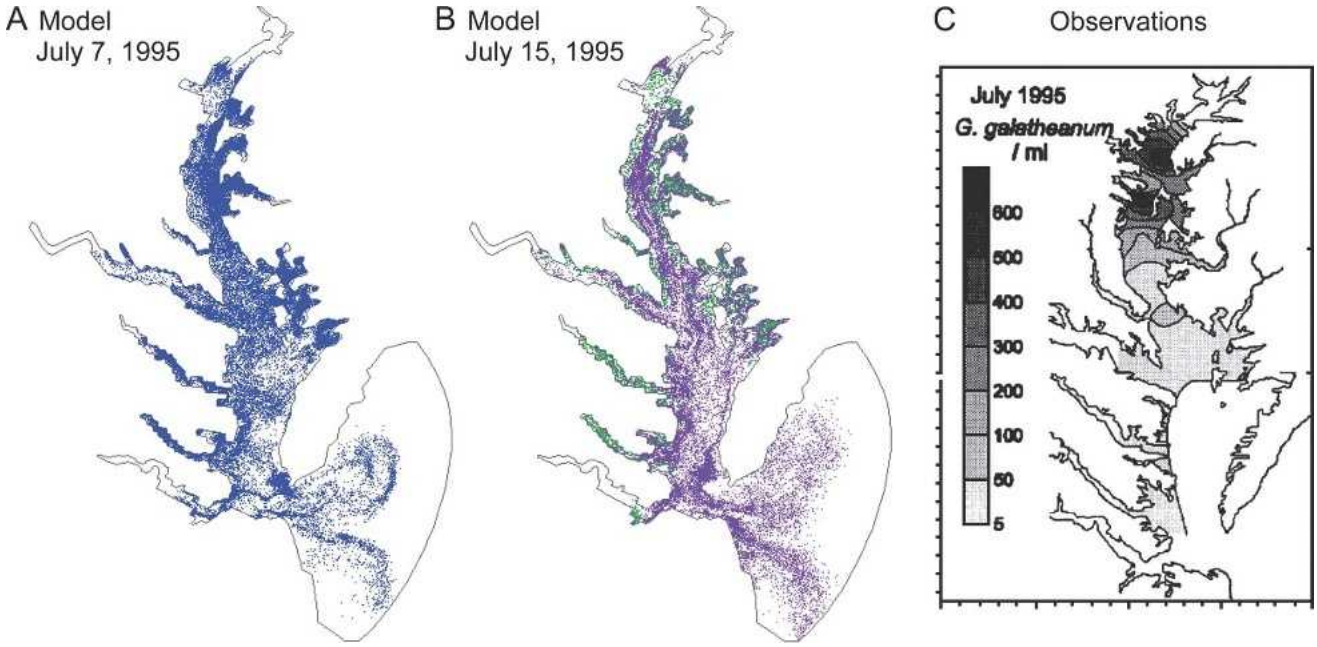

Figure 4. Comparison of oyster larval transport model output (panels A, B) and *K. veneficum* observation data (panel C) for 1995 in Chesapeake Bay. In the model output, particles started from 2,776 oysters reefs and spend ~2 wk in the water column as veliger larvae (indicated as blue symbols). If they become pediveligers (purple symbols) and encounter suitable habitat, they "settle" and stop moving (green symbols). Snapshots represent days 8 (panel A) and 16 (panel B) of a simulation that started on June 29, 1995. The observational data are from a cruise in July 1995 reported by Li et al. (2000). Note that *K. veneficum* was reported as *Gyrodinium galatheanum*. Panel C reproduced from Li et al. (2000) with permission of the publisher.

Wikfors 2005), and algal growth stage appears to be important in this regard. Interestingly, Brownlee et al. (2005) found that *P. minimum* blooms (10^4 cells mL^{-1}) from the Patuxent River, a tributary of the Chesapeake Bay, had positive effects on growth of eastern oyster spat in 12-day laboratory experiments. Wikfors and Smolowitz (1995), however, demonstrated that larvae had poorer survival and lower settling success with *P. minimum* in the diet, at concentrations as low as 3×10^3 cells mL^{-1}. In experiments involving juvenile *C. virginica* oysters, Luckenbach et al. (1993) demonstrated that 100% of oysters died in 14 days when fed a diet containing 100% *P. minimum* at a density of 1.6×10^3 cells mL^{-1}. Survival improved when the diet of *P. minimum* was reduced to 33% of natural bloom density and mixed with other phytoplankton. At 5% natural bloom density of *P. minimum*, all oysters survived. These findings, along with other laboratory experiments, were synthesized by Wikfors (2005), who speculated that these dinoflagellates are more toxic when in a stage of growth decline than those that are rapidly growing. In Australia, the related HAB species, *P. rhathymum* has been associated with mortalities of spat of the Japanese or Pacific oyster, *C. gigas* (Pearce et al. 2005).

These results underscore the need to consider the relationships between eutrophication and the restoration of *C. virginica*, or the establishment of *C. ariakensis*. These findings suggest that HABs may have been a factor in reduction in recruitment over the past several decades coincident with general declines in water quality. Increasing frequency and intensity of the eutrophication-related blooms of *K. veneficum* and other harmful algal species in Chesapeake Bay likely will impact our ability to achieve the ultimate goal of restoring or establishing vibrant, healthy oyster populations in some regions. Any reduction in natural recruitment either spatially or temporally from HABs further complicate the already complex challenges for establishing significant, self-sustaining oyster populations.

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