COMPETITION FOR ORGANIC RESOURCES: BACTERIA VERSUS *Aureococcus anophagefferens*

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Blooms of the brown tide pelagophyte, *Aureococcus anophagefferens*, occur seasonally along the eastern seaboard of the USA, where high concentrations of dissolved organic nitrogen (DON) relative to dissolved inorganic N (DIN) are thought to fuel their growth. High dissolved organic C (DOC)/DON ratios are also thought to contribute to bloom formation. In addition to providing N, dissolved organic matter (DOM) can also provide carbon to cells and previous work has illustrated that *A. anophagefferens* assimilates both N and C from amino acids and dipeptides. While this may be advantageous for out-competing other phytoplankters when cell densities are high (e.g., 10^6 cells/ml) and self-shading becomes significant, there are new competitive challenges since bacteria are thought to be the primary consumers of DOM in nature. During 2002 and 2003, we have experienced intense brown tide blooms in Chincoteague Bay, MD and VA (2003 only). *A. anophagefferens* cell densities exceeded 1 million cells/ml during these blooms. During the 2002 bloom, we observed that photosynthetically-mediated uptake of bicarbonate and organic C uptake during the daylight was insufficient to meet the cellular C demand based on N uptake (C:N uptake ratio of 0.87). Therefore, during 2003, we examined inorganic and organic C and N uptake over diel cycles to see whether nighttime uptake of organic C could significantly reduce this imbalance. We compare uptake in the two size fractions, in order to correct for the bacterial contribution to organic C uptake. Preliminary results suggest that during the day, bacteria are responsible for most of the organic C uptake while *A. anophagefferens* primarily takes up bicarbonate. By contrast, during the night, uptake of organic C by the *A. anophagefferens* size fraction is substantial. These results suggest that photosynthesis during the daylight and DOM uptake at night combine to meet the C demands of *A. anophagefferens* in nature.

TOXIC MODE OF ACTION OF KmTx 2, A NEW FISH-KILLING TOXIN FROM *Karlodinium micrum* (DINOPHYCEAE)

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Blooms of the estuarine dinoflagellate *Karlodinium micrum* have been associated with fish kills worldwide since the 1950's. However, in US mid-Atlantic states prior to the late 1990's, *K. micrum* was often misidentified as other similarly sized gymnodinoid dinoflagellates, such as *Gyrodinium estuariale* and *Pfiesteria* spp. For several years, our goal has been to establish the potential ichthyotoxicity of *K. micrum* in the United States.

To be presented will be a detailed description of the cytotoxic mode of action of KmTx 2, a newly described fish-killing toxin from *K. micrum*. KmTx 2 was first isolated during a fish kill in South Carolina, USA, and has subsequently been identified in US Atlantic coast isolates from North Carolina to Florida. A similar compound (KmTx 2-like) has recently been isolated from water samples collected during a fish kill in Western Australia associated with a large, persistent, bloom of *K. micrum*.

KmTx 2 is toxic to all mammalian cell types tested, including epithelial cells, neurons, fibroblasts, cardiac myocytes, and lymphocytes. Whole-cell voltage-clamp and single-cell microfluorimetry studies revealed that cytotoxicity occurs through permeabilization of the plasma membrane to cation fluxes, which results in osmotic cell lysis. This study also reveals mechanisms that underlie the historical association between *K. micrum* blooms and fish kills in the marine environment: KmTx 2 is lethal to zebrafish (*Danio rerio*) at environmentally relevant concentrations, while sub-lethal doses severely damage gill epithelia. In addition, KmTx 2 is toxic to representative fungal and dinoflagellate species, but is not toxic to *K. micrum* itself. Membrane sterol composition appears to be critical in determining both cellular susceptibility to KmTx 2 toxicity, and immunity of *K. micrum* from the membrane disrupting properties of its own toxins.

In US Atlantic coastal states, *K. micrum* co-occurs with, and has been confused with, the ichthyocidal dinoflagellate *Pfiesteria piscicida*, but has been reported as non-toxic under ecologically realistic conditions. This study, in conjunction with recent work from our laboratory, proves that *K. micrum* is far from being benign, and is in fact highly toxic. This work confirms the association between high densities of this organism and fish kills that have been observed worldwide for decades.

LONG-DISTANCE AND LOCAL-SCALE TRANSPORT OF *Aureococcus* BY SHIPS AND SMALL BOATS IN FRESH AND MARINE WATERS

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It is well established that cyst-forming phytoplankton species are transported in ships' ballast tanks. However, there is increasing evidence that other phytoplankton species, ones which do not encyst, are capable of surviving ballast transit. These species have alternative modes of nutrition (hetero- or mixotrophy) or are able to survive long-term darkness, or both. In our studies of NOBOB (No-Ballast-On-Board) vessels arriving to the Great Lakes, we tested for the harmful algal bloom (HAB) species *Aureococcus anophagefferens* (brown tide) in residual (i.e., unpumpable) ballast water using polymerase chain reaction based methods.

The brown tide organism was detected in 10 out of 20 residual water samples (50%) following transit from foreign ports. Not only this, it was detected after 10 days of ballast-tank confinement during a vessel transit in the Great Lakes (Port of Hamilton, Lake Ontario; Ports of Windsor and Detroit, Lake Erie; Port of Burns Harbor, Lake Michigan). This result is significant, given the large disparity between the salinity tolerance of *Aureococcus* (>22 ppt; Cosper et al., 1989) and the low salinity of the residual ballast water (approximately 2 ppt).

We also investigated the potential for smaller recreational vessels to transport and distribute *Aureococcus*. During the summer of 2002, 11 boats were sampled at boat ramps as they emerged from the Delaware Inland Bays and Maryland Coastal Bays. Brown tide was detected in the bilge water at the bottom of small trailered boats in 8 out of 10 cases, as well as the one live well sample we collected. Work is currently underway to quantitatively assess *Aureococcus* abundance and metabolic activity in these samples. However from our results to date, it appears that commercial ships and small recreational boats are significant vectors for long-distance transport and local-scale dispersal of *Aureococcus*.

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VIRUSES AS POTENTIAL REGULATORS OF REGIONAL BROWN TIDE BLOOMS CAUSED BY THE ALGA, *Aureococcus anophagefferens* AND THE ASSESSMENT OF BROWN TIDE BLOOMS AND RELATED ENVIRONMENTAL FACTORS IN COASTAL WATERS OF NEW JERSEY (2000-2002)

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The objective of the first study was to determine whether viral-like particles (VLPs) infected and lysed natural populations of *A. anophagefferens* in coastal bays of New Jersey and in New York in 2002 with the same frequency as in 1999-2000 and especially, at the termination of the bloom. The results indicated that intracellular VLPs continued to infect *A. anophagefferens* throughout the bloom event. Similar to previous years, the percentage of visibly infected cells was higher at the beginning of the bloom than during the height of the bloom. For the first time, our results confirmed that the highest percentage (60%) of VLP-infected cells occurred at the termination of the brown tide bloom in New Jersey in 2002. The intracellular VLPs in natural populations of *A. anophagefferens* were consistent in size and shape (approximately 140 nm in diameter) and comparable to those in previous studies. Concentrated viral isolates, prepared from waters during brown tide blooms in New York and New Jersey in 2002, infected healthy laboratory *A. anophagefferens* cultures in vitro. The viral isolates associated with the highest laboratory viral activity (lysis positive) were concentrated from water samples having the highest viral and bacteria concentrations. Moreover, the intracellular viruses in these virally infected laboratory cultures of *A. anophagefferens* were similar in size and shape to those found in natural populations. The successful isolation of an *A. anophagefferens* specific virus from a brown tide bloom in the field, the similarity of ultrastructure of VLPs infecting both natural populations and laboratory infected cultures, and the pattern of VLP infection during bloom activity in combination with the observed high percentage of VLP-infected cells during bloom termination, supports the hypothesis that viruses may be a major source of mortality for brown tide blooms in regional coastal bays of New Jersey and New York.

The second study was conducted in Barnegat Bay-Little Egg Harbor (BB/LEH), New Jersey (USA) (2000-2002) by the New Jersey Department of Environmental Protection, Division of Science Research and Technology (DSRT), in cooperation with several partners, to assess the spatial and temporal extent of *A. anophagefferens* blooms and associated environmental factors and analyze the potential risk of these algal blooms to submerged aquatic vegetation (SAV) communities. Water samples were collected by boat and helicopter at coastal stations from 2000-2002. *A. anophagefferens* were enumerated and associated environmental factors were measured. *A. anophagefferens* abundances were classified using the Brown Tide Bloom Index and mapped, along with sampled parameters, to their geo-referenced location using the ArcView GIS. To determine the possible risk that brown tide blooms pose to the BB/LEH submerged aquatic vegetation (SAV) communities, the GIS was used to determine the spatial coincidence between locations of high *A. anophagefferens* abundances or duration and the mapped location of seagrass habitat. The results indicated that the highest *A. anophagefferens* abundances ($>10^6$ cells ml^{-1}), including Category 3 blooms ($\geq$ 200,000 cells ml^{-1}) and Category 2 blooms ($\geq$ 35,000 to $\leq$ 200,000 cells ml^{-1}), recurred during each of the three years of sampling and covered significant geographic areas of the estuary, especially in

Little Egg Harbor. While Category 3 blooms were generally associated with warmer water temperatures (> 16^{o}C) and higher salinity (> 25-26 ppt), these factors were not sufficient alone to explain the timing or distribution of *A. anophagefferens* blooms. There was no significant relationship between brown tide abundances and dissolved organic nitrogen measured in 2002 but this was consistent with other studies. However, there was a significant difference in Secchi disk depth between the three bloom categories. Extended drought conditions, with corresponding low freshwater inputs and elevated bay water salinities, occurring during this time were conducive to blooms. *A. anophagefferens* abundances were well above the reported threshold levels that have been reported for negative impacts on shellfish. For the first time, it was shown that 35% of the SAV habitat located in Barnegat Bay/Little Egg Harbor was categorized as having a high frequency of Category 2 or 3 blooms for all three years.

CULTIVATION AND CHARACTERIZATION OF AMOEBOID PROTISTS IMPLICATED IN THE PUTATIVE LIFE CYCLE OF *Pfiesteria piscicida* AND RELATED DINOFLAGELLATES

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This study was undertaken to assess whether or not amoebae, common in mesohaline environments, are stages in the life cycles of *Pfiesteria piscicida*, and *Pfiesteria*-like dinoflagellates. Primary isolations were made from water and sediment samples from five tributaries of the Chesapeake Bay. Enrichment cultivation methods used for the isolation of dinoflagellates and amoebae were performed in parallel. Recovered protists that were morphologically indistinguishable from putative life cycle stages of *Pfiesteria piscicida*, and *Pfiesteria*-like dinoflagellates were studied in xenic and clonal cultivation systems. Cultures of amoebae cloned from laboratory aquaria where fish mortality had been attributed to *P. piscicida* and *Pfiesteria*-like dinoflagellates were determined to belong to the genera *Korotnevella* and *Vannella*. Species of these genera were also recovered from environmental samples with *Pfiesteria*-like dinoflagellates, as were species of at least six other genera of gymnamoebae. Morphologically, all could be confused with putative life cycle stages of *P. piscicida* or *Pfiesteria*-like dinoflagellates. Based upon environmental sampling, cultivation methodologies, long-term observations of established cultures and light and electron microscopy, ubiquitous gymnamoebae of mesohaline environments are not related to *P. piscicida* or *Pfiesteria*-like dinoflagellates that co-occur within the natural environment. The phylogenetic analyses of the SSU rRNA gene data corroborate our conclusions based upon morphological data using key informative features. The SSU rRNA gene has now been sequenced from representatives of many of the major genera of mesohaline gymnamoebae and it is clear that these amoebae are not remotely related to Pp/PLD or other dinoflagellates (Amaral-Zettler et al., 2000; Peglar et al., 2003). Based upon our combined morphological and gene analyses, the assertion that many mesohaline amoebae are stages in the life cycle of dinoflagellates is clearly erroneous. This conclusion is further supported by other work involving laboratory aquaria, where all putative forms of *Pfiesteria* are present during fish-kills, but the amoebae are unrelated to the dinoflagellates (Litaker et al., 2002). Hence we find no support for the ambush predator hypothesis as a model explaining fish death in the natural environment.

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A SURVEY OF PELAGOPHYTE STEROLS AND A STEROL PALEOCHRONOLOGY OF THE BROWN TIDE ALGA *Aureococcus anophagefferens* IN LONG ISLAND WATERS

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The rare marine sterol 24-propylidenecholesterol is produced by algae of the class Pelagophyceae. While the E-isomer of this sterol has been found in other algae in this class, the Z-isomer was considered specific to *Aureococcus anophagefferens*. We analyzed the sterols of 44 algae representing all strains of *Aureococcus* and all of the Pelagophyte algae that are cultured at Bigelow Lab (CCMP). The chemotaxonomic categories compare well with rRNA based taxonomic analysis. The results show that all strains of *Aureococcus* contain both the E- and Z-isomers in ratios ranging between 1.3 and 4.2 with an average of 3.2. Seawater samples taken during a brown tide bloom on Great South Bay contained large amounts of 24-propylidenecholesterol as a 4:1 mixture of the E- and Z-isomers. Analysis of surface sediments from the same location showed a 2:1 ratio of the isomers. A dated sediment core was obtained from Peconic Bay and was submitted to sterol analysis. A 2:1 ratio of the E and Z isomers of 24-propylidenecholesterol was clearly evident in the top six 2 cm sections of the core, representing the past 120 yrs. These data strongly suggest that *Aureococcus* has been present in Long Island waters for at least a century.

DIRECT UPTAKE OF INORGANIC AND ORGANIC NITROGEN BY *Pfiesteria piscicida AND P. shumwayae*

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Although *Pfiesteria piscicida* and *P. shumwayae* are heterotrophic dinoflagellates, and obtain the bulk of their nutrition via grazing, they can take up nutrients directly. The capacity for nitrogen uptake, nitrogen nutritional preferences, and the extent to which these vary with genus and physiological state, has been the subject of debate, and are important in modeling nitrogen flow and population dynamics. Our objective in this study was to compare the rates and kinetics of uptake of different forms of nitrogen, both inorganic and organic, by both different strains of *P. piscicida* and *P. shumwayae* and cells in different physiological states. All strains under all growth conditions were capable of direct uptake of nitrogen.

Of the nitrogenous substrates investigated (nitrate, ammonium, urea, glycine, and glutamic acid), the highest absolute uptake rates for all cultures were found for ammonium and glutamic acid. Relative to ambient nitrogen availability, all forms of organic nitrogen (urea and the amino acids) were consistently preferred over inorganic nitrogen forms both at near ambient levels and at levels sufficient for uptake saturation. When different cultures were compared, these preferences were most pronounced for both *P. piscicida* and *P. shumwayae* recently removed from active fish killing conditions compared to those that had been maintained on algal food long-term.

The kinetics of uptake differed by substrate, but general patterns were consistent across cultures. The lowest half saturation concentrations (e.g. highest affinity) for uptake were found for glycine and glutamic acid. Uptake of urea did not appear to saturate over the concentration range measured (up to 25 mg at N l^{-1}) and uptake kinetics for this substrate were generally linear rather than hyperbolic.

In regions where *Pfiesteria* spp. are found, such as upper estuaries and aquaculture systems, nutrient enrichment – including organic enrichment - is common. For example, in several of the tributaries of Chesapeake Bay where these species are commonly found, urea concentrations can exceed 10 mg at N l^{-1}. Such high levels result from agricultural runoff of fertilizers and manures. The high uptake rates of, and preferences for, organic forms of nitrogen documented in this study suggest that direct uptake of nitrogen by *Pfiesteria* spp. in nutrient enriched waters may be more significant than previously thought.

IMPACTS OF VIRUSES ISOLATED FROM NEW YORK WATERS ON GROWTH OF THE BROWN TIDE ALGA, *Aureococcus anophagefferens*: A FIELD AND LABORATORY ASSESSMENT

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Viruses may be an important source of mortality for brown tides caused by the picoplanktonic pelagophyte *Aureococcus anophagefferens*, particularly since other biological mortality mechanisms such as benthic and pelagic grazing are disrupted during intense blooms. During 2002, we used cross flow filtration techniques to concentrate and isolate high molecular weight (HMW: 30 kDa – 0.2 μm) material from New York estuaries which commonly host blooms. Since concentrated HMW material contains elevated densities of viruses, this material was used to conduct field-based viral enrichment experiments and to isolate *A. anophagefferens*-specific lytic viruses. During 48 hr bottled, field experiments, we found that enriching background levels of viruses episodically enhanced net growth rates of *A. anophagefferens* compared to control treatments. This result suggests that activity within total viral community (bacteriophage, cyanophage, algal viruses) may indirectly enhance brown tide growth during blooms by regenerating dissolved organic matter or by altering the composition of microbial assemblages. Screening of HMW viral concentrates isolated from two estuaries during 2002 indicated that viruses able to lyse *A. anophagefferens* cultures were present on nine dates within both bays, only one of which hosted a brown tide. Results from pulsed field gel electrophoresis (PFGE) used to compare the viroplankton communities collected from these sites before and after serial passage through axenic *A. anophagefferens* cultures will be presented. The nine viral isolates have been serially propagated through laboratory cultures of *A. anophagefferens* weekly since the summer of 2002. Ultrastructural analysis of *A. anophagefferens* cultures infected with isolated viruses revealed the presence of intracellular viral capsids similar to those found previously in field populations. The viral isolates are chloroform sensitive, and hence likely have lipid associated with their protein coat. The viruses also appear to be species specific, as they do not lyse 10 other classes of algae and are only able to lyse *A. anophagefferens* within the pelagophyte class (three other pelagophytes screened including the Texas brown tide, *Aureoumbra lagunensis*). Isolated viruses are able to lyse clonal cultures of brown tide originating from multiple NY and NJ estuaries, as well as axenic cultures of *A. anophagefferens*. The ability of propagated viruses to lyse only a portion of clonal brown tide cultures isolated from the same date and location (Great South Bay, NY; 6 May 1998) suggests that there may be a greater degree of clonal diversity within brown tide field populations than has previously been hypothesized. The experimental additions of laboratory propagated viruses to NY bloom waters demonstrated that some, but not all, of the viral isolates were capable of significantly reducing the abundance of *A. anophagefferens* during five-day, field bottle experiments. This result suggests lab propagated, *A. anophagefferens*-specific viruses may be a viable biological control agent for brown tides. Finally, preliminary experiments indicate that viral lysis of *A. anophagefferens* is delayed in the dark, suggesting these viruses may partly depend on host photosynthetic pathways to replicate. If confirmed, this finding would suggest that the low light conditions which prevail during peak abundances of dense brown tide blooms may reduce the ability of viruses to replicate and cause mortality in *A. anophagefferens*.

RELATIVE CONTRIBUTION OF TOXIN AND MICROPREDATION TO ICTHYOTOXICITY OF TWO STRAINS OF *Pfiesteria shumwayae*

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Controversy exists regarding the mechanisms by which *Pfiesteria shumwayae* kills fish. Several studies have implicated a *Pfiesteria*-associated exotoxin in fish mortality while other studies indicate that direct attack of dinoflagellates on fish (micropredation) and not exotoxin is responsible. Many microorganisms, including dinoflagellates, exhibit variability in toxin expression and/or composition among strains of the same species. Accordingly, we examined the ichthyotoxicity of two strains of *P. shumwayae* (CAAE 101272 and CCMP 2089) in a bioassay system that was designed to expose test fish to *P. shumwayae* cultures both with and without direct contact between fish and dinoflagellate cells. Cell free supernatants from both strains were also tested for toxicity. CAAE 101272 has previously been associated with toxin production while CCMP 2089 has been reported to kill by micropredation alone.

Juvenile tilapia (*O. nilauticus*) were exposed to cultures of each *P. shumwayae* strain in 10 liter aquaria. Controls consisted of tanks that were not inoculated with *Pfiesteria* cultures. In each experiment four fish were placed directly into test tanks allowing direct contact between the dinoflagellates and the fish. Four additional fish were placed into aerated containers constructed from tissue culture flasks and polycarbonate membrane (3 μm). These containers were placed into the same tank as the non-contained fish. Fish were exposed for 48 h. Dead fish were tallied after 24 and 48h. Dinoflagellates were counted inside and outside of the containers and water quality parameters were monitored. In separate experiments cultures from highly toxic aquaria (killing fish in ca. 4 hr) were centrifuged (9,635xg 20 min) and fish were exposed to the resultant dinoflagellate-free supernatants.

The results indicate that direct contact between *P. shumwayae* and fish generally enhances fish mortality with both strains. Time to death was quicker and percent of fish killed was higher outside of the containers except in two of 22 trails. Mortality of fish exposed to CAAE 101272 cultures but protected from direct contact with *Pfiesteria* cells ranged from 0 to 100% of exposed fish in individual trials. In contrast only one contained fish (of 36) exposed to CCMP 2089 cultures died. No deaths were observed in control tanks inside or outside of the containers. Supernatants obtained from cultures in highly toxic condition (killing all fish in ca. 4 hr.) killed fish when obtained from cultures of CAAE 101272 but not when obtained from CCMP 2089.

These results suggest that production of *Pfiesteria*-associated toxin varies among strains of *P. shumwayae* and with time in cultures of toxin-producing strains. The toxin(s) can kill fish when present at sufficiently high concentration but dissolved toxin production is apparently not required for fish mortality. The dominant and most consistent mechanism of fish mortality in these cultures required direct contact between fish and *Pfiesteria* cells in both of the strains we examined.

NUTRIENT EFFECTS ON *Pfiesteria* SPP. GROWTH RATES

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Nutrient enrichment associated with cultural eutrophication is believed to be a primary factor in regulating environmental abundances of *Pfiesteria* spp. Laboratory experiments which quantify the effect of nutrient enrichment on *Pfiesteria* spp. growth rates are complicated by a.) nutrient utilization and subsequent growth stimulation of algal prey, which in turn support enhanced *Pfiesteria* spp. growth and b.) alteration of nutrient utilization and subsequent growth as a result of kleptoplastidy by *Pfiesteria* spp. Utilizing a procedure in which algal prey are incapable of nutrient utilization and growth, we investigated the direct growth response of *Pfiesteria* spp. to enrichment with both inorganic and organic nutrients. The effect of kleptoplastidy on the growth response to various nutrient enrichments by *Pfiesteria* spp. was also evaluated. The results of this study, which may have significant implications for observations of *Pfiesteria* spp. abundance in the natural environment, are reported.

ECOLOGY OF *Pfiesteria* spp. AND RAPHIDOPHYTE BLOOMS IN SOUTH CAROLINA

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Initial HAB research efforts in South Carolina targeted tidal creeks and open estuaries. Overall, *Pfiesteria piscicida* and *P. shumwayae* were concluded to be widespread in these systems, but never observed in high (potentially toxic) abundances. From more recent efforts (after spring 2001) targeting brackish stormwater detention ponds, we now know that "blooms" of *Pfiesteria* spp. can occur in these lagoonal systems, in some cases associated with fish kills. Also, positive results from fish mortality bioassays have been demonstrated. Based on real-time PCR, *P. piscicida* is prevalent in the sediments of these ponds (~60% of ponds sampled and 47% of samples taken from adjoining tidal creeks). Assuming that these are cysts or amoebae, these results have implications for *Pfiesteria* dispersion through exchange with adjacent tidal creeks and sediment dredging.

Brackish pond sampling has also revealed the near-ubiquitous occurrence of raphidophyte blooms by four species, *Heterosigma akashiwo, Chattonella subsalsa, Fibrocapsa japonica,* and *C. verruculosa.* Axenic cultures of local isolates of the former three species have been obtained. Through distributional associations with environmental conditions and bioassays with field and culture material, we are testing the following attributes of raphidophytes as explanation for the prevalence of blooms in brackish lagoonal ponds: a high mixotrophic ability, resistance to herbicides, high metal (e.g. Fe) uptake capabilities, and antipredator strategies. In addition, we present documentation of an anomalous April 2003 *H. akashiwo* bloom that extended from Bulls Bay estuary to 5 miles offshore (Fig. 1), and was associated with a fish kill and physiological stressful effects on shellfish. We present data on *Pfiesteria* and raphidophyte distribution and association with environmental variables that suggest that these numerous hypereutrophic, low-flow lagoons are favorable environments for proliferation of these HABs.

Fig. 1. *Heterosigma akashiwo* bloom in nearshore waters just outside of Bulls Bay (29 April 2003).

STRONG DEPENDENCE OF *Pfiesteria piscicida* GROWTH ON THE ABUNDANCE OF PREY

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Growth dependence of the heterotrophic dinoflagellate *Pfiesteria piscicida* on its prey was investigated using batch and cyclostat cultures and the cryptomonad *Rhodomonas* sp. as prey. Extremely rapid grazing was observed in video recordings. Predator-prey dynamics were investigated using batch cultures and various feeding schemes, ranging from adding a small amount of the prey alga *Rhodomonas* sp. to a large population of *P. piscicida* to adding a small amount of *P. piscicida* to dense *Rhodomonas* sp. cultures. In most cases, *Rhodomonas* sp. was depleted within a day, and this was accompanied by an increase in *P. piscicida*. The magnitude of *P. piscicida* increase was dependent on the initial prey concentration and prey/grazer. Only in cases where few *P. piscicida* were added to *Rhodomonas* sp. cultures, i.e. under very high initial prey/predator ratio (50:1 or higher), was *Rhodomonas* depleted more slowly (up to 5 days). On the contrary, *P. piscicida* populations did not increase when the initial *Rhodomonas* to *Pfiesteria* ratio was very low (<1). In a 2-stage cyclostat with hourly supply of prey, growth of *P. piscicida* increased with grazing rate. Grazing by *P. piscicida* caused *Rhodomonas* to decline exponentially in both the batch and the cyclostat cultures. The dependence of *P. piscicida* growth on *Rhodomonas* sp. was further verified with cell size measurements and flow cytometric analysis of the cell cycle. In the starved culture, cell size and S-phase cells decreased markedly, with most *P. piscicida* cells apparently arrested at the G1 and the G2 phases. Re-supply of *Rhodomonas* sp. released the cell cycle arrest of *P. piscicida* and led to a rapid increase in the number of S-phase cells as well as cell size. Regression analysis revealed a significant positive correlation between *P. piscicida* yield and *Rhodomonas* concentration. The maximum and the 5-day-averaged growth rates also exhibited a Michaelis-Menton functional response to *Rhodomonas* sp. concentration and prey/predator ratio. Our results demonstrate that *P. piscicida* is a voracious grazer with its population being regulated directly by the availability of prey algae, and thus should be considered an obligate heterotroph.

MICROPREDATORY BEHAVIOR AND PATHOGENICITY IN *Pfiesteria*-LIKE DINOFLAGELLATES

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Pfiesteria piscicida and *Pfiesteria shumwayae*, members of the "toxic *Pfiesteria* complex" (TPC), are reported to secrete potent exotoxins in response to the presence of fish or fish excreta. These toxins are believed to be responsible for fish pathology and mortality as well as human illness. Recently, however, *P. shumwayae* was demonstrated to cause fish mortality through the process of micropredatory feeding upon the epidermal tissues of fish rather than through the action of a toxin. Other morphologically similar dinoflagellate species frequently occur in the same environment as *Pfiesteria* spp. Many of these organisms are heterotrophic and, like *Pfiesteria* spp., feed upon algal prey through a peduncle that attaches to the prey cell and extracts the cell contents into a food vacuole. We hypothesize that these other heterotrophic, penduncle-feeding *Pfiesteria*-like dinoflagellates may also have the ability to cause pathology to fish in a manner similar to what has been demonstrated for *P. shumwaye*. Using a larval fish bioassay we tested the ability of several species of heterotrophic dinoflagellates to cause pathology and mortality in larval cyprinodontid fishes. Additionally, we used membrane insert studies to evaluate the effects of direct vs. indirect contact. Results indicate that all species tested exhibited some degree of micropredatory attack behavior and several species caused fish pathology or mortality. None of the species tested caused pathology or mortality when not in direct contact with fish. The results of these studies indicate that, under controlled laboratory conditions, *P. shumwayae* is not unique in its ability to cause fish pathology or mortality as a result of micropredatory feeding upon fish epidermal tissues.

MEDIATION OF BENTHIC-PELAGIC COUPLING BY MICROPHYTOBENTHOS: AN ENERGY- AND MATERIAL-BASED MODEL FOR INITIATION OF BLOOMS OF *Aureococcus anophagefferens*

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Prior research has demonstrated that growth of the brown Tide pelagophyte, *Aureococcus anophagefferens,* is stimulated by organic nutrients. However, even under nutrient-replete conditions, it also has a constitutively low maximum growth rate and growth becomes light-saturated at a low irradiance when compared to other estuarine species. This is so when grown with either nitrate or urea as the sole nitrogen source. It is therefore unlikely to out-compete the other species, except at low light, regardless of the availability of organic nutrients. Further, its high Chl*a*-specific absorption cross-section, large PSU size and very low ratio of photoprotective to photosynthetic carotenoids make it susceptible to photoinhibition. We argue that it is most likely to bloom in shallow waters when high turbidity provides a refuge from sustained exposure to bright light.

The light environment in many shallow bays is dominated by the dynamics of benthic resuspension. This is mediated by the presence of microphytobenthos (MPB), which stabilize the sediment matrix and resist transfer of material into the overlying water. The MPB also modulate benthic fluxes of nutrients and are capable of altering both the magnitude and the direction of flux. Photosynthesis and growth of the MPB is frequently correlated with the intensity of light penetration to the sediment surface. We present a model of bloom initiation in response to benthic coupling that is based on these three observations. The exchange is modulated by the MPB through one of two positive feedback cycles.

1) In one cycle, a dense assemblage of MPB reduces sediment-water exchange, maintaining high water clarity and low nutrient efflux. Increased water clarity sustains high growth rates of the MPB, reinforcing the condition. The high water clarity and low nutrient levels do not favor growth of *Aureococcus*. This condition will prevail only if shear stress at the sediment surface is relatively low.

2) In the other cycle, an initial resuspension event reduces water clarity. The consequent reduction in growth rate of the MPB causes a reduction in their density as grazing continues. This results in a reduction in their ability to stabilize the sediment matrix, leading to continued resuspension and a further reduction in water clarity. The increased turbidity and release of organic material as the MPB are grazed create the conditions that favor growth of *Aureococcus.* This condition will occur only if shear stress at the sediment surface is high enough to erode the initial population.

We present a suite of data in support of the model: the physiological responses of cells in culture; observations of the dynamics of resuspension under controlled conditions in a microcosm; and field observations from Quantuck and Peconic Bays, Long Island, NY. Our field data were collected before and after a moderate-sized bloom and in a non-bloom year. A comparison of bi-weekly cell counts collected from Quantuck Bay during the bloom by the Sussex County Department of Health Services with National Weather Service wind data recorded at an adjacent site indicate that the bloom occurred after a 2-day period of prolonged wind stress. This is the condition in which the transition from a low to high probability of bloom development is predicted by the model (i.e. erosion of high MPB density by increased shear stress).

ISOLATION AND STRUCTURAL INFORMATION ON A WATER SOLUBLE TOXIN DERIVED FROM *Pfiesteria piscicida*

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The structure and function of marine biotoxins are inextricably linked. To measure, characterize or chemically modify the activity of a particular toxin, its molecular structure must be determined unambiguously. Bioassay guided extraction and fractionation schemes have yielded discrete water soluble toxic fractions from *Pfiesteria piscicida*. Small sample sizes, molecular degradation processes and a consistent loss of activity over the time period required for purification and structural elucidation have required the use of innovative chromatographic methods coupled to large scale culturing techniques in our efforts to complete structural analysis as quickly as possible. We have been able, using rapid novel purification methods, to provide partial MS and NMR data on active fractions prior to molecular degradation.

Though obtained on microgram quantities from purified fractions, the data obtained to date has provided interesting structural information, providing clues to the identity of functional groups associated with the toxin(s). Identification of these functional groups in turn provides us opportunities to chemically stabilize the toxin(s) allowing the design of preparative scale isolation and purification schemes. Up to date 13C, 1H NMR as well as MS data are reported.

OBSERVATIONS ON THE SEXUAL LIFE CYCLES OF *Pfiesteria piscicida* AND CRYPTOPERIDINIOPSOIDS (DINOPHYCEAE)

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The occurrence of meiosis is fundamentally important in defining the life history of organisms with a sexual cycle. Video photography and photographic series were used to document sexual life cycle events in *Pfiesteria piscicida* as well as closely related cryptoperidiniopsoid dinoflagellates. Individual pairs of fusing gametes were isolated, and their development was followed in single-drop microcultures with cryptomonad prey. The observed patterns of zygote development and postzygotic divisions were similar in these related taxa. Isolated, motile gamete pairs each typically fused within 30 minutes to produce a rapidly swimming planozygote with a typical dinoflagellate shape and two trailing flagella. Planozygotes grew in size as they fed repeatedly on cryptomonads. In < 12 hours in most cases, each planozygote formed a transparent-walled nonmotile cell (cyst) with a single nucleus. The prominent chromosome movements of nuclear cyclosis occurred in the nucleus of the mature zygote. Nuclear cyclosis is believed to coincide with the homologous chromosome pairing of meiotic prophase in dinoflagellate zygotes, prior to meiotic division. In *P. piscicida* it was determined that nuclear cyclosis occurred in either the mature planozygote or in the zygotic cyst formed by it. A single cell division occurred in the *P. piscicida* zygotic cyst, and two offspring were produced that emerged from the cyst as biflagellated cells. In the cryptoperidiniopsoids, nuclear cyclosis occurred in the zygotic cyst formed by the planozygote. Following nuclear cyclosis, a single cell division occurred and two biflagellated offspring were produced. In both taxa, the two flagellated offspring formed by division of the zygote were motile and fed on cryptomonads before each forming a cyst. A single cell division in these cysts produced two biflagellated offspring that also fed before encysting for further reproduction. This pattern of zygote development and postzygotic divisions was confirmed in examples (n > 9) from different isolates of each taxon. Thus it was determined that in both *P. piscicida* and cryptoperidiniopsoids: 1) planozygotes were phagotrophic before encysting for cell division; 2) no lengthy dormancy occurred in zygotic cysts under these conditions; 3) nuclear cyclosis occurred in the zygote nucleus prior to nuclear division; and 4) the two divisions that followed zygote formation were spatially and temporally uncoordinated, unlike the pair of consecutive divisions that occur in the conventional pattern of meiosis. These results are discussed and compared to the sexual cycles of other dinoflagellates. Meiosis has only rarely been demonstrated in dinoflagellates, and has not yet been proven in *P. piscicida* or cryptoperidiniopsoids. The methods and results of this study provide new information toward clarifying the sexual cycle, including meiosis, in these dinoflagellates. Recent discrepancies in the literature regarding the sexual and asexual reproductive cycles in *P. piscicida* are also addressed.

HARMFUL ALGAL TOXINS AND STEROLS: NOT SO STRANGE BEDFELLOWS?

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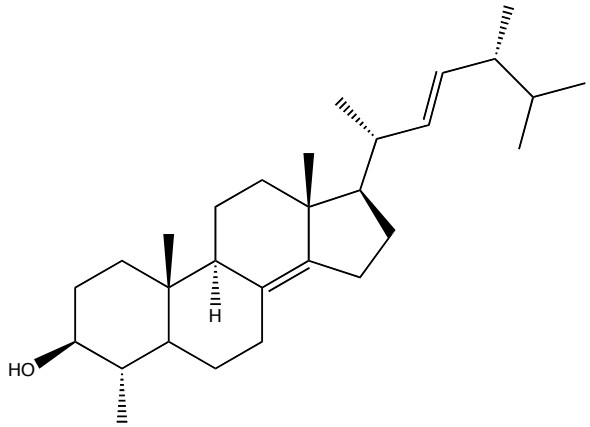
Recently, the unique sterols of *Karenia brevis* (Davis) Hansen & Moestrup were characterized by Giner et al., (J. Phycol. 39, 315-319, 2003) with the two predominant sterols being (24*R*)-4a-methy-5a-ergosta-8(14),22-dienol and (24*R*)-4a-methy-27-nor-5a-ergosta-8(14),22-dienol, called **gymnodinosterol** and **brevesterol**, respectively. These same two sterols were shown by Leblond and Chapman (J. Phycol., 38, 670-682, 2002) to also be the dominant sterols in *Karenia mikimotoi* and *Karlodinium micrum.*



Gymnodinosterol
(24S)-4α-methyl-5α-ergosta-8(14), 22-diene-3β-ol

Each of these groups produce polyketide toxins (brevetoxins, gymnocins, and a newly descried suite of toxins putatively called karlotoxins) which function by insertion into biological membranes. Our recent work on the mode of action of karlotoxins has found that sterol type effects toxin activity, with cholesterol being the most effective. Purified **gymnodinosterol** from *K. micrum* appears to be least effective in effecting karlotoxin activity. This finding is consistent with our observation that karlotoxins have little effect on *K. micrum* yet cause death or growth retardation in cells with cholesterol and ergosterol.

Two of the unique structural characters of these sterols are the 4-a methyl group and the 8,14 double bond in the C ring. These same characters are found in the major sterol, 4a-methyl-24-methylene-cholesta-8(14)-3b-ol, (amphisterol) of all species tested of the dinoflagellate genus *Amphidinium,* including *A. klebsii* which is known to produce a series of polyhydroxy-polyene compounds named **amphidinols**. Amphidinols exhibit a variety of biological actions such as antifungal, hemolytic, cytotoxic and icthyotoxic activites, identical to the activities we have found with karlotoxins. Moreover, this bioactivity is enhanced when cholesterol is present in the membrane.

Giner et al., (J. Phycol. 39, 315-319, 2003) proposed a possible function for these unusual sterols whereby the structural modifications render the sterols non-nutritious to marine invertebrates, reducing predation and thereby enhancing the ability of the dinoflagellates to form blooms. We would like to suggest one additional function. **The presence of these sterols in the dinoflagellate renders them impervious to the membrane disrupting effects of their own toxins.**

We will present our findings on toxin binding to artificial surfaces containing different sterols using surface plasmon resonance as a direct measure of interaction.

MESOZOOPLANKTON GRAZING OF MICROZOOPLANKTON: THE AFFECT OF FOOD WEB DYNAMICS ON THE POTENTIAL FOR HARMFUL ALGAL BLOOM FORMATION

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The impact of mesozooplankton grazing on protozoan microplankton and the subsequent impact on the phytoplankton biomass was measured in laboratory experiments. The calaniod copepod *Acartia* spp. was fed natural assemblages of microzooplankton collected from the Patuxent River, a tributary of the Chesapeake Bay, during mid and late April of 2002 and 2003. The percentage of ciliates lost to total *Acartia* spp. community grazing per day (g) was much greater in 2003 (43.5 and 26.2%) than in 2002 (0.69 and 1.7%). This corresponded with preliminary data showing an increased heterotrophic dinoflagellate concentration in April of 2003 above that of 2002, which suggests top-down control of ciliates as a factor influencing heterotrophic dinoflagellate biomass. *Acartia* spp. abundance was 1 to 2 fold greater in April of 2003 (2.3 - 2.7 L^{-1}) than in 2002 (0.04 – 0.15 L^{-1}). However, the differences in g were not a function merely of increased copepod abundance as the percentage of *Acartia* spp. body carbon ingested, as a function of ciliate biomass, was slightly greater during 2003. These results suggest that top-down control of microzooplankton communities (specifically herbivorous ciliates), resulting from increased copepod abundances and greater grazing rates, may be an important factor affecting the formation of algal blooms in the Chesapeake Bay.

PROTEOLYTIC ACTIVITY DURING DINOFLAGELLATE BLOOMS IN THE CHOPTANK RIVER, MARYLAND (U.S.A.)

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The leucine aminopeptidase (LAP) assay is a measure of extracellular exopeptidase activity. We measured the LAP activity using the artificial substrate L-leucine **7**-amido-4-methyl-coumarin (Leu-AMC) during dinoflagellate blooms in spring 2003 in the Choptank River, a tributary of the Chesapeake Bay. In March and April the blooms were dominated by *Heterocapsa rotundatum* which reached high densities ($88x10^3$ cells ml^{-1} in March and $67x10^3$ cells ml^{-1} in April). *Prorocentrum minimum* and *Karlodinium micrum* were dominant during the blooms that occurred in May.

LAP activity ranged from 1.9 to 7.5 mmoles AMC l^{-1} h^{-1} with 2 to 34% of the total activity associated with the >2 mm fraction. The percentage of LAP activity associated with the >2 mm fraction was positively correlated with dinoflagellate concentration (Fig.1), but we did not find any significant correlation between the density of dinoflagellates and the activity in the <2 mm fraction. LAP activity and ammonium concentration were inversely correlated.

LAP activity in seawater is usually ascribed to bacterial activity, but, during blooms, dinoflagellates could be an important source of proteolytic activity. Leucine aminopeptidase degrades polypeptides, releasing amino acids which may be directly taken up by dinoflagellates or oxidized to ammonium which can be assimilated. This proteolytic activity may play a role in nutrition of mixotrophic dinoflagellates and enhance carbon flow through the microbial loop.

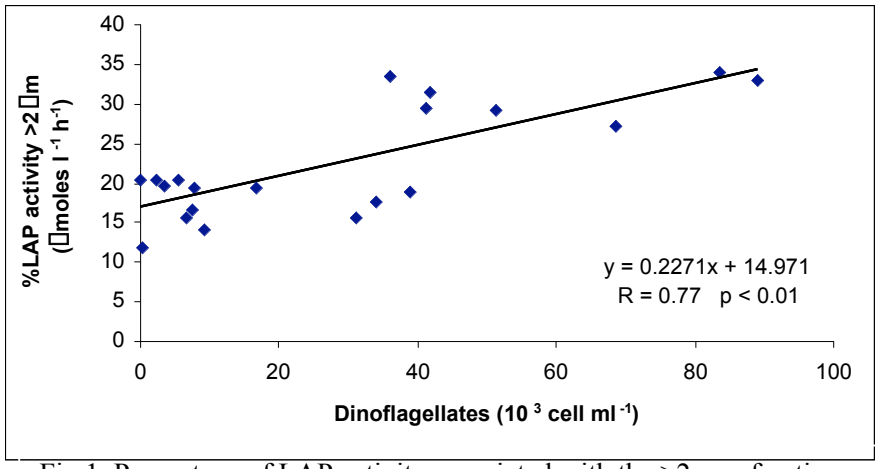

Fig.1. Percentage of LAP activity associated with the >2 mm fraction and dinoflagellate density in the Choptank River, spring 2003.

ALTERED FOS EXPRESSION AS A BIOMARKER OF NEURONAL STRESS ASSOCIATED WITH HARMFUL ALGAL BLOOM EXPOSURE IN MUMMICHOG, *Fundulus heteroclitus*

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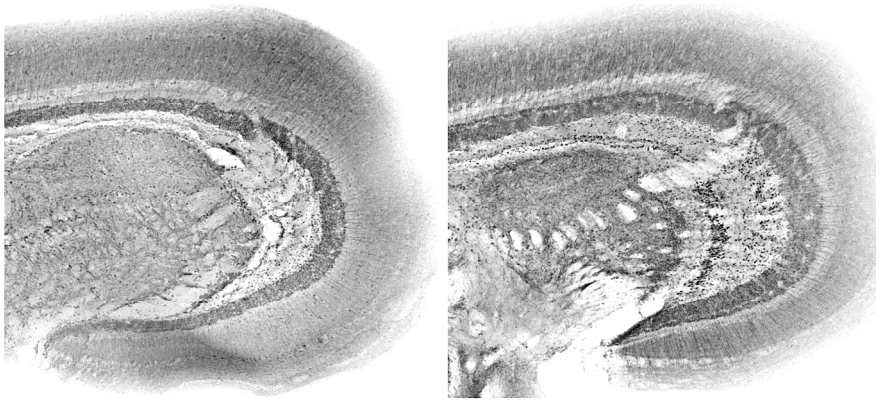
Behavioral changes in organisms can result from complex alterations at the biochemical and physiological levels of organization. The goal of this work was to develop and test the utility of Fos expression using immunocytochemistry as a biomarker of stress exposure in mummichog, *Fundulus heteroclitus*. c-Fos, an immediate early gene, and its protein product Fos, are induced in neurons as a result of neuronal stimulation. This study examined alterations in the brains of mummichog exposed to different harmful algal bloom (HAB) stressors including brevetoxin, domoic acid and *Pfiesteria shumwayae*. Brains of exposed fish were removed, sectioned and stained, and neurons expressing Fos were quantified. HAB-exposed fish brains showed increased neuronal Fos labeling compared to control fish brains. A dose response relationship was observed in *P. shumwayae* exposed fish, with increased labeling in fish exposed to higher dinoflagellate cell counts. Areas of the brain with increased labeling included optic lobes, midbrain and portions of the medulla. Alterations in swimming and respiratory behaviors were observed during all HAB exposures and may be associated with the increased regional neuronal activity. Alterations in Fos labeling as a biomarker of exposure may link quantifiable changes in fish swimming behavior associated with HAB exposure to changes in brain activity. General alterations in brain activity, as well as knowledge of specific, stress-activated regions within the brain, can provide valuable insights into the neural control of fish behavior as well as sublethal effects of chemical and physical HAB stressors.



Optic lobes of typical control (left) and *Pfiesteria*-exposed (right) mummichog brains. Brains of *Pfiesteria*-exposed animals reveal a higher density of dark, focal neuronal c-fos staining.

MICROZOOPLANKTON GRAZING AND THE POPULATION DYNAMICS OF HABS

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Harmful algal blooms (HAB) are evidence of uncoupling of predator:prey dynamics. Why does this happen? Microzooplankton grazing is usually tightly coupled to phytoplankton growth in the sea. Are the interactions between HAB species and microzooplankton an exception to grazing control? Three types of interactions between HABs and microzooplankton can be distinguished. Type I interactions involve HAB species that are an appropriate size for most microzooplankton but are not grazed. Blooms of these species tend to persist and disrupt planktonic food webs. Examples are brown tide, and toxic blooms of *Chrysochromulina spp.* and *Prymnesium parvum* (Gobler et al., 2002; Jakobsen et al., 2001; John, 2002; Tillman, 2003). Type II interactions involve relatively large cell size HAB species that have specialized microzooplankton grazers. Grazing often contributes to the decline of these blooms. In some cases, micro- and mesozooplankton can serve as vectors for toxin transfer to higher trophic levels. Examples are *Chattonella antiqua, Heterosigma akashiwo* and some of the large cell size HAB dinoflagellates (Nakamura et al., 1992; Jeong et al., 2003; Matsuyama et al., 1999). Type III interactions involve small and medium cell-size HAB species that are susceptible to grazing when at relatively low densities mixed with other phytoplankton species. However, at bloom densities, these species often are not grazed or have a detrimental affect on grazer populations. Many HAB dinoflagellates appear to have this type of interaction with grazers (Johnson et al., 2003; Hansen, 1995; Kamiyama, 2000; Stoecker and Gustafson, 2002; Rosetta and McManus, 2002). In type III interactions, microzooplankton grazing has important role in populations dynamics. Initiation of blooms may be suppressed or regulated by microzooplankton grazing. However, microzooplankton grazing may have little effect on dense blooms of these algae.

"Windows" of low grazing pressure that coincide with appropriate algal growth conditions are necessary for Type III blooms. Experimental studies of three small dinoflagellates (*Pfiesteria piscicida*, *Prorocentrum minimum* and *Karlodinium micrum)* that are harmful or nuisance species in the Chesapeake Bay region have shown that potential microzooplankton grazing is usually greater than potential growth rate, but that temporal and spatial "windows" of low grazing pressure occur (Johnson et al., 2003; Stoecker et al., 2000; Stoecker and Gustafson, 2002). Hypotheses for "window" formation include top down control of microzooplankton by mesozooplankton after the spring diatom bloom, freshets that flush-out resident microzooplankton populations or create conditions unsuitable for their rapid growth, and alterations in microzooplankton species composition due to eutrophication and/or pollution.

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THE PRESENCE OF DOMOIC ACID IN *Pseudo-nitzschia* FROM THE CHOPTANK RIVER, A CHESAPEAKE BAY TRIBUTARY

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Pseudo-nitzschia is a toxic diatom that produces domoic acid (DA), the neurotoxin responsible for Amnesic Shellfish Poisoning (ASP) and Domoic Acid Poisoning (DAP) which affects humans through contaminated shellfish and marine mammals and birds through contaminated fish. Three clones of *Pseudo-nitzschia* were isolated from the Choptank River, one in November 2002 and two in April 2003. All three clones were tested for domoic acid activity using the receptor binding assay and ASP direct cELISA test kits. Only the November clone was found to be toxic, displaying 0.08 pg DA-equl/cell in exponential phase and .438 pg DA per cell in stationary phase. While the presence of *Pseudo-nitzschia* has been documented in the lower Bay since the early 1980's, this is the first record of toxic *Pseudo-nitzschia* in the Chesapeake Bay area. Historical data suggests that *Pseudo-nitzschia* abundances have been increasing and spreading throughout the Bay over the past five years. Based on algal community monitoring by Maryland Department of Natural Resources between 2000-2002, 50% of the water samples containing *Pseudo-nitzschia* had concentrations above levels requiring mandatory testing of shellfish meats in Denmark and New Zealand. No known toxic events have occurred in Maryland, however, increasing *Pseudo-nitzschia* abundances and the presence of domoic acid in the food chain could have an impact on the future of the Cheapeake Bay shellfish industry, wild vertebrate populations, and public health.

OBSERVATIONS ON MIXOTROPHIC FEEDING BY THE TWO AUTOTROPHIC HAB SPECIES *Amphidinium carterae* AND *Prymnesium parvum* IN ESTUARINE WATERS

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Clonal cultures of the two HAB species *Amphidinium carterae* and *Prymnesium parvum* were established from natural samples and studied in batch culture. *Amphidinium carterae,* originally isolated from a sample taken in the coastal Yucatan Peninsula, Mexico, and a *P. parvum* clone isolated from an aquaculture facility having large fish kills in North Carolina, were both grown in F/2 medium in 6 well tissue culture plates. Cultures were further conditioned to phosphorus limiting media by transferring them to F/2 having $10\mu M \cdot L^{-1}$ PO_4-P. Cell growth was monitored over a 21 day period and observations were made relative to their behavior when ciliates or dinoflagellates were introduced. For the *A. carterae* cultures, cells of the ciliate *Euplotes* sp., abundant in an original raw sample, were introduced into wells when *A. carterae* densities of 5×10^4 cells$\cdot L^{-1}$ occurred. Within a few hours, *A. carterae* cells were found aggregating near the *Euplotes* and while the ciliate kept moving away from the mass of algal cells, a trailing stream of *A. carterae* continued to follow the ciliate eventually surrounding it and rendering it immobile. The mass of *A. carterae* cells eventually totally enveloped the *Euplotes* appearing to attach to the surface of the ciliate. Within four hours, the ciliate was completely devoid of cellular material with a remaining empty cast. After consumption of the *Euplotes, A. carterae* cells dispersed and were found regularly distributed throughout the volume of the wells.

For *Prymnesium parvum*, phosphorus limited cells were also conditioned in the manner described above and attained densities of 6.6×10^5 cells$\cdot L^{-1}$. When these densities were achieved, cells of a clonal culture of *Gyrodinium instriatum* were introduced into the *P. parvum* wells and followed for a period of 6 days. The *G. instriatum* cells were highly motile, exhibited normal morphology and for the first day showed no effects of *P. parvum*. Within 48 hours, however, enlarged *G. instriatum* cells showing a rounded morphology began to appear in the cultures with at first few (2-5) *P. parvum* cells attached. The enlarged spherical infected cells continued to be motile but had slower motion than those showing no symptoms of attachment by *P. parvum*. By the third day of incubation, numerous *G. instriatum* showed the unusual morphology, no motility as completely spherical cells with attached swellings of *P. parvum* plainly visible. These cells persisted for a few days before they were lysed releasing the *P. parvum* to the media as motile cells. By the end of the 6 day study, over 80% of the *G. instriatum* cells had been infected and the *P. parvum* cells had increased to 3.3×10^6 cells$\cdot L^{-1}$.

Both organisms studied naturally co-occur with the prey items tested. In the case of *A. carterae*, a sand dwelling dinoflagellate, *Euplotes* is present in its immediate environment in the highly saline waters of the Gulf of Mexico. In contrast, *P. parvum*, found at salinities as low as 4, co-occurred with *G. instriatum* competing for the same resources. Infection of a co-dominant under the conditions of the aquaculture ponds offered an effective nutrient gathering method as well as reduction in competition for any available resources. Both organisms when nutrient stressed exhibited the same strategy of mixotrophy to allow them to maintain their densities and bloom populations.

PATHOGENICITY OF *Pfiesteria piscicida* AND *P. shumwayae* TO FISH: DOSE RESPONSE STUDIES

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Pfiesteria piscicida and *P. shumwayae*, members of the toxic *Pfiesteria* complex (TPC), are reported to secrete potent exotoxins responsible for fish pathology and mortality as well as human illness in mid-Atlantic estuaries. We recently demonstrated that *P. shumwayae* causes it's adverse fish health effects through the process of micropredatory feeding on the epidermis rather than by secretion of a potent exotoxin, as previously claimed. We have observed a similar mechanism of fish killing in *P. piscicida* and several related lightly armored heterotrophs. However, the biological, "toxicological" and ecological determinants that modulate pathogenicity of these organisms are not well understood and urgently require clarification. Using our new larval fish bioassay, we therefore conducted dose response studies with *Pfiesteria* spp. to clarify issues affecting time-to-fish-death, with particular focus on the role of zoospore density. Cell density was observed to be a critical determinant of time-to-death, with higher initial zoospore concentrations resulting in more rapid time to fish-death for all cultures tested. Toxigenicity (e.g., exotoxin secretion) was not a determinant of fish pathogenicity for any species or strain tested, however, ability to kill fish varied tremendously among the two species.

THE RELATIVE IMPORTANCE OF DOM UPTAKE, GRAZING AND PHOTOSYNTHESIS OVER DIEL CYCLES IN THE MIXOTROPHIC DINOFLAGELLATE *Akashiwo sanguinea*, IN THE LAFAYETTE RIVER, A TRIBUTARY OF THE CHESAPEAKE BAY

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The Lafayette River, a tributary of the Chesapeake Bay, experienced dense concentrations (6,000 cells/ml) of the mixotrophic dinoflagellate, *Akashiwo sanguinea,* during the summer in both 2002 and 2003. Mixotrophic species such as *A. sanguinea* may have a competitive advantage over strictly autotrophic species that can only fix carbon during the day through photosynthesis. In previous studies, we determined that *A. sanguine*a has the capacity to take up organic N and C and that both contribute substantially to their growth. Urea and amino acid C were major sources of C for *A sanguinea* and their uptake exceeded photosynthetic C-uptake during blooms in 2002. At the beginning of this bloom, total C uptake measured during the day was insufficient for meeting cellular C demands based on the observed N uptake. Later in the bloom, uptake of organic C could almost completely satisfy the C demand based on the observed N uptake and the molar C:N ratio. Grazing and nighttime uptake of organic C were not considered in this first study, nor were changes in the physiological state of cells as blooms progress. So, in order to determine the relative N and C contributions from dissolved organic matter (DOM) uptake, grazing and photosynthesis over the progression of blooms, we measured uptake of dually-labeled ^{15}N- and ^{13}C organic compounds (urea, amino acids and dipeptides), ^{15}N-labeled nitrate and ammonium, and ^{13}C-labeled bicarbonate and glucose at 6-12 hour intervals over several 24-hour periods during which natural populations were increasingly dominated by *A. sanguinea*. In addition, we estimated grazing rates by feeding them cryptophyte prey and examining changes in prey concentrations using microscopy. Results suggest that *Akashiwo sanguinea* takes up organic C and grazes to supplement photosynthetic C acquisition and that rates of these processes vary on diel time scales and as blooms initiate and develop.

COMPLEX GENE STRUCTURE OF THE FORM II RUBISCO IN THE DINOFLAGELLATE *Prorocentrum minimum* (DINOPHYCEAE)

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We analyzed the unusually complex organization of the nuclear-encoded (form II) ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) gene in the potentially harmful dinoflagellate *Prorocentrum minimum* (Parvillard) Schiller by intensive genomic DNA and cDNA sequencing and Western blotting. Over ten transcribed units (TUs) were detected, which varied dramatically in their 3' untranslated region. Each TU appeared to contain four tandem copies of the Rubisco coding region (1.46 kb each; coding unit, or CU) interspersed by a 63-bp spacer; the four CUs in each TU were co-transcribed and apparently co-translated to a tetrameric polyprotein that may undergo successive cleavage steps to yield mature Rubisco. By means of Real-Time PCR analysis, it was estimated that each of the *P. minimum* genome harbored 148 ± 16 CUs. Although nucleotide sequences varied by 1-9% among the detected CUs, their inferred amino acid sequences were essentially identical. Our results suggest that the complex structure of *Pmrbc* has been derived from extensive and repeated gene duplications, an evolutionary process that has also been observed for other dinoflagellate genes.

NUTRIENT EFFECTS ON GROWTH AND PHOTOSYNTHESIS OF *Chattonella subsalsa* (RAPHIDOPHYCEAE) ISOLATED FROM INLAND BAYS, DELAWARE (U.S.A)

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The Inland Bays of Delaware are shallow, highly eutrophic embayments that have been plagued by frequent and severe harmful algal blooms. During the past several years, novel blooms of the toxic Raphidophytes *Chattonella*, *Heterosigma* and *Fibrocapsa* have caused fish kills and raised concerns for human and ecosystem health. We examined the effects of nitrate, phosphate, ammonium and urea availability on the biomass yield, growth rate, and photosynthesis of unialgal *Chattonella subsalsa* cultures isolated from the bays, using classic Monod-type experiments. This isolate was unable to grow on urea, suggesting that it does not possess a urease enzyme and may require inorganic sources of nitrogen. Values for μ_{max} (maximum nutrient-saturated growth rates) in the various experiments ranged from 0.64 to 0.7 d^{-1}. Half-saturation concentrations for growth ($K_{1/2}$) were 8 μM for nitrate, 3 μM for ammonium, and 0.7 μM for phosphate, suggesting that this species has a high requirement for inorganic nitrogen and should thrive in the highly N-enriched environment of the Inland Bays. Experiments also examined photosynthetic efficiency as a function of nutrient availability, supporting the conclusions from the growth rate determinations. We are currently comparing the nutrient requirements of *Chattonella subsalsa* with several sympatric Raphidophyte species and other local algal isolates to assess the effects of eutrophication on competition and succession both within the Raphidophyte group, and in comparison to other groups such as diatoms and dinoflagellates.

THE GROWTH AND SURVIVAL OF THE COASTAL DINOFLAGELLATE, *Gyrodinium instriatum*, IN DIFFERENT SALINITIES

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Harmful algal blooms (HABs) are a specific problem and concern for the delicate coastal ecosystem. These blooms, or the toxins they produce, result in fish kills and some have been shown to cause adverse effects on human populations. The dinoflagellate *Gyrodinium instriatum* Freudenthal and Lee was selected for this study because it commonly forms extensive blooms in temperate estuaries. This species, identified in waters with salinities ranging from 2 to 36, formed blooms in estuaries along the eastern seaboard of the United States, as well as worldwide. These estuaries varied in salinity and in location, including some in Florida, North Carolina, New York, Ecuador, and Japan. The apparent extraordinary tolerance to variations in salinity stimulated this study. Growth studies were conducted, to examine the capacity for growth of the species, by performing visual counts on cultures grown in a series of salinities, from 5 to 30. Growth was seen in all six of the salinities, 5, 10, 15, 20, 25, and 30, indicating that this species is very tolerant to salinity, but the most dense growth was seen at the lower salinities of 5 and 10. In order to mimic what might be expected in natural environmental systems, cultures were preconditioned in each of the six salinities and then transferred to the salinity series (5-30). Growth was monitored daily for a period of 8 days by measuring in vivo fluorescence. Cultures grown at the lower salinities, 5 and 10, increased biomass when introduced to the highest salinities, 25 and 30. When cultured in the intermediate salinities, 15 and 20, growth occurred in all salinities tested, from 5-30. *Gyrodinium instriatum* grown at the highest salinities of 25 and 30 showed low growth when introduced to the salinity range. Adjustment of this organism to changes in salinity seems to most readily take place at salinities below 20, with the most rapid growth occurring at extremely low salinity levels. The species is definitely estuarine in nature, with a remarkable capacity to adjust to salinity changes of estuarine environments; this specific trait is essential for it's capacity to form blooms. Along with tolerance to salinity changes, the morphology of the species shows a great deal of plasticity with the shapes ranging from broadly oval, to rotund, to completely spherical. When monitored by microscopic observation, stressed *G. instriatum*'s morphology changed drastically. The cells changed shape from a fairly uniform oval to a very spherical shape. This suggests that *G. instriatum* has difficulty osmoregulating, yet adapts its shape to the salinity in which it resides. This observation agrees with the field studies involving this species, in samples from Delaware, Maryland, and North Carolina where highly spherical cells were collected. Once these cells are isolated and placed into an appropriate media, they resume the shape described for the species. These morphological observations may help to explain the confusion encountered when identifying *G. instriatum* and why it may be confused with a similarly described species, *G. uncatenum*. Further investigation is needed to determine if these species are indeed different and, if so, what characteristics should be used to distinguish between the two species.