

# **Fulfilling Data Needs for Assessing Numeric CHLa Criteria of the Lower James River Estuary: Microscopic and molecular genetic analyses of blooms, and determination of bloom impacts on aquatic life**

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**Introduction:** In addition to impacting human and animal health, harmful algal blooms (HABs) can affect aquatic food webs, commercial fisheries and aquaculture, and recreational water use. Recent increases in the frequency, severity and distribution of algal blooms have occurred worldwide and the threats posed by emerging HAB species due to global climate change are predicted to increase (HARRNESS, 2005). Several HAB species have produced significant blooms in Chesapeake Bay for the past several years (Marshall et al 2005, Marshall and Egerton 2009, Reece 2012, Reece et al. 2012). Many of these HAB species have been associated with finfish or shellfish mortalities and have impacted recreational water usage locally in the Bay and at other sites around the world (Gates and Wilson 1960, Marshall 1995, Deeds 2003). Marshall et al. (2008) listed 37 potentially toxic/harmful phytoplankton species within the Bay and its tributaries. These include diatoms, notably many *Pseudo-nitzschia* species (Anderson et al. 2010), dinoflagellates including *Karlodinium veneficum* (Pate 2006, Place et al. 2008), *Cochlodinium polykrikoides* (Vargas-Montero et al. 2006, Richlen et al. 2010), *Scrippsiella trochoidea* (Hallegraeff 1992, Licea et al. 2004), *Heterocapsa rotundata*, *H. triquetra* (Sato et al. 2002, Marshall et al. 2005, Marshall & Egerton is 2009), *Akashiwo sanguinea* (Cardwell et al. 1979, Botes et al. 2002, Jessup et al. 2009), *Prorocentrum minimum*, *P. micans* (Grzebyk et al. 1997, Heil et al. 2005) and *Alexandrium monilatum* (May et al. 2010, Reece et al. 2012); raphidophytes *Chattonella veruculosa* and *Heterosigma akashiwo* (Keppler et al. 2005, 2006, Zhang et al. 2006) and cyanobacteria (Codd et al. 2003, Wiegand and Pflugmacher 2005) including the species *Microcystis aeruginosa*, *Anabaena* spp. and *Oscillatoria* spp. found primarily in freshwater and the lower salinity portions of estuaries. Blooms of these species could represent a significant emerging threat to the Bay ecosystem.

The primary purpose of the studies described herein was to provide data characterizing the phytoplankton species composition of water samples, particularly those collected during blooms,

and to establish quantitative linkages between algal blooms and deterioration of designated uses in the lower James River. The overall goal of the project is to provide information that is vital to evaluate existing numeric criteria for the tidal James River system. This work focuses on the two objectives “Characterizing Algal Blooms” and “Characterizing Impairments Associated with Algal Blooms”, and the related subtasks “Subtask 1.2—CHLa, diagnostic pigments and the occurrence of harmful algae” and “Subtask 2.1—Determining linkages between algal blooms and impairments”, which were specifically identified by the scientific advisory panel for the lower James River (Bell et al. 2011). Two tasks were undertaken for this study during 2013: 1) Determining the occurrence and density of harmful dinoflagellates, raphidophytes, cyanobacteria and diatoms in the lower James River region through microscopic and molecular genetic analyses and 2) Examining linkages between bloom species, cell densities and adverse health impacts on aquatic life through laboratory dose-response bioassays with clonal cultures of HAB species. These studies are an integral part of the effort to provide scientific data for the water quality standards rulemaking process, which may result in revisions to nutrient allocations contained in the Chesapeake Bay TMDL. Specific questions addressed in 2013-February 2014 were:

1. What were the timing, intensity, duration, and spatial extent of algal blooms in the oligohaline and mesohaline regions of the James River in 2013?
2. What was the phytoplankton community composition and cell density for recognized harmful algal bloom (HAB) species during blooms throughout this region?
3. What are the levels of CHLa in bloom samples and what is the probability of adverse effects on aquatic life during blooms (i.e. high chlorophyll levels)?
4. What diagnostic microscopic and molecular genetic profiles (i.e. types and densities of harmful phytoplankton) are linked to decline in designated uses including fishing and recreational activities?
5. Which harmful algal bloom organisms (species/strains) have biological impacts in this system?

During the course of this multi-year study we have undertaken a three-tiered framework for assessing the probability of adverse effects on aquatic life due to harmful algae in the lower

James River. Within this framework, CHLa was routinely monitored at likely “hotspots” using fixed station continuous monitoring (ConMon) and real-time mapping approaches such as comprehensive on-board and underway monitoring (DATAFLOW; see [www.VECOS.org](http://www.VECOS.org) and Moore et al. 2014). Samples were analyzed to determine phytoplankton community composition and cell density via microscopy and/or molecular-genetic approaches, and we have assessed risk of adverse impacts to aquatic life using both field and laboratory studies. Oysters were used as a sentinel species for the analysis of the harmful effects of algal blooms as the James River oyster fishery is an important source of this product in Virginia and oyster aquaculture is a rapidly growing industry in the state. In addition, numerous oyster restoration projects are underway throughout the state and these efforts could be hindered by bloom events. Laboratory toxicity bioassays were done with both bloom samples and some laboratory cultures in 2012 and with laboratory cultures in 2013-2014. From 2012-2014 toxicity bioassays were done with clonal isolate cultures of organisms that bloom in Virginia estuarine waters to evaluate potential adverse health impacts through quantitative measurements of morbidity, and particularly mortality. During 2012 brine shrimp, *Artemia salina* nauplii were exposed to bloom samples and to live cells or lysates of HAB organisms maintained at VIMS as clonal isolate cultures. Dose response assays in 2013-2014 were done with *Crassostrea virginica* veligers, *Cyprinodon variegatus* larvae and *Ceriodaphnia dubia* neonates.

## **Materials and Methods:**

### *Collection of water samples*

Two replicate 100 ml water samples were collected from the VIMS ConMon station at least every other week and during DATAFLOW cruises (for additional details on the VIMS ConMon and DATAFLOW cruises see Moore et al. 2014). Samples were transported to VIMS in a cooler with insulating material between the water sample and blue ice. Sampling sites are listed in Table 1 and indicated on the map (Fig. 1).

### *Microscopic examination of samples*

Visual microscopic identifications of dominant dinoflagellate, raphidophyte and cyanobacteria species from one of the replicate water samples were done as described in the SOP for the Reece and Vogelbein laboratories.

### *Establishment and maintenance of clonal cultures*

Bloom samples containing species of interest were retained and used to establish *in vitro* cultures as described in the SOP. Cultures were maintained as described in the SOP for the Reece and Vogelbein laboratories in L1.5 medium containing 50% more nitrate than the typical L1 medium (Guillard and Hargraves 1993) and supplemented with vitamins (Guillard & Ryther 1962, Guillard 1975).

### *DNA Purification*

One replicate 100 ml water sample was filtered and processed to extract DNA as described in the SOP for the Reece and Vogelbein laboratories. Beginning in April, 20 ml from most samples were filtered onto a 0.22 or 0.45 um filter for DNA extraction and analysis for the presence of *M. aeruginosa* DNA as described in the SOP for the Reece and Vogelbein laboratories. DNA was stored at 4°C for up to 24 hours and then at -20°C for long-term storage.

### *PCR amplification of extracted DNA*

Ribosomal RNA gene regions were amplified for each species of interest using the assays developed and/or optimized in the Reece laboratory according to the protocols listed in the SOP for the Reece and Vogelbein laboratories including the *Microcystis aeruginosa* assay developed during this study.

All bloom samples were immediately processed to determine the cell counts using the specific quantitative real-time PCR assay. Samples were screened using standard PCR assays for specific

species of interest. All samples that were positive using standard PCR were screened with the corresponding quantitative real-time PCR assay. DNA extracted from a known number of cells from control material cultures was used as a positive control for each assay. Real-time PCR standard curves were generated by serially diluting the DNA to achieve a range of cell number equivalents that were reliably measured by the specific assay.

#### *Cloning and sequencing for species verification*

PCR products of stock culture ribosomal RNA gene regions ITS, LSU and SSU were cloned for sequencing when a new culture was received or a bloom sample was used to establish a new culture, and periodically during culture maintenance to verify the culture was the species expected. PCR products of standard PCR species-specific assays were periodically cloned for sequencing to verify the specificity of the assays. PCR products were ligated into the plasmid pCR<sup>®</sup>4-TOPO<sup>®</sup> and transformed into TOP10 *Escherichia coli* using a TOPO<sup>®</sup>TA Cloning<sup>®</sup> kit (Life Technologies) following the manufacturer's protocol. Transformed colonies were screened by PCR using the M13 forward and reverse primers provided in the kit. Individual colonies were inoculated into 20 µl of sterile water and lysed by boiling for 10 minutes at 95°C. PCR reactions (15 µl) contained 1× PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.5 µM of each primer, 0.025 U of *Taq* DNA Polymerase (Life Technologies), and 1 µl of lysed cells as template. The thermal cycling parameters included an initial denaturation of 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 1 minute with a final extension of 72°C for 5 minutes. PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized under a UV light.

PCR products from at least 4 clones containing the correct insert size were identified and enzymatically treated with shrimp alkaline phosphatase and exonuclease I (Amersham Biosciences) to degrade nucleotides and primers that would interfere with the sequencing reactions. These PCR products were then sequenced bidirectionally using the Big Dye Terminator Kit v3.1 (Life Technologies) following the manufacturer's protocol and the M13 forward and reverse primers originally used to screen the colonies. The sequencing reactions

were then electrophoresed using a 3130xl Genetic Analyzer (Life Technologies). After analyzing the resulting electropherograms using MacVector 12.7.1 the sequences were compared to those deposited in GenBank using BLAST (basic local alignment search tool) searches (Altschul et al. 1990) of the National Center for Biotechnology Information (NCBI) database, and those compiled previously by our laboratory.

### *Field exposures*

Small market-sized oysters (~75mm) were deployed in a cage (n=175) near the ConMon station (Moore et al. 2014) in the mesohaline region of the James River on May 28, 2013. Oysters were also deployed near the ODU ConMon station from a pier at the Norfolk Yacht Club in the Lafayette River on May 30, 2013. Five oysters were processed for histopathological analysis before deployment and 15 oysters were collected and processed from each site as a baseline (i.e. before elevated chlorophyll levels) on June 12-18, 2013. During bloom events 15 oysters were collected from the cage, mortalities were recorded and the live animals were processed for histological analyses. Briefly, the oysters were shucked and the tissue was fixed in Davidson's Fixative and then processed using standard methods for paraffin histology. Six-micron sections were cut and mounted on slides. The slides were stained with hemotoxilyn and eosin for pathological evaluation.

### *Laboratory toxicity bioassays*

Toxicity bioassays were conducted using clonal cultures that were established from bloom samples and are being maintained long-term (Table 2). Dose response studies were conducted with both clonal isolate live cell and cell lysate material.

Toxicity bioassays were generally conducted as outlined in the SOP protocols for the Reece and Vogelbein laboratories. Live cell treatments were established by counting and diluting cell numbers to desired concentrations with L1.5 media or, if higher cell concentrations were desired, filtration of the cultures was used. For preparation of the lysates cells were harvested from each culture isolate prior to the live cell assays and frozen until use. These materials were thawed and

subsequently lysed on ice using a Misonix Microson™ ultrasonic cell disruptor at full power for 20 to 30 seconds, in 5 second bursts, to prevent the possibility of heat damage to any toxins from microbursts. The lysate was then diluted to the desired corresponding cell concentrations with L1.5 media. For the duration of the assay (i.e. up to ~96hr) both mortality and animal activity (swimming, feeding) versus lack of activity were noted in order to determine the condition during the exposures. To assess morbidity/mortality of oyster veligers activity (swimming, feeding) versus lack of activity (closed and not feeding) was noted in addition to movement of the animals within the shell (if lying closed on the beaker bottoms) in order to determine the condition of the veligers during the exposures. In assays where the veligers remained closed and inactive, movement within the shell, presence of active hemolymph circulation, heart beat, movement of the vellum cilia, and the refractile appearance of live tissue were used to judge viability. Mortality was determined based on the lack of these criteria and obvious tissue degradation, in addition to the appearance of bacterial growth and increased numbers and activity of non-dinoflagellate protozoa inside the shells. For *Cyprinodon* larvae reduction or cessation of swimming, blood circulation, heartbeat and pectoral fin, opercular or mouth movement were used as indications of morbidity/mortality.

## **Results**

### *Occurrence of harmful algae as determined through microscopic and molecular genetic analyses.*

A total of 115 samples were collected for microscopic and molecular analyses from the lower James River system in 2013 including two Elizabeth River samples, four Lafayette River samples, two from Cypress Creek off of the Pagan River in Smithfield and one from the Hampton River (Table 1). There was general congruence between the visual and molecular identifications and counts, although some HAB cells could not be confidently identified to the species level through microscopic visualization and molecular cell counts were higher than visual counts when cells were rapidly multiplying and visual counts were higher than molecular if cells were rapidly lysing. Results of the microscopic examinations and molecular assays are given in Appendix 1 (spreadsheet). Mid-February through early April 2013 we received several

bloom samples of *Heterocapsa triquetra* from the mesohaline region. This organism was found at bloom concentrations (~5K– >35K cells/ml) with blooms often comprised of a mixed phytoplankton community that included *H. rotundata*, *Gymnodinium* sp., *Gyrodinium* sp., *Karlodinium veneficum*, *Protoperidinium* sp. and/or *Cryptoperidiniopsis brodyi* (Appendix 1). Several samples had high CHLa concentrations with the highest being 380.00 ug/L in a sample collected in the late afternoon at station JMS017.96. The *Cochlodinium polykrikoides* bloom in the meso and poly-haline regions started around August 9 and continued until early September. In the samples analyzed by PCR for *C. polykrikoides*, concentrations ranged from 72 – 20,137 cells/ml. The highest concentrations were found on August 26 in samples from the James River. One sample was collected by HRSD at station JMSMH-2 (16,928 cells/ml) and the other was collected by VIMS personnel at Huntington Beach in Newport News (20,137 cells/ml). *Luciella* species were also detected in several water samples by PCR. We developed a standard PCR assay in 2012 to detect *Microcystis aeruginosa* and developed a quantitative PCR (qPCR) assay in 2013. The qPCR assay indicated that *M. aeruginosa* cells were present in many of the samples collected during the DATAFLOW cruises (Moore et al. 2013) in the oligohaline region of the James, but it was also present in samples from the mesohaline region. The concentrations ranged from <1-2,205,000 cells/ml (Table 3).

Cultures were established for several of the James River bloom species including *Akashiwo sanguinea*, *Heterosigma akashiwo*, *Scrippsiella trochoidea* and genetic analysis including DNA sequencing confirmed the species identification of these cultures. Fifteen *M. aeruginosa* clonal cultures were established from a bloom sample collected in 2011 from Lake Rooty, and live cell and lysate bioassays were run with one of these isolates (see below). The current list of *in vitro* isolate cultures of HAB organisms maintained at VIMS is provided in Table 2.

#### *Determining linkages between blooms and adverse effects on aquatic life.*

No notable pathology was observed in sentinel oysters, *Crassostrea virginica*, prior to deployment at the VIMS and ODU ConMon stations in the mesohaline region of the James River and the Lafayette River, respectively. This is the summary of results from the deployed sentinel oysters at each of the sites.

James River ConMon (Oysters deployed on May 28, 2013). They were deployed for a total of 167 days and the remaining oysters were retrieved on November 11, 2013. CHLa was monitored for 157 of those days. The CHLa ranged from 2.6 - ~500 ug/L, with an average level of 15.98 ug/L.

1. Twelve oysters were collected on 6/18/13 - Total deployment time = 21 days; There were no days when the CHLa level exceeded 50 ug/L; Oyster pathology: MSX and dermo disease were not observed. *Rickettsia*-like bacteria (RLO) were found in three oysters, four oysters had *Sphenophrya*-like ciliates, four were infected by the ciliate *Stegotricha enterikos*, one had an oocyte microsporidian, one had viral gametocytic hypertrophy. One oyster had focal hemocytosis at the mantle.
2. Fifteen oysters were collected on 8/6/13 - Total deployment time = 70 days; 6 days with CHLa >50 ug/L and 3 days >100 ug/L. Oyster pathology: MSX and dermo disease were not observed, however, one oyster had an oocyte microsporidian, another had *S. enterikos* and one had an RLO. One oyster had hemocytosis in the digestive gland and another had disrupted stomach epithelia.
3. Fifteen oysters were collected on 9/14/13 - Total deployment time = 109 days; 18 days with CHLa levels > 50 ug/L and 10 days >100 ug/L and 1 day >200 ug/L. Oyster pathology: MSX and dermo disease were not observed, however, 3/15 oysters had RLOs and one was infected with *S. enterikos*. One oyster had a localized area of increased hemocytosis.
4. Fifteen oysters were collected on 9/27/13 - Total deployment time = 122 days; 18 days with CHLa levels > 50 ug/L and 10 days >100 ug/L and 1 day >200 ug/L (Note: no change from above, as there were no days with CHLa >50 ug/L after 9/5/2013). Oyster pathology: MSX and dermo disease were not observed. Two oysters had *Sphenophrya*-like ciliates, while one oyster exhibited gill erosion and another was emaciated with breakdown of the stomach and intestinal epithelium.
5. The final group of fifteen oysters was collected on 11/11/13 – Total deployment time = 167 days; 18 days with CHLa levels > 50 ug/L and 10 days >100 ug/L and 1 day >200 ug/L (Note: no change from above, as there were no days with CHLa >50 ug/L after 9/5/2013). Oyster pathology:

There were no pathological signs that could be clearly attributed to HAB exposure.

Lafayette River ConMon (Oysters deployed on May 30, 2013)

1. Fourteen oysters were collected on 6/12/13 - Total deployment time = 13 days; There were no days when the CHLa level exceeded 50 ug/L; Oyster pathology: MSX and dermo disease were not observed, however, one oyster had a *Nematopsis* sp. infection, two oysters had RLO infections, five were infected with *S. enterikos* and one with *Sphenophrya*-like ciliate. One oyster demonstrated three focal areas of increased hemocytosis.

2. Fifteen oysters were collected on 8/20/13 - Total deployment time = 82 days; 16 days with CHLa levels > 50 ug/L and 11 days >100 ug/L and 6 days >200 ug/L, 4 days >300 ug/L, 1 day >400 ug/L; Oyster pathology: Two oysters had heavy, systemic MSX infections resulting in general hemocytosis. No dermo disease was observed, however four oysters had *Nematopsis* sp. infections, two oysters had RLO infections, two were infected with *S. enterikos*, one had *Sphenophrya*-like ciliates and one had viral gametocytic hypertrophy. Two oysters had localized areas of hemocytosis and a third had disruption of the intestinal epithelium.
3. Fifteen oysters were collected on 9/25/13 after the *Cochlodinium polykrikoides* bloom. Total deployment time = 118 days; 40 days with CHLa levels > 50 ug/L and 26 days >100 ug/L and 14 days >200 ug/L, 9 days >300 ug/L, 3 days >400 ug/L, 3 days >500 ug/L; Oyster pathology: Two oysters had light MSX infections and one had a rare dermo infection. Four oysters were infected with *Nematopsis* sp. and one oyster had three focal areas of increased hemocytosis.
4. The final group of 15 oysters was collected on 11/18/13. Total deployment time = 172 days; 40 days with CHLa levels > 50 ug/L and 26 days >100 ug/L and 14 days >200 ug/L, 9 days >300 ug/L, 3 days >400 ug/L, 3 days >500 ug/L (Note: no change from above, as there were no days with CHLa >50 ug/L after 9/5/2013); Oyster pathology:

Observed histopathology in oysters could not be attributed to bloom exposure in any of the field-deployed samples. Several common parasites were observed, but they occurred in both localities and at all sampling times.

Laboratory bioassays were conducted with a few field bloom samples during the *Heterocapsa triquetra* bloom in February (Table 4, Figs. 2-4). Two assays were done with *Artemia salina* as the test organism and in one of these both lysate and live cells were tested. A sample collected on Feb. 19, 2013 from the ConMon station in the mesohaline region of the James River had 8,500 cells/ml (CHLa=70 ug/L). Both lysate and live cells were tested and very low mortality was observed in the controls and both treatments (<1%) (Fig. 2). A sample collected on Feb. 25, 2013 from the James River near the idle fleet was tested on *Cyprinodon variegatus*. The sample had an *H. triquetra* cell concentration of 13,000 cells/ml and a CHLa concentration of 94.6 ug/L. Mortality in the controls was <5% and the fish exposed to the sample suffered 15% mortality (Fig. 3). On Feb. 27, 2013 a mixed species bloom sample was collected. It contained *Heterocapsa triquetra*, *H. rotundata* and *Cryptoperidiniopsis brodyi* and had a CHLa concentration of 191 ug/L. A bioassay was done with *A. salina* and less than 1% mortality was

observed for the fed control animals and for those exposed to the bloom sample. Unfed control animals exhibited ~25% mortality (Fig. 4).

In addition to the three bioassays with field samples, several assays were done with *in vitro* clonal isolate cultures. Before starting these assays we did a dilution series for CHLa analysis and found that there was a linear correlation between CHLa concentration and cell number as determined by qPCR for isolate cultures (Figure 5). Four dose response bioassays were done using a clonal *Prorocentrum minimum* culture (2 live cell and 2 lysate) established at VIMS from a James River sample. The test organisms for these assays were larval sheepshead minnows, *Cyprinodon variegatus*, for two assays and *Crassostrea virginica* veligers for the other two. The *P. minimum* dose response assays were done at the following concentrations: 10,000 cells/ml, 5,000 cell/ml, 2,500 cells/ml and 1,000 cells/ml. The CHLa concentration for the high dose treatment was 28.59 ug/L. In the live cell assay with oyster veligers ~22% mortality was observed in the 10,000 cells/ml treatment after 120hr (Fig. 6). Little to no mortality was observed in the other treatments or in the controls. In addition, at 96hr the cumulative mortality in the 10,000 cells/ml treatment was ~3%, so that most of the mortality occurred during the last 24hr of the experiment. In the lysate treatments mortality was higher with >90% cumulative mortality in the 10,000 cell/ml lysate and ~23% mortality in the 5,000 cells/ml lysate treatment after 120hr (Fig. 7). Mortality in the lower concentration treatments and in the controls was less than 10%. As with the live cell assay, most mortality occurred during the last 24hr of the experiment. In the *P. minimum* live cell and lysate bioassays with *C. variegatus* as the test organism little to no mortality was observed after 96hr in any of the treatments or the controls (Figs. 8 &9). We typically conduct the veliger assays for 120hr and larval fish assays for 96hr. We have found that it usually takes longer for veligers to respond to treatments. For these *P. minimum* assays, however, we were concerned that the water quality, particularly the dissolved oxygen (DO) concentration, may have been compromised in the high dose wells near the end of the bioassay. It is not clear why only the high dose treatments were impacted, but the cell biomass in those wells was highest, so possibly DO would drop with cell degradation during the assay. Therefore, we are purchased a microprobe to more routinely measure DO in the microtiter plate wells in the recent assays. There have been no indications of low DO during the assays conducted to date with the microprobe.

Dose response bioassays were done with a VIMS *Microcystis aeruginosa* culture (live cell and lysate, each 3X) using *C. variegatus* as the test organism. The first assay was done in August 2013 at the following concentrations for both the live cell and lysate assays:  $5.9 \times 10^7$  cells/ml,  $5.9 \times 10^6$  cells/ml,  $5.9 \times 10^5$  cells/ml and 59,000 cells/ml. The CHLa concentration for the high dose treatment was ~1,500 ug/L and ~150 ug/L for the lowest concentration. In both the live cell and lysate assays mortality was observed at the high dose of  $5.9 \times 10^7$  cells/ml, however, little to no mortality (<10%) was observed in the controls and lower doses (Figs. 10 & 11). Cumulative mortality in the high dose live cell treatment was ~60% after 96hr, while in the high dose lysate treatment there was 100% mortality by 48hr. There is a concern that the *M. aeruginosa* cells may not have been efficiently lysed to make the lysate for this assay.

Another set of *M. aeruginosa* (live cell and lysate) dose response bioassays were done in October 2013 using *C. variegatus* as the test organism. The cell concentrations ranged from  $1.375 \times 10^6$  to  $1.9 \times 10^6$  cells/ml. The CHLa concentration for the high dose treatment was 481.44 ug/L. High mortality (~65%) was observed in the high dose lysate treatment (Fig. 12) when the wells were first observed 3-4 hrs post-initiation. At 24 hr ~85% and 100% mortality were observed in the high dose live cell (Fig. 13) and lysate treatments, respectively. There was also 100% mortality in the  $5.5 \times 10^6$  cells/ml lysate treatment after 24 hr. At the end of the assay (i.e. 96hr) mortality was >60% in both the  $2.75 \times 10^6$  cells/ml and  $1.375 \times 10^6$  cells/ml lysate treatments. ~35% mortality was observed in the  $5.5 \times 10^6$  cells/ml live cell treatment and there was about 20% mortality in the  $2.75 \times 10^6$  cells/ml live cell treatment after 96 hr. ~12% mortality was observed in the controls after 96 hr.

A third set of dose response bioassays with *M. aeruginosa* and *C. variegatus* was done in March 2014. The cell concentrations for this assay ranged from  $1.5 \times 10^6$  -  $1.2 \times 10^7$  cells/ml. The CHLa concentration in the highest dose was 827.77 ug/L. Mortality for this assay was very low with the highest mortality observed in the lysate representative of  $1.2 \times 10^7$  cells/ml (Figs. 14 & 15).

One set of *Karlodinium veneficum* (live cell and lysate) dose response bioassays was done using *C. variegatus* as the test organism in October 2013 with cell concentrations ranging from 2,500 - 70,500 cells/ml. The CHLa concentration in the high dose treatment was 37.13 ug/L. During this assay ~60% mortality occurred in the high dose lysate (68,700 cells/ml) treatment within the first 3 hours of the 96 hr assay (Fig. 16), therefore, the remaining moribund animals were fixed for histopathology. No mortality was observed in the other lysate treatments (2,500, 5,000 and 10,000 cells/ml) after 96hr. In the live cell treatments (Fig. 17) ~80% mortality was observed in the 5,000 cells/ml treatment, but it is not clear whether there was a confounding factor in that treatment. ~10-20% mortality was observed at concentrations of 70,500, 10,000 and 2,500 cells/ml by 96hr.

Another set of *K. veneficum* dose response bioassays was done using *C. virginica* as the test organism in June 2013 with cell concentrations ranging from 2,500 cells/ml – 126,000 or 133,000 cells/ml for the live cell and lysate assays, respectively. The CHLa concentration in the 126,000 cell/ml treatment was 251.66 ug/L and 4.98 ug/L in the 2,500 cells/ml. 100% mortality was observed in the high dose live cell treatment after 120hr and ~82% mortality was observed in the 10,000 cell/ml live cell treatment. The live cell 2,500 – 5,000 cell/ml treatments had 60-70% mortality. The mortality was lower in the lysate treatments. 40% mortality was observed after 120hr in the high dose lysate treatment. <10% mortality was observed in all other lysate treatments and the controls (Fig. 18).

Four dose response bioassays were done with a culture of *Gyrodinium instriatum* that was isolated from a James River sample. The cell concentrations ranged from 1,000 – 10,000 cells/ml and the CHLa concentration in the high dose treatment was 318.22 ug/L. One set with live cells and lysates used *C. virginica* veligers as the test organism (Fig. 19) and another set used *C. variegatus* (Fig. 20 & 21). Very low mortality (<2%) was observed in the assays with *C. variegatus*, while after 120 hr there was mortality in the lysate assay with *C. virginica* veligers. Mortality was ~55% in the samples after 120 hr with lysate concentrations equivalent to 2,500, 5,000 and 10,000 cells/ml. Mortality in the lysate sample equivalent to 1,000 cells/ml was ~40%. Mortality in the control and live cell treatments was less than 10% (Fig. 19).

A culture of *Gymnodinium aureolum* was established from a James River sample. Bioassays with live cells and lysates with cell concentrations ranging from 1,000 to 10,000 cells/ml were conducted with *C. variegatus* larvae and *C. virginica* veligers. The CHLa concentration was 71.03 ug/L for the 10,000 cells/ml in the *C. virginica* assays and 96.36 for 10,000 cells/ml in the *C. variegatus* assays. Mortality was <1% for controls and all cell concentrations with both *C. virginica* and *C. variegatus* (Figs. 22, 23 & 24).

A culture of *C. polykrikoides* was established from a Lafayette River sample. A bioassay with *C. variegatus* as the test species was conducted with cell concentrations ranging from 1,000 - 10,000 cells/ml. The CHLa concentration was 79.08 ug/L in the high dose treatment. Low mortality was observed in all lysate treatments (<3%) (Fig. 26). In the live cell treatments mortality was low for all treatments except the 10,000 cells/ml where at 96hr there was ~33% mortality (Fig. 25). In a previous assay with *C. virginica* veligers as the test organism, exposure to lysate equivalent to 10,000 cells/ml of a York River *C. polykrikoides* isolate resulted in ~50% mortality after 120hr (Fig. 27 A, B). Lower mortality (~22%) was observed in the live cell exposure of 10,000 cells/ml. Interestingly, exposure to live cells and lysates of a *C. polykrikoides* isolate from Florida resulted in 80-100% mortality after 120hr (Fig. 27 C, D)

## **Discussion**

The human and animal health impacts of most of the organisms that are found to bloom in the James River system, including *C. polykrikoides*, have not been adequately assessed. Many of the species are reported to produce harmful toxins under certain conditions and have demonstrated effects on marine life based on studies conducted in other estuarine systems. These organisms exert their harmful effects by several different mechanisms including mechanical disruption or clogging of respiratory organs or production of potent neuro- or hepatotoxins that can result in gastrointestinal distress, respiratory failure, neurologic symptoms and in some cases, death. Additionally, they can impact aquatic organisms indirectly by causing hypoxia/anoxia of waters in which blooms are dying and decomposing (Cardwell et al. 1979, Hallegraeff 1992, Grzebyk et al. 1997, Botes et al. 2002, Sato et al. 2002, Codd et al. 2003, Licea et al. 2004, Heil et al. 2005, Wiegand and Pflugmacher 2005, Vargas-Montero et al. 2006, Marshall et al. 2008, Marshall & Egerton is 2009, Jessup et al. 2009,

Richlen et al. 2010). The studies described herein are aimed at trying to understand the linkages, if any, between aqueous chlorophyll a (CHLa) levels in the James River system, cell concentrations of specific phytoplankton species and biological impacts.

Bioassays in 2013 were focused on using laboratory cultures established from James River samples. However, during the “spring” 2013 *H. triquetra* bloom three field samples were tested in bioassays. Although high cell (8,500 -13,000 cells/ml) and CHLa (70-191 ug/L) concentrations were observed in these field samples, bioassays exposing both *A. salina* and *C. variegatus* exhibited negligible mortality.

Mild and largely non-specific histopathological endpoint were observed in adult oysters deployed at the VIMS James River and ODU Lafayette River ConMon stations from May through November, however, these changes could not be clearly attributed to exposure to *C. polykrikoides* blooms. Oysters collected in November from both sites demonstrated less hemocytosis and disruption of gills and intestinal epithelia than was observed in the early samples. Hemocytosis is known to occur in bivalve molluscs exposed to HAB challenges (e.g., Hégaret and Wikfors 2005, Harmful Algae 4:201-209), and while this often is focused around the gut and digestive gland (Galimany et al. 2008, Harmful Algae 7:702-711), exposure to at least one algal species (*Alexandrium monilatum*) has produced marked hemocytosis and erosion in gills in *C. virginica* in experimental systems (Carnegie, Reece, and Vogelbein, unpublished). It is possible, however, that this pathology was due to other factors including parasites, viruses or other unidentified stressors.

We established cultures of *Microcystis aeruginosa* and did three bioassays with *C. variegatus* as the test organism. *M. aeruginosa* is known to be able to produce a suite of toxic molecules including protein phosphatase inhibitors known to be potent hepatotoxins in vertebrates (Codd et al. 2003, Wiegand and Pflugmacher 2005). During the bioassays we conducted there was large variation in the mortality rates observed (Figs. 10-15) and the results did not correlate with the concentration of *M. aeruginosa* used in the assays. Therefore, we hypothesize that there are unclear differences in the culture state and in the amount of toxin that is being produced. Interestingly, we did an ELISA assay on a sample of the culture that was used for bioassay #2.

Microcystin was not detected, however, the ELISA assay is unable to efficiently detect all forms of microcystin, so it is possible that toxin was present.

The bioassays with different HAB species have demonstrated wide variation in the biological impacts at comparable cell and CHLa concentrations. For example, no or very low mortality was observed in the bioassays with *C. variegatus* larvae exposed to *P. minimum*, *G. aureolum* and *G. instriatum* at CHLa concentrations of 20.52 ug/L, 96.36 and 243.54 ug/L, respectively. However, 100 % mortality occurred in the bioassay with *C. variegatus* exposed to *K. veneficum* at lysate equivalent to a cell concentration of ~70,000 cells/ml and a CHLa concentration of 37.13 ug/L. Also, at very low CHLa concentration (~1.3 ug/L) and a cell concentration of 2,500 cells/ml, 60-70% mortality occurred in oyster veligers exposed to live *K. veneficum* cells. *K. veneficum* is known to produce toxins that have been associated with fish kills (Deeds et al. 2006) and many studies have already demonstrated harmful effects on finfish and shellfish (Deeds et al. 2002, Kempton et al. 2002, Stoecker et al. 2008).

Based on the field observations and results of the laboratory bioassays with both field samples and clonal *in vitro* isolate cultures the current threats to organisms in the lower James River system include the very dense *C. polykrikoides* blooms characteristic of this region, as well as even low density blooms of *K. veneficum*. In the near future, *Alexandrium monilatum* may become established in the system, as it has been detected in several samples and it appears to be expanding its range from the York River system. This organism would present a serious threat with substantial biological impacts, as many field exposures and laboratory studies have demonstrated harm to invertebrates and vertebrates (Harding et al. 2009, May et al. 2010, Reece et al. 2012).

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**Table 1:** Sampling site information for samples analyzed at VIMS during 2013.

JR oligohaline				
1	JMS050.74	1-VECOS	37.21335	-76.91730
2	JMS048.03	5-VECOS	37.23980	-76.87915
3	JMS043.78	4-VECOS	37.22775	-76.79147
5	JMS042.92	2-VECOS	37.20294	-76.78219
7	JMS032.59	3-VECOS	37.20297	-76.64833
31	Hog Island	VECOS	37.19206	-76.67853
32	James River	VECOS	37.189783	-76.63282
JR mesohaline				
8	Meso1	HRSD	36.9373	-76.4606
9	JMS017.96	VECOS CMON	37.04892	-76.504404
10	Meso2	HRSD	37.002770	-76.52387
13	Huntington Beach *	VIMS	37.016969	-76.456644
30	Idle Fleet	VECOS	37.11975	-76.64627
36	Cypress Creek #	VIMS	36.982974	-76.62029
37	JMS-16AM	HRSD/VECOS	36.9297	-76.41670
38	JMS-1AM (LE.5.3)	HRSD/VECOS	36.99	-76.46000
39	JMS-20AM (LE.5.2)	HRSD/VECOS	37.056	-76.59310
JR polyhaline				
21	Lafayette River #	CBF	36.905373	-76.306761
33	Hampton River #	CBF	37.021774	-76.34206
34	Elizabeth River #	VIMS	36.807555	-76.29307
35	Elizabeth River #	VIMS	36.809107	-76.28711

\* Lat/Long is approximate. Replicate samples were collected from this site.

# Lat/Long is approximate. Event samples were collected from blooms.

**Table 2:** HAB clonal cultures currently maintained at VIMS.

<b>Organism</b>	<b>Origin</b>	<b>Identifier</b>
<i>Alexandrium andersoni</i>	Eastham, Mass	CCMP1718
<i>Alexandrium affine</i>	Ria de Vigi, Spain	CCMP112
<i>Alexandrium catenella</i>	Monterey Bay, CA	CA-Ac
<i>Alexandrium fundyense</i>	Bay of Fundy, New Brunswick, Canada	Can-Af
<i>Alexandrium hiranoi</i>	Kanagawa, Japan	CCMP 2215
<i>Alexandrium insuetum</i>	Uchiumi Bay, Japan	CCMP2082
<i>Alexandrium leei</i>	Singapore Straits	CCMP2955
<i>Alexandrium luritanicum</i>	Laguna Obidos, Portugal	CCMP1888
<i>Alexandrium minutum</i>	Ria de Vigo, Spain	CCMP113
<i>Alexandrium monilatum</i>	York River, VA	YR-Am
<i>Alexandrium monilatum</i>	Mississippi	CCMP3105
<i>Alexandrium ostenfeldii</i>	Baltic Sea	CCMP1718
<i>Alexandrium peruvianum</i>	Pamlico Sound, NC	NC-Ap
<i>Alexandrium tamarense</i>	Delray Beach, CA	CA-At
<i>Alexandrium tamarense</i>	Da-ya Bay, Canton, China	CCMP1598
<i>Chattonella subsalsa</i>	Delaware	CCMP 2191
<i>Chloromorom toxicum</i>	Delaware	DE-Ct
<i>Cochlodinium polykrikoides</i>	Lafayette River	LafR-Cp
<i>Cochlodinium polykrikoides</i>	York River, VA	YR-Cp
<i>Cochlodinium polykrikoides</i>	Florida	FL-Cp
<i>Gymnodinium aureolum</i>	James River, VA	JR-Ga
<i>Heterosigma akashiwo</i>	Delaware	CCMP 2393
<i>Karlodinium veneficum</i>	Chesapeake Bay, VA	CCMP 1974
<i>Karlodinium veneficum</i>	James River, VA	JR-Kv
<i>Microcystis aeruginosa</i>	Cypress Creek, VA	CC-Ma
<i>Microcystis aeruginosa</i>	Lake Rooty, VA	LR-Ma
<i>Phaeocystis globosa</i>	Galapagos Island, Ecuador	CCMP1528
<i>Phaeocystis globosa</i>	Surinam, Caribbean Sea	CCMP628
<i>Prorocentrum minimum</i>	Choptank River, MD	CR-Pm
<i>Prorocentrum minimum</i>	Colonial Beach, VA	CB-Pm
<i>Prorocentrum minimum</i>	James River, VA	JR-Pm
<i>Scrippsiella trochoidea</i>	Lafayette River isolate	LafR-St-1
<i>Scrippsiella trochoidea</i>	Lafayette River isolate	LafR-St-2

**Table 3:** Chlorophyll a concentrations and *Microcystis aeruginosa* cell counts in water samples as determined by qPCR.

<b>Sample Name</b>	<b>Collection Date</b>	<b>Site</b>	<b>Cells/ml</b>	<b>Chlorophyll a</b>
4-JR213	2/18/13	James River Jamestown 4H	24.1	2.59
2-JR313	2/25/13	James River Swann's Point	<1	2.44
3-JR313	2/25/13	James River buoy 36	22.2	2.84
4-JR313	2/25/13	James River Jamestown 4H	<1	5.50
5-JR313	2/25/13	James River near Chickahominy	182	2.34
E213	2/25/13	James River, Hog Island	126	61.10
E313	2/25/13	James River, Idle Fleet	179	94.60
6-JR213	2/26/13	James River CMON	25.8	223.80
E613	3/11/13	James River mesohaline	134	191.00
E1413	4/18/13	Cypress Creek, Smithfield	39.4	no data
1-JR713	4/18/13	James River south shore	4.4	7.56
2-JR713	4/18/13	James River Swann's Point	115	9.53
3-JR713	4/18/13	James River buoy 36	16.4	4.80
4-JR713	4/18/13	James River Jamestown 4H	<1	13.15
5-JR713	4/18/13	James River near Chickahominy	<1	5.47
6-JR613	4/25/13	James River CMON	8.4	15.10
6-JR713	5/7/13	James River CMON	103	12.80
1-JR813	5/15/13	James River south shore	560	5.89
2-JR813	5/15/13	James River Swann's Point	<1	27.43
3-JR813	5/15/13	James River buoy 36	128	20.68
4-JR813	5/15/13	James River Jamestown 4H	60.6	30.07
5-JR813	5/15/13	James River near Chickahominy	102	26.43
6-JR813	5/22/13	James River CMON	<1	18.20
6-JR913	6/4/13	James River CMON	62.6	12.50
1-JR913	6/13/13	James River south shore	3,350	9.63
2-JR913	6/13/13	James River Swann's Point	3,675	23.17

3-JR913	6/13/13	James River buoy 36	1,098	19.78
4-JR913	6/13/13	James River Jamestown 4H	625	16.89
5-JR913	6/13/13	James River near Chickahominy	3,550	9.85
6-JR1013	6/18/13	James River CMON	16.5	9.90
6-JR1213	7/9/13	James River CMON	1,501	21.90
1-JR1013	7/11/13	James River south shore	75,500	25.61
2-JR1013	7/11/13	James River Swann's Point	189,750	32.07
3-JR1013	7/11/13	James River buoy 36	67,250	15.49
4-JR1013	7/11/13	James River Jamestown 4H	68,000	12.98
5-JR1013	7/11/13	James River near Chickahominy	56,500	45.41
6-JR1313	7/17/13	James River CMON	234	11.00
6-JR1413	7/24/13	James River CMON	<1	12.20
6-JR1513	7/31/13	James River CMON	14.8	10.60
6-JR1613	8/6/13	James River CMON	<1	14.20
1-JR1113	8/7/13	James River south shore	93,000	50.82
2-JR1113	8/7/13	James River Swann's Point	51,000	37.76
3-JR1113	8/7/13	James River buoy 36	1,105	22.46
4-JR1113	8/7/13	James River Jamestown 4H	13,000	87.76
5-JR1113	8/7/13	James River near Chickahominy	46,000	65.64
6-JR1713	8/14/13	James River CMON	1,386	13.60
6-JR1813	8/21/13	James River CMON	0	13.10
6-JR1913	8/28/13	James River CMON	2,205,000	32.70
6-JR2013	9/3/13	James River CMON	<1	41.20
E10613	9/3/13	Lafayette River	<1	no data
6-JR2113	9/10/13	James River CMON	0	7.20
1-JR1213	9/12/13	James River south shore	0	16.66
2-JR1213	9/12/13	James River Swann's Point	26,300	14.43
3-JR1213	9/12/13	James River buoy 36	106,500	8.42
4-JR1213	9/12/13	James River Jamestown 4H	7,650	24.60
5-JR1213	9/12/13	James River near Chickahominy	4,492	15.80

6-JR2313	9/24/13	James River CMON	10,100	9.20
6-JR2413	10/3/10	James River CMON	0	11.40
6-JR2513	10/8/13	James River CMON	28.2	6.80
1-JR1313	10/16/13	James River south shore	355	11.29
2-JR1313	10/16/13	James River Swann's Point	2.6	9.33
3-JR1313	10/16/13	James River buoy 36	1.4	4.58
4-JR1313	10/16/13	James River Jamestown 4H	37.8	11.99
5-JR1313	10/16/13	James River near Chickahominy	81.2	24.41
6-JR2613	10/17/13	James River CMON	<1	6.00
6-JR2713	11/1/13	James River CMON	207	13.50

**Table 4:** Summary of bioassay data including CHLa ( $\mu\text{g/L}$ ) and cell concentrations, %mortality observed in high dose live cell and lysate treatments of fed and unfed animals, and in the control animals.

BIOASSAY DATE	HAB SPECIES	TEST ORGANISM	ISOLATE SOURCE or FIELD LOCATION	Salinity (PSU)	CHLa ( $\mu\text{g/L}$ ) High Dose	Cell Count (cells / mL) High Dose	LIVE CELL % Mortality High Dose Unfed Treatment	LYSATE % Mortality High Dose Unfed Treatment)	LIVE CELL % Mortality High Dose Fed Treatment)	LYSATE % Mortality (High Dose Fed Treatment	% Mortality Unfed Control	% Mortality Fed Control
5/7/12	<i>Cochlodinium polykrikoides</i>	<i>Crassostrea virginica</i>	York Rr.	20	ND	10,000	.....	.....	22.5	50.5	11.4	2.4
8/17/11	<i>Cochlodinium polykrikoides</i>	<i>Crassostrea virginica</i>	Florida	22	ND	10,000	.....	.....	100	.....	82	20
4/7/14	<i>Cochlodinium polykrikoides</i>	<i>Cyprinodon variegatus</i>	Lafayette Rr.	20	79.08???	10,000	33	2.1	.....	.....	0	.....
7/10/13	<i>Prorocentrum minimum</i>	<i>Crassostrea virginica</i>	Isolate James Rr. Bloom	20	28.59	10,000	.....	.....	22	>90	1.7	0.8
7/30/13	<i>Prorocentrum minimum</i>	<i>Cyprinodon variegatus</i>	Isolate James Rr. Bloom	20	20.52	10,000	0	2	.....	.....	0	.....
6/12 & 6/19/2013	<i>Karlodinium veneficum</i>	<i>Crassostrea virginica</i>	Isolate James Rr. Bloom	20	71.03(126k)	126k LC 133k LY	.....	.....	100	41	1.6	7.4
10/15/13	<i>Karlodinium veneficum</i>	<i>Cyprinodon variegatus</i>	Isolate James Rr. Bloom	16	37.13	70.5k LC 68.7k LY	22	100	.....	.....	0	.....
5/15/13	<i>Gyrodinium instriatum</i>	<i>Crassostrea virginica</i>	Isolate James Rr. Bloom	20	318.22	10,000	.....	.....	4	54	0.8	0.8
1/14/14	<i>Gyrodinium instriatum</i>	<i>Cyprinodon variegatus</i>	Isolate James Rr. Bloom	20	243.54	10,000	0	0	.....	.....	0	.....
6/19/13	<i>Gymnodinium aureolum</i>	<i>Crassostrea virginica</i>	Isolate James Rr. Bloom	20	71.03	10,000	.....	.....	0	0.8	0	0

**Table 4:** Summary of bioassay data including CHLa ( $\mu\text{g/L}$ ) and cell concentrations, %mortality observed in high dose live cell and lysate treatments of fed and unfed animals, and in the control animals.

BIOASSAY DATE	HAB SPECIES	TEST ORGANISM	ISOLATE SOURCE or FIELD LOCATION	Salinity (PSU)	CHLa ( $\mu\text{g/L}$ ) High Dose	Cell Count (cells / mL) High Dose	LIVE CELL % Mortality High Dose Unfed Treatment	LYSATE % Mortality High Dose Unfed Treatment)	LIVE CELL % Mortality High Dose Fed Treatment)	LYSATE % Mortality (High Dose Fed Treatment	% Mortality Unfed Control	% Mortality Fed Control
4/14/14	<i>Gymnodinium aureolum</i>	<i>Cyprinodon variegatus</i>	Isolate James Rr. Bloom	20	96.36	10,000	0	0	.....	.....	0	.....
2/26/13	<i>Heterocapsa triquetra</i>	<i>Cyprinodon variegatus</i>	Field Sample James Rr., Idle Fleet	8	94.6	13,000	16	.....	.....	.....	< 5	.....
2/20/13	<i>Heterocapsa triquetra</i>	<i>Artemia salina</i>	Field Sample James Rr., CMON	10	70	8500	< 1	.....	< 1	.....	0	0
2/27/13	<i>Heterocapsa triquetra-mixed bloom</i>	<i>Artemia salina</i>	Field Sample James Rr., MESO	8	191	ND-mixed	1.5	.....	.....	.....	25	2.4
8/16/13	<i>Microcystis aeruginosa</i>	<i>Cyprinodon variegatus</i>	Industrial Park, Richmond	0	1500	5.90E+07	62.5	100	.....	.....	0	.....
10/24/13	<i>Microcystis aeruginosa</i>	<i>Cyprinodon variegatus</i>	Industrial Park, Richmond	0	481.44	1.90E+06	94	100	.....	.....	12.5	.....
3/19/14	<i>Microcystis aeruginosa</i>	<i>Cyprinodon variegatus</i>	Industrial Park, Richmond	0	862.77	1.20E+07	0	23.5	.....	.....	9.4	.....
PENDING	<i>Microcystis aeruginosa</i>	<i>Ceriodaphnia dubia</i>	Industrial Park, Richmond	0								

**Figure 1:** Map of sampling sites. Sites names corresponding to the numbers are given in Table 1.



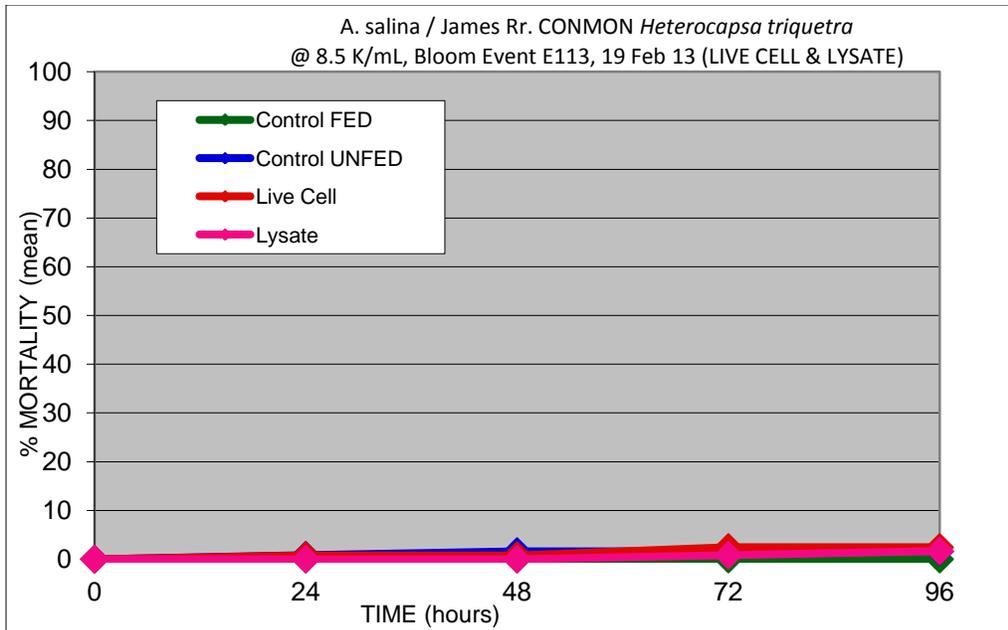


Figure 2: Percent cumulative mortality observed in a bioassay using *A. salina* nauplii exposed to a *Heterocapsa triquetra* bloom sample (live cell and lysate) with ~8,500 cells/ml. CHLa concentration = 70 ug/L.

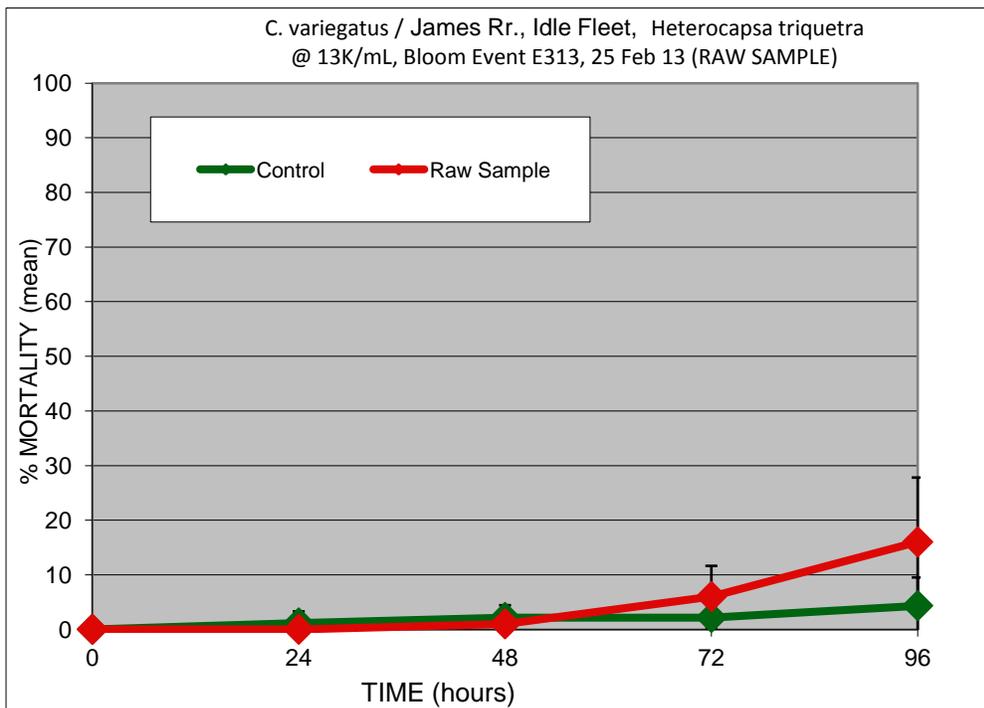


Figure 2: Percent cumulative mortality observed in a bioassay using *Cyprinodon variegatus* larvae exposed to a *H. triquetra* bloom sample with approximately 13,000 cells/ml. CHLa concentration = 94.6 ug/L.

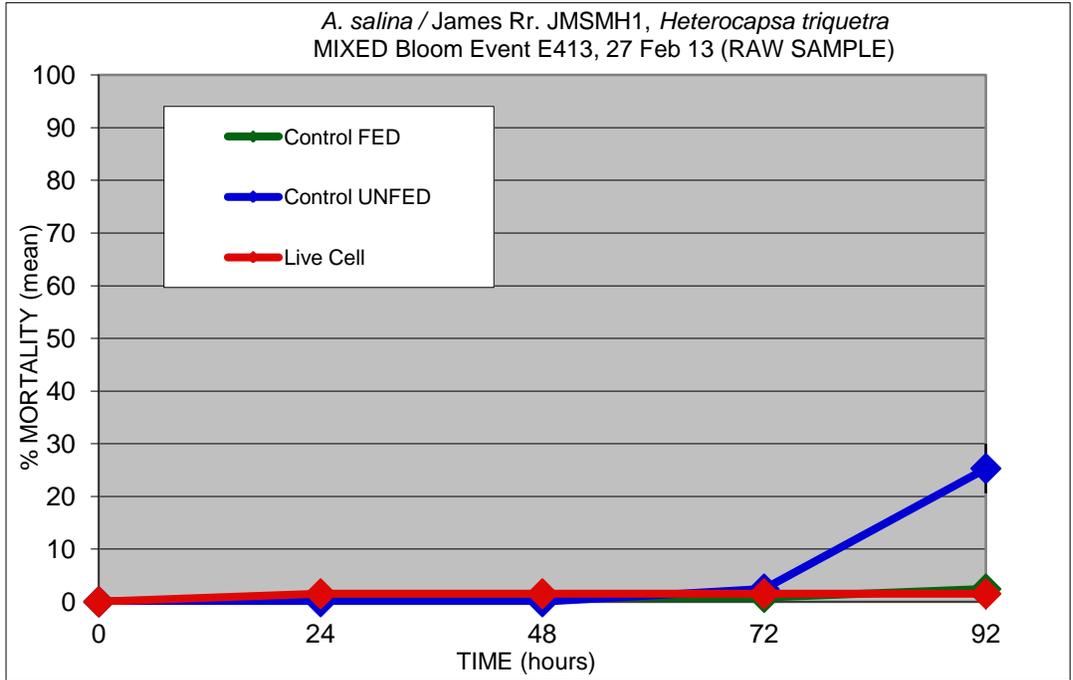
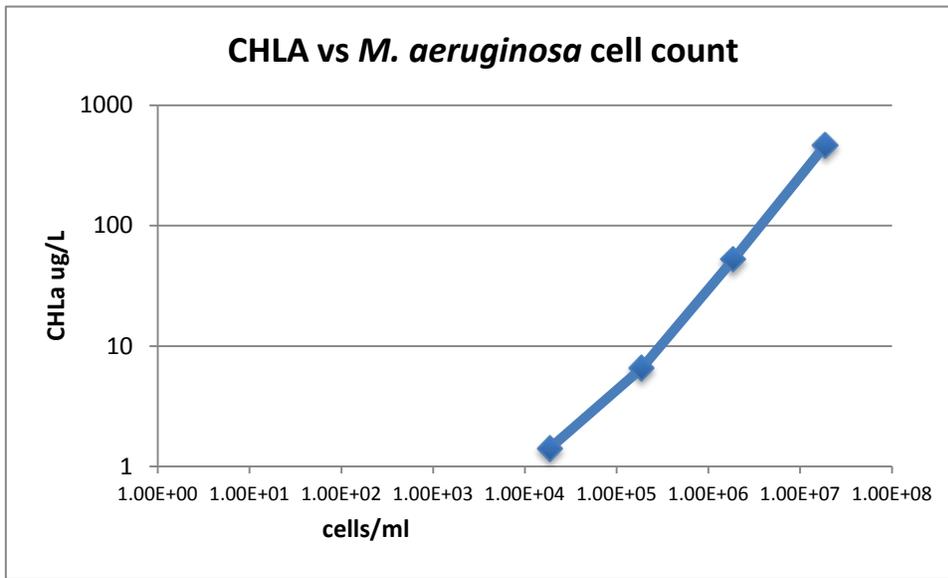
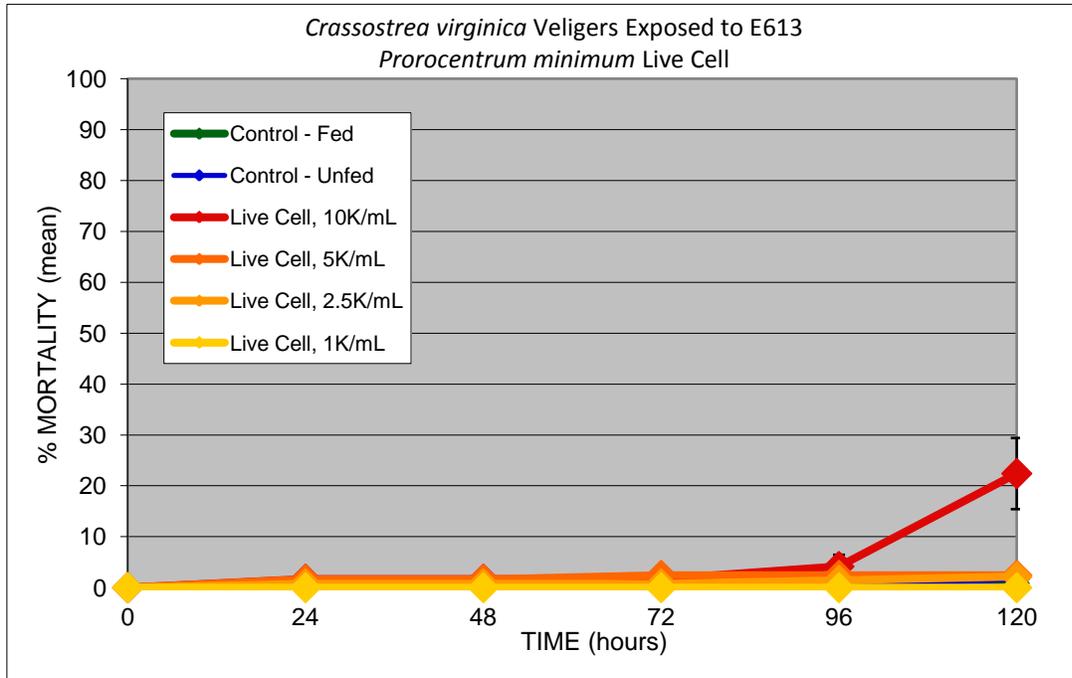


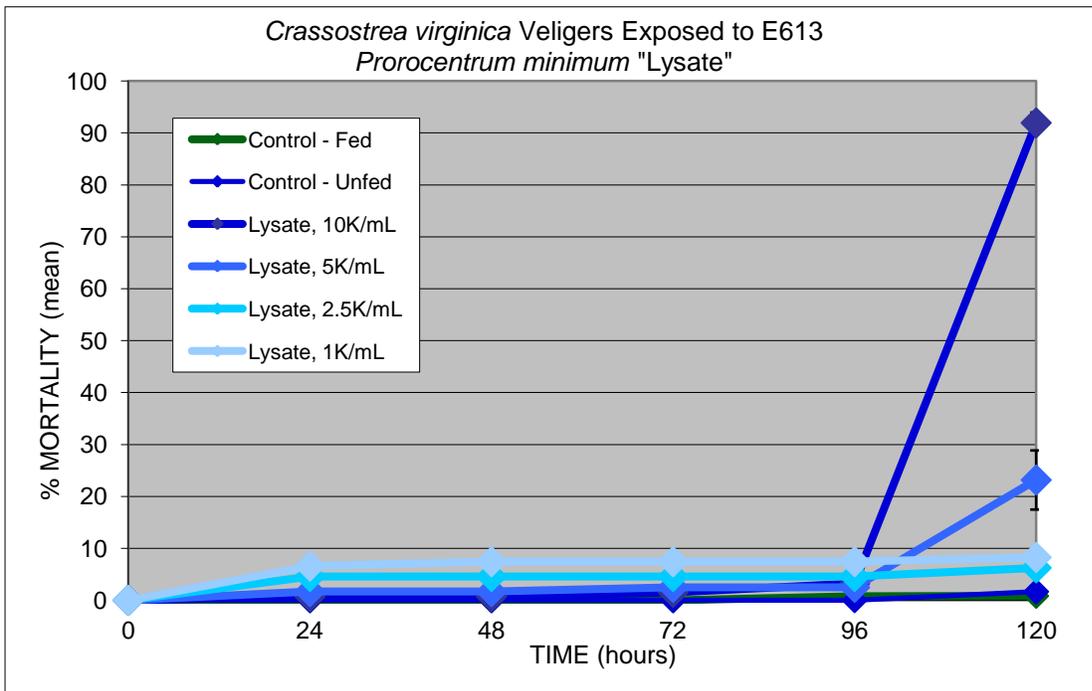
Figure 4: Percent cumulative mortality observed in a bioassay using *A. salina* nauplii exposed to a mixed bloom sample with 1,000's of cells/ml of *H. triquetra*, *H. rotundata* and *Cryptoperidiniopsis brodyi*. CHLa concentration = 191 ug/L.



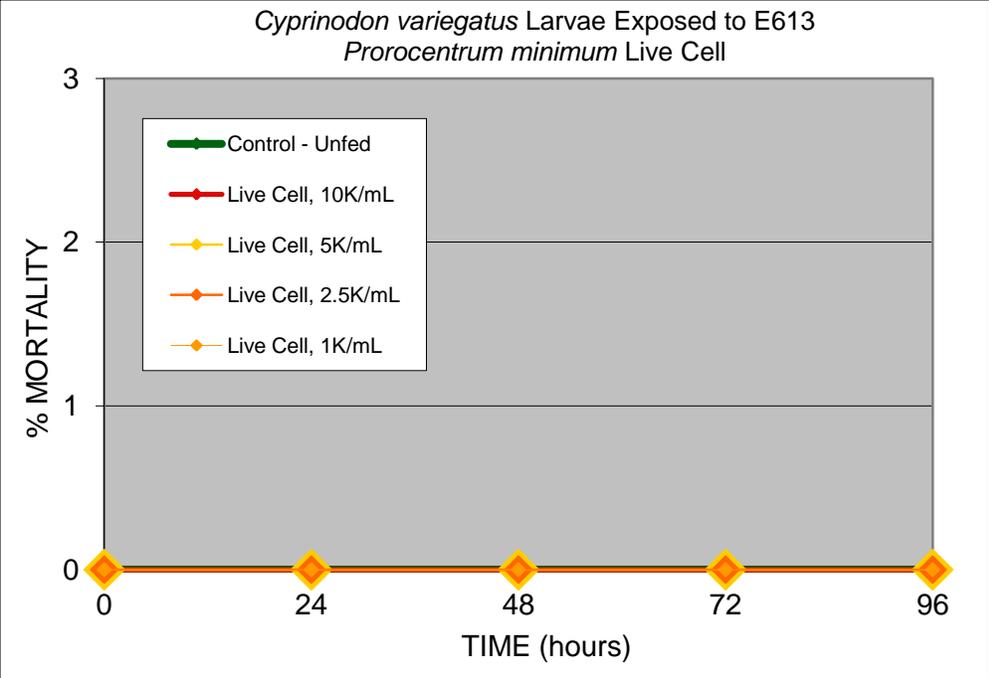
**Figure 5:** CHLa concentrations as a function of *M. aeruginosa* cells/ml. Values are plotted on log scales.



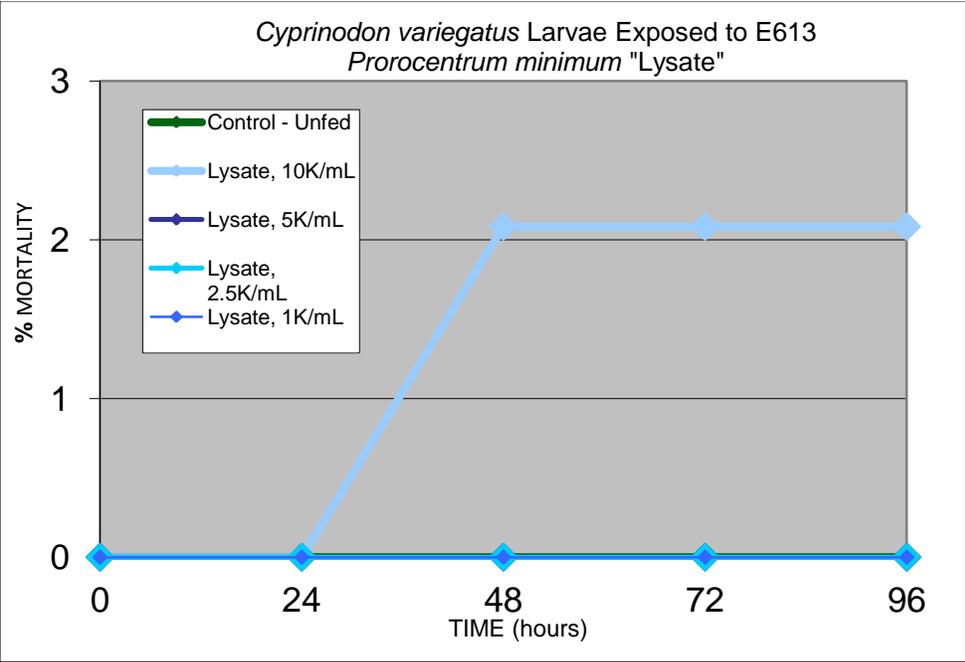
**Figure 6:** Percent cumulative mortality observed in a dose response bioassay using *Crassostrea virginica* veligers exposed to *Prorocentrum minimum* cells at concentrations ranging from 1,000 – 10,000 cells/ml.



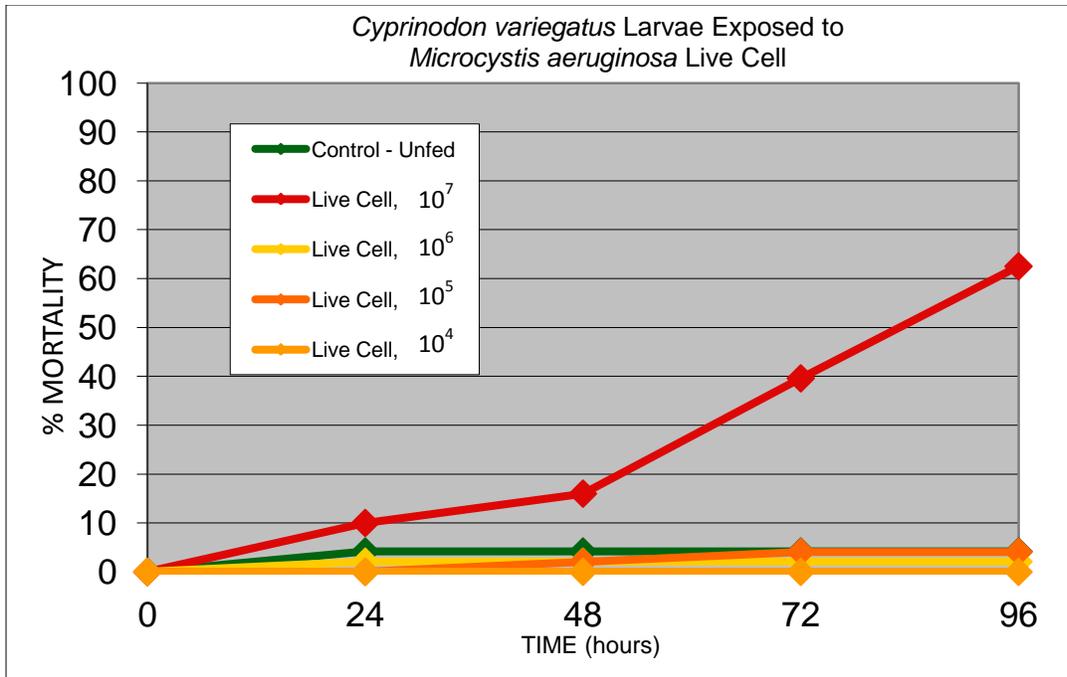
**Figure 7:** Percent cumulative mortality observed in a dose response bioassay using *Crassostrea virginica* veligers exposed to *Prorocentrum minimum* cell lysate at concentrations ranging from 1,000 – 10,000 cells/ml.



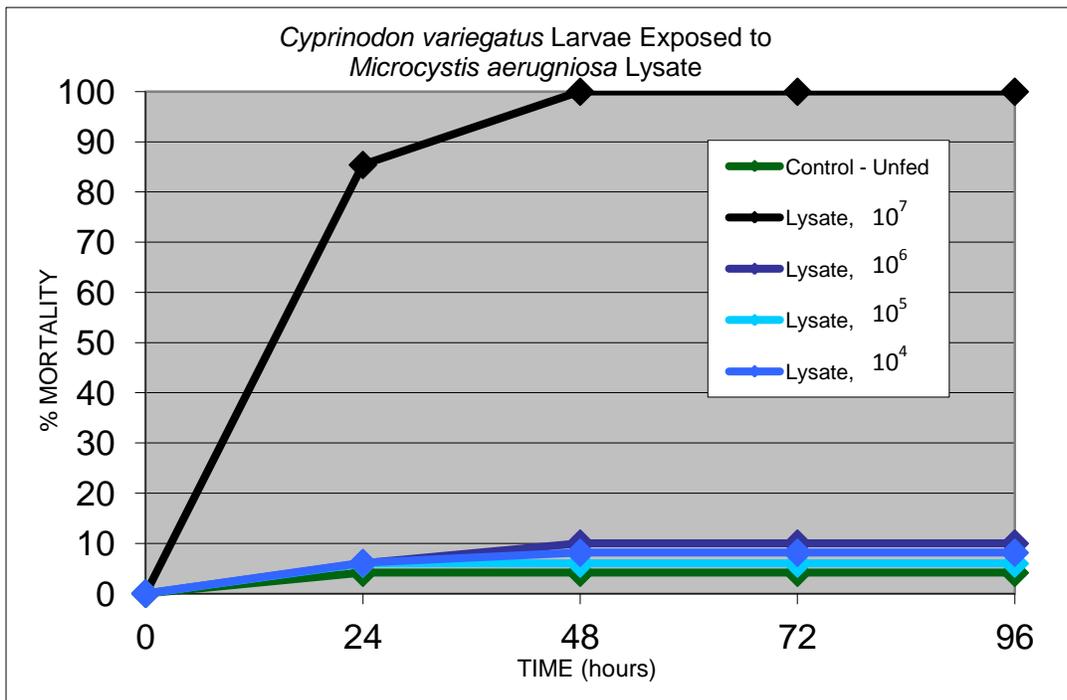
**Figure 8:** Percent cumulative mortality observed in a dose response bioassay using *Cyprinodon variegatus* larvae exposed to *Prorocentrum minimum* cells at concentrations ranging from 1,000 – 10,000 cells/ml. CHLa = X for XXX cells/ml



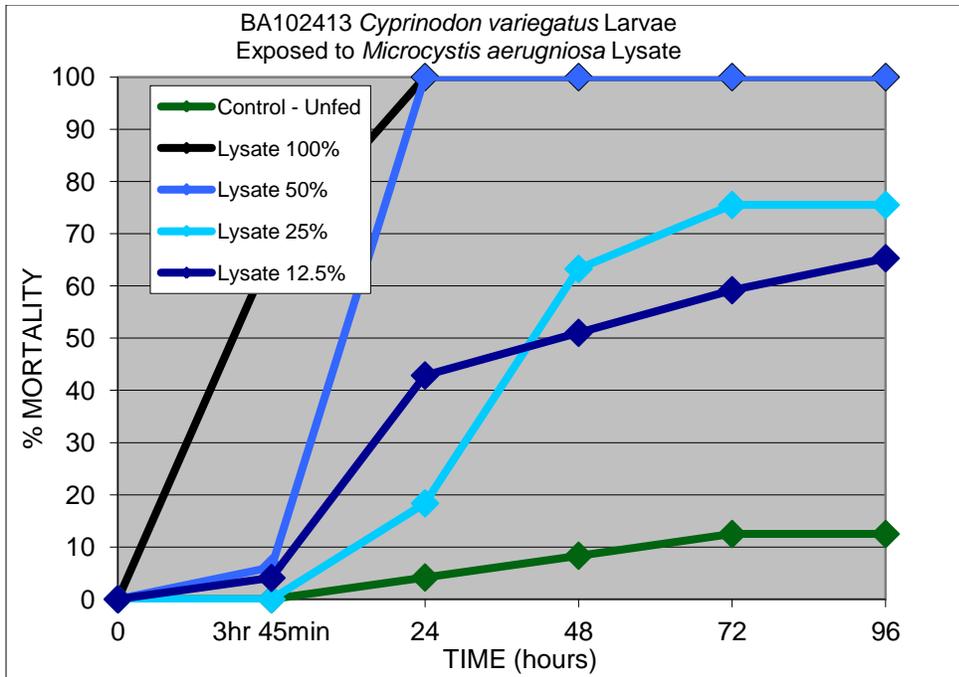
**Figure 9:** Percent cumulative mortality observed in a dose response bioassay using *Cyprinodon variegatus* larvae exposed to *Prorocentrum minimum* cell lysate at concentrations ranging from 1,000 – 10,000 cells/ml. CHLa = X for XXX cells/ml



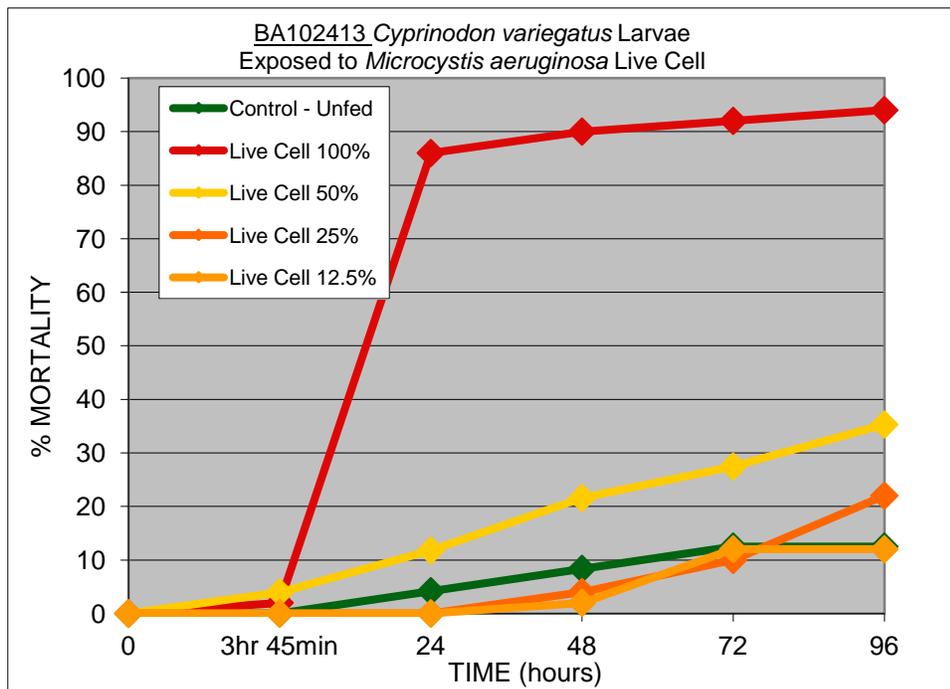
**Figure 10:** Percent cumulative mortality observed in a dose response bioassay using *Cyprinodon variegatus* larvae exposed to *Microcystis aeruginosa* cells at concentrations ranging from  $5.9 \times 10^4$  –  $5.9 \times 10^7$  cells/ml.



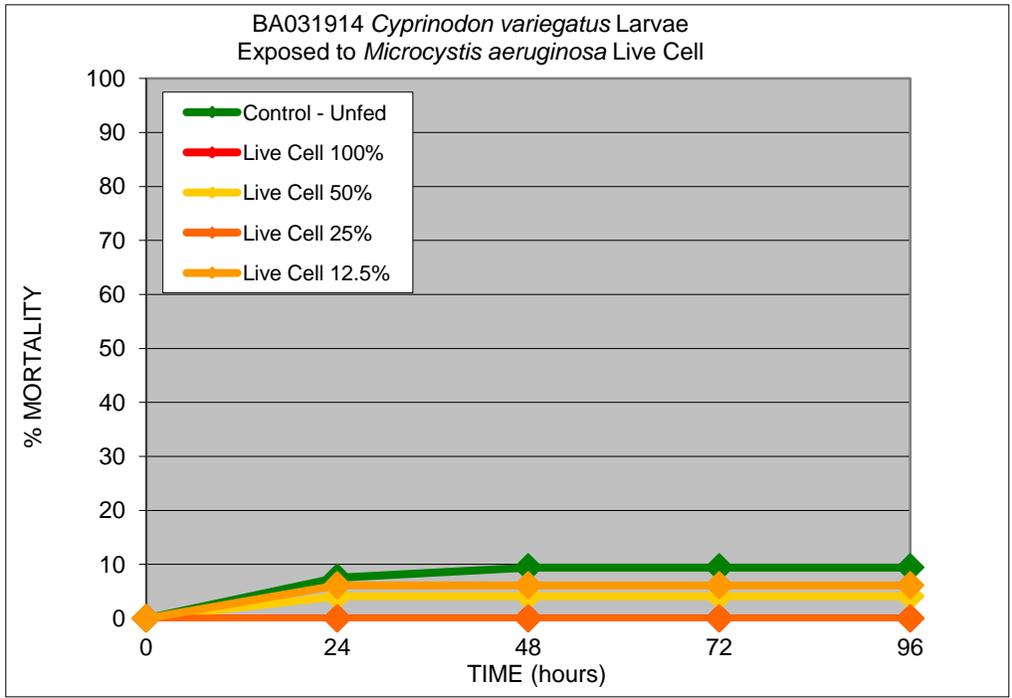
**Figure 11:** Percent cumulative mortality observed in a dose response bioassay using *Cyprinodon variegatus* larvae exposed to *Microcystis aeruginosa* cell lysate at concentrations ranging from  $5.9 \times 10^4$  –  $5.9 \times 10^7$  cells/ml.



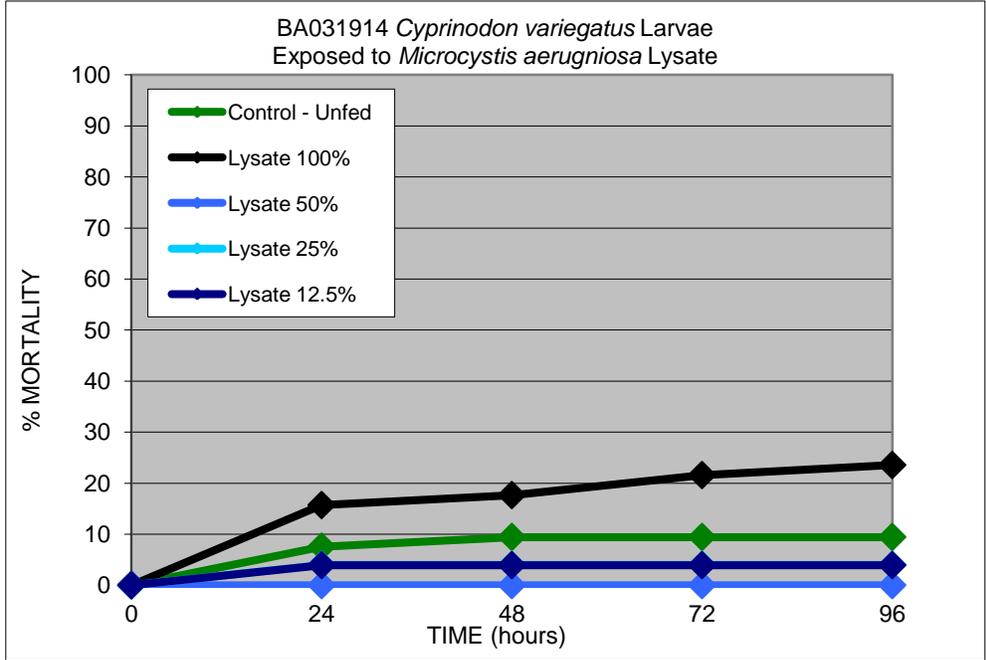
**Figure 12:** Percent cumulative mortality observed in a dose response bioassay using *Cyprinodon variegatus* larvae exposed to *Microcystis aeruginosa* lysate ranging concentrations equivalent to  $1.375 \times 10^6$  to  $1.1 \times 10^7$  cells/ml.



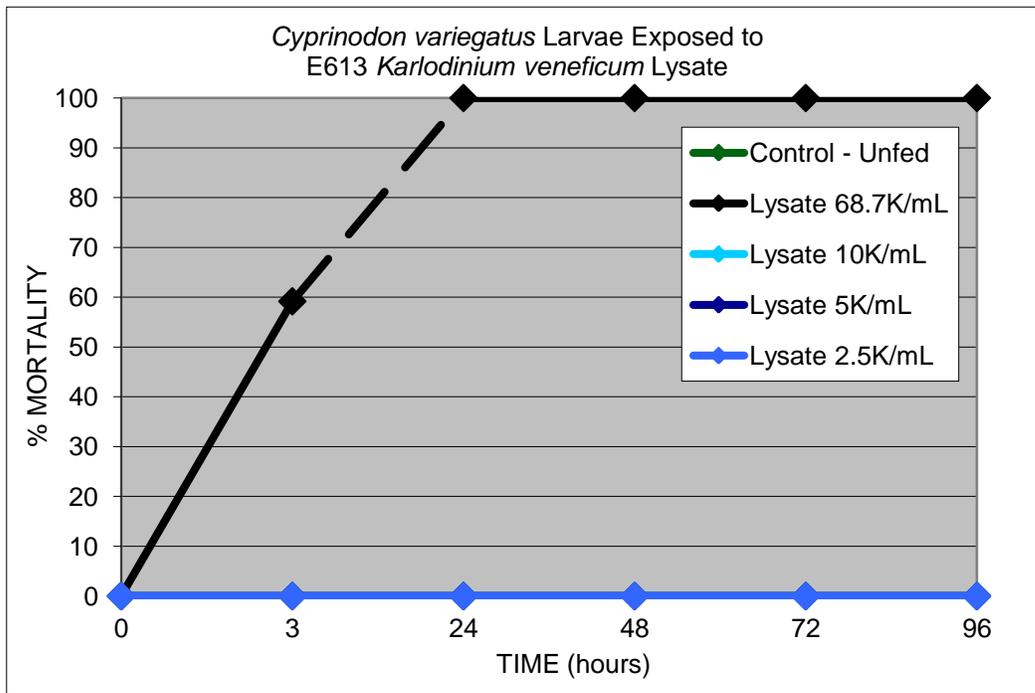
**Figure 13:** Percent cumulative mortality observed in a dose response bioassay using *Cyprinodon variegatus* larvae exposed to *Microcystis aeruginosa* live cells at concentrations ranging from  $1.375 \times 10^6$  to  $1.1 \times 10^7$  cells/ml. CHLa = X for XXX cells/ml



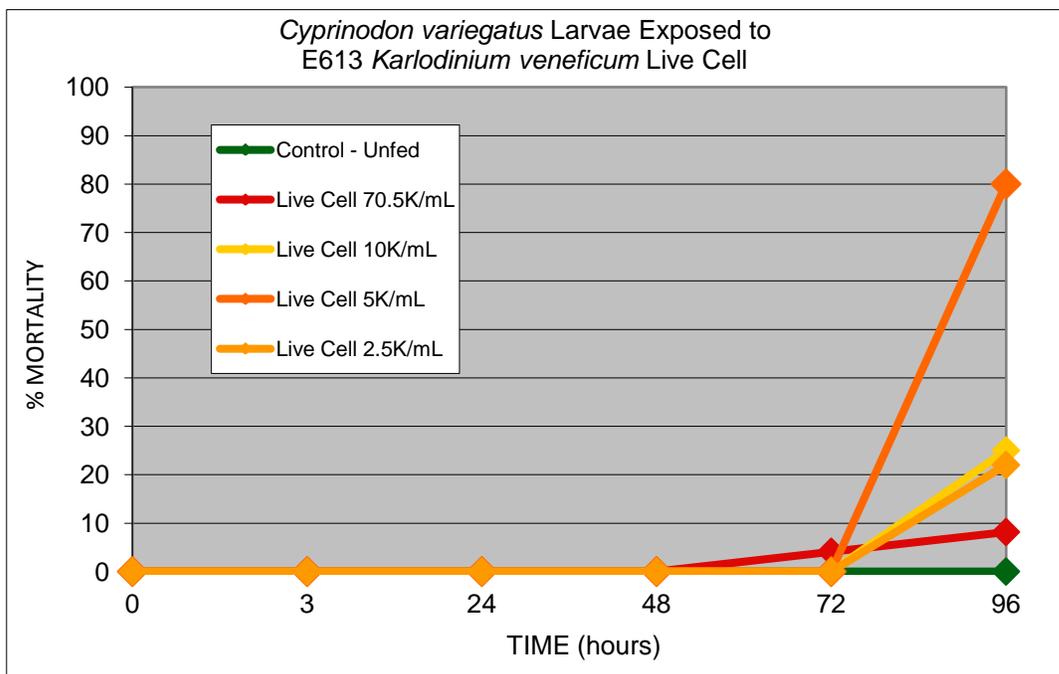
**Figure 14:** Percent cumulative mortality observed in a dose response bioassay using *Cyprinodon variegatus* larvae exposed to *M. aeruginosa* live cells ranging in concentrations from  $1.5 \times 10^6$  –  $1.2 \times 10^7$  cells/ml. CHLa = X for XXX cells/ml.



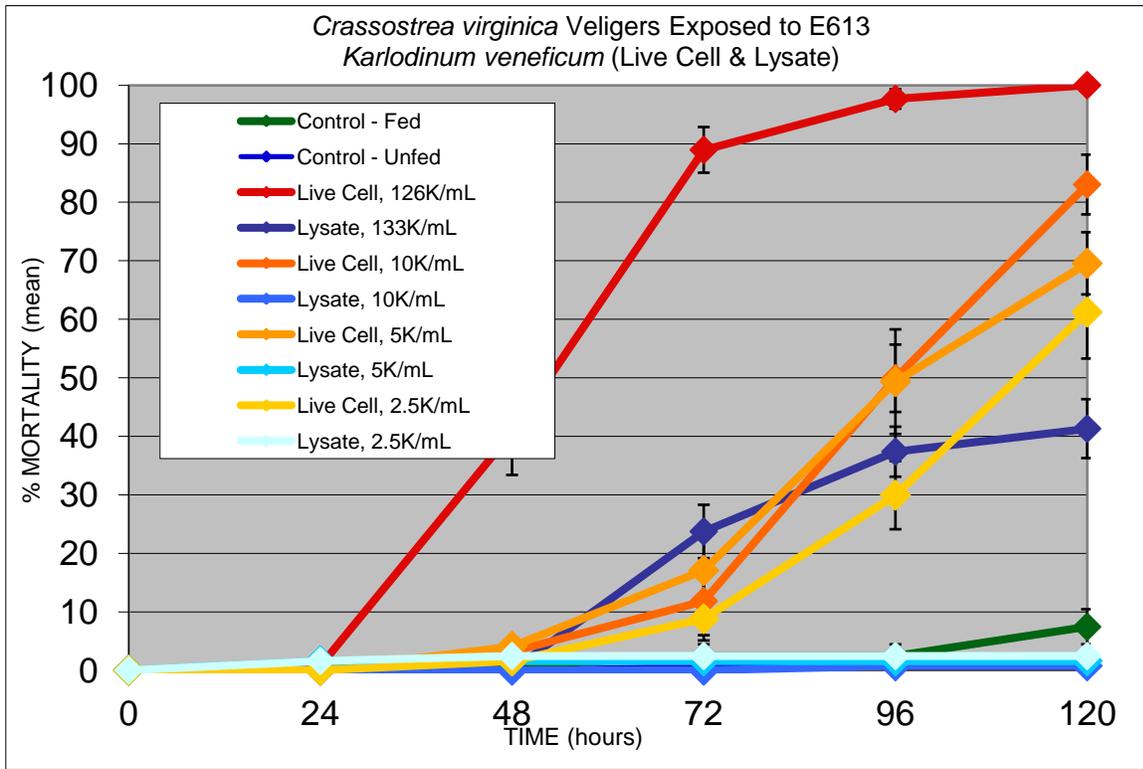
**Figure 15:** Percent cumulative mortality observed in a dose response bioassay using *Cyprinodon variegatus* larvae exposed to *M. aeruginosa* lysate ranging in concentrations equivalent to  $1.5 \times 10^6$  –  $1.2 \times 10^7$  cells/ml. CHLa = X for XXX cells/ml.



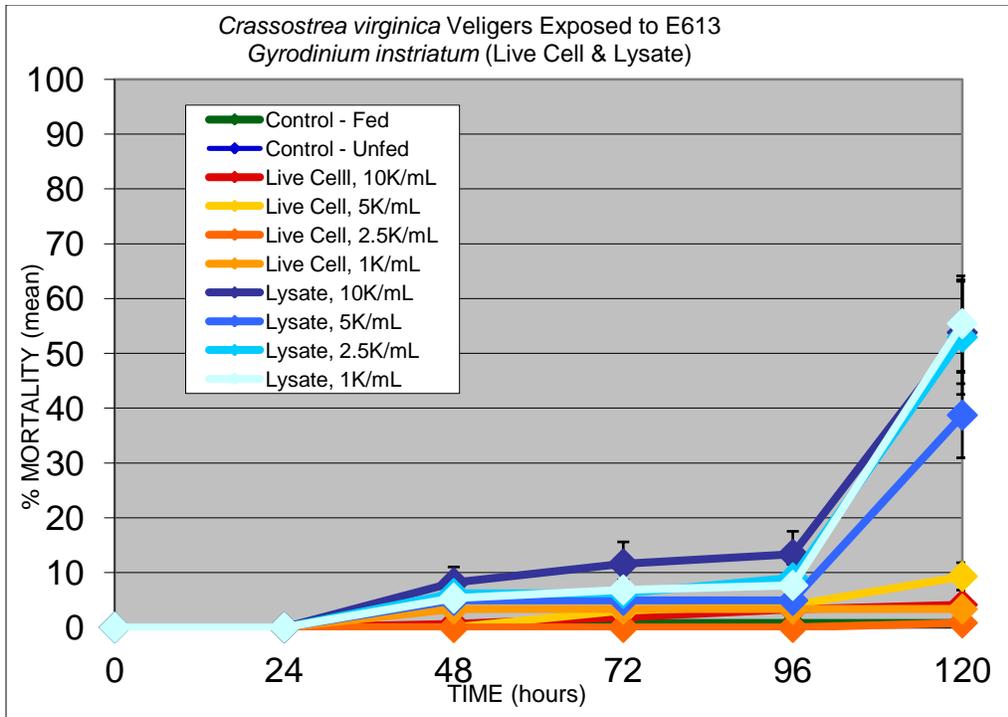
**Figure 16:** Percent cumulative mortality observed in a dose response bioassay using *Cyprinodon variegatus* larvae exposed to *Karlodinium veneficum* lysate ranging concentrations equivalent to 2,500 – 68,700 cells/ml.



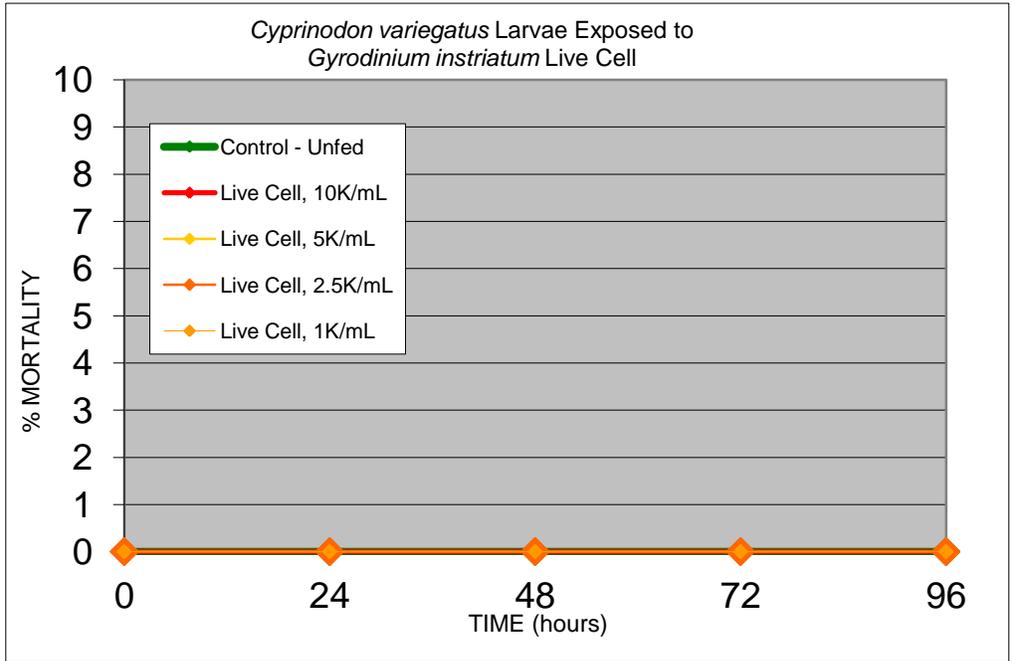
**Figure 17:** Percent cumulative mortality observed in a dose response bioassay using *Cyprinodon variegatus* larvae exposed to *Karlodinium veneficum* live cells at concentrations ranging from 2,500 – 70,500 cells/ml.



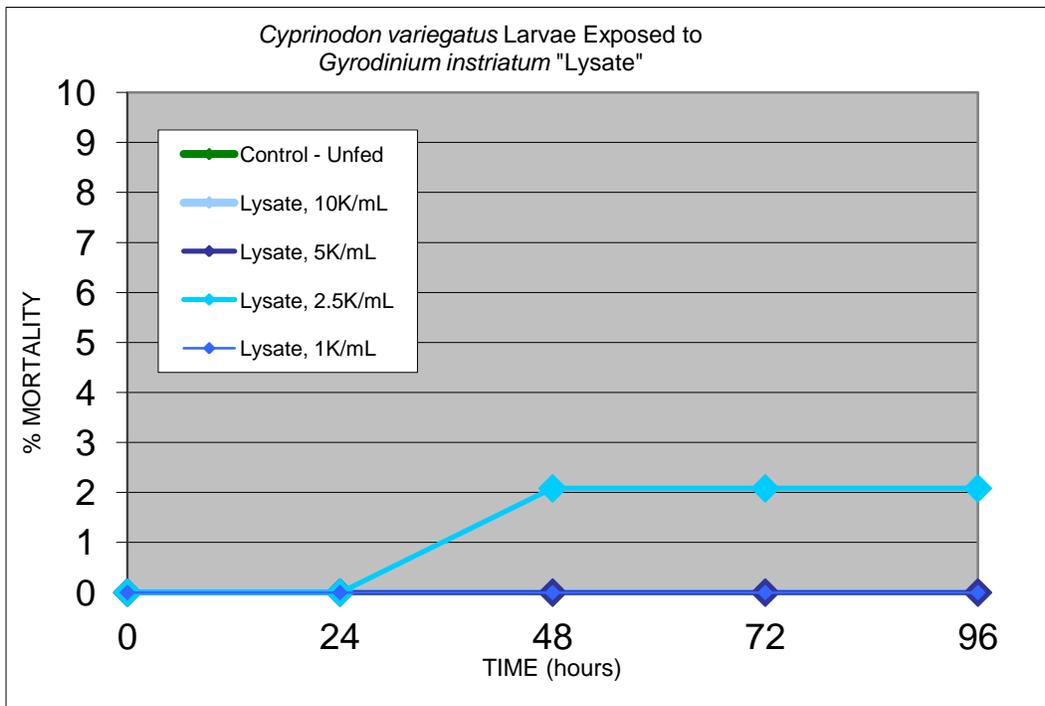
**Figure 18:** Percent cumulative mortality observed in a dose response bioassay using *C. virginica* veligers exposed to *Karlodinium veneficum* live cells at concentrations ranging from 2,500 – 126,000 cells/ml and lysate equivalent to 2,500 – 133,000 cells/ml.



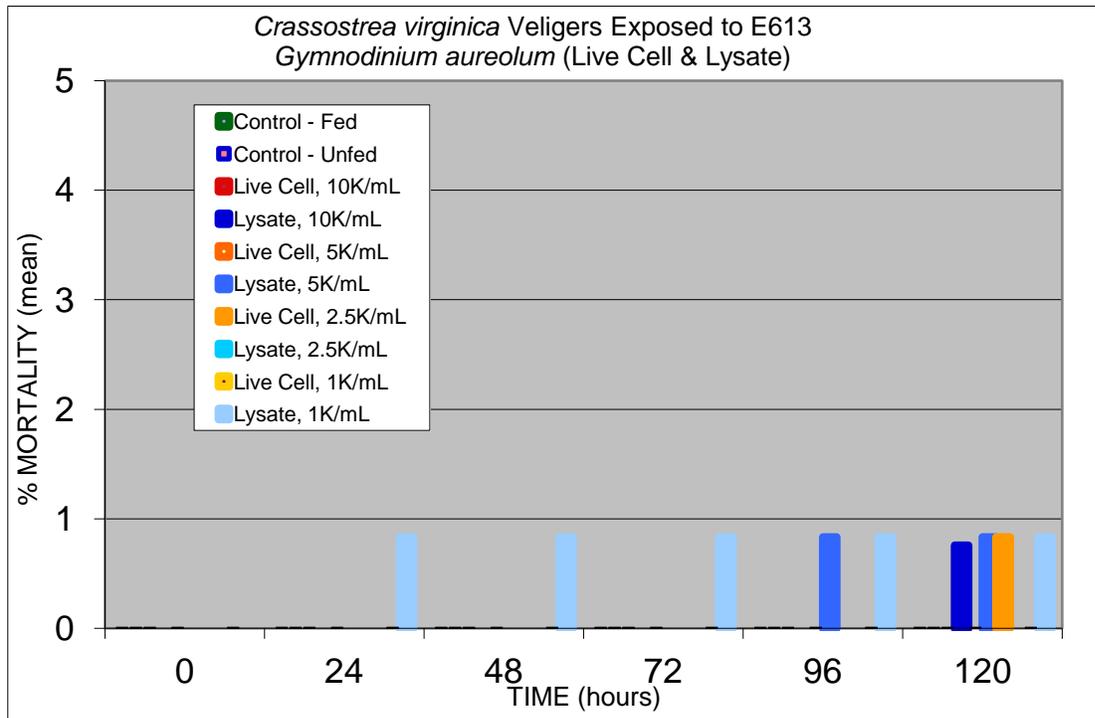
**Figure 19:** Percent cumulative mortality observed in a dose response bioassay using *C. virginica* veligers exposed to *Gyrodinium instriatum* live cells and lysates at concentrations ranging from 1,000 – 10,000 cells/ml.



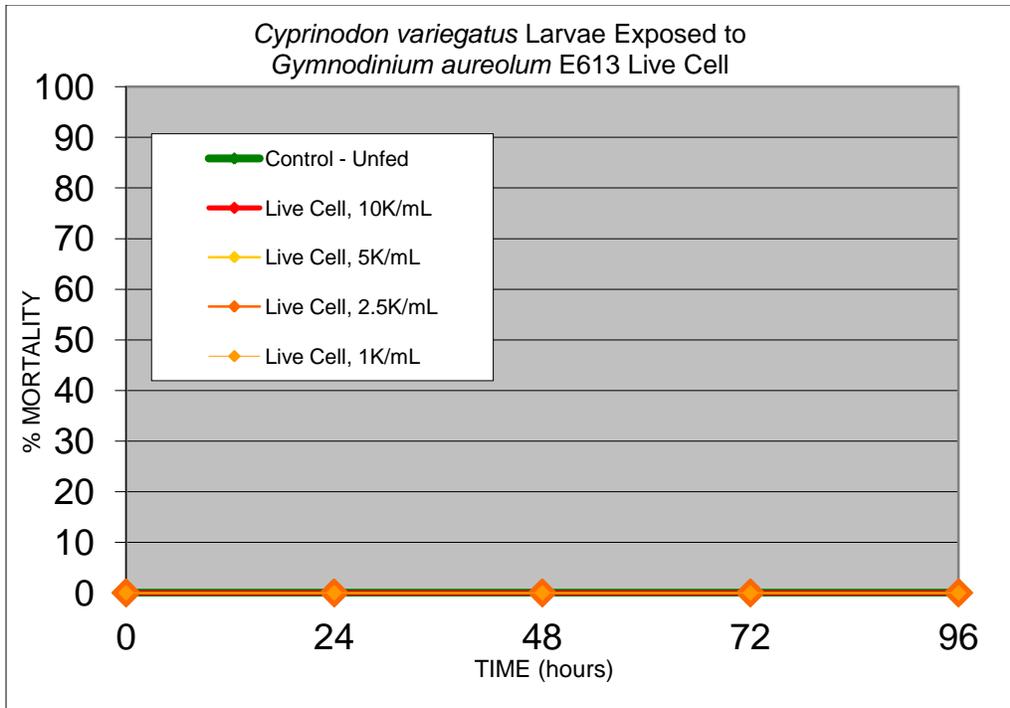
**Figure 20:** Percent cumulative mortality observed in a dose response bioassay using *C. variegatus* larvae exposed to *Gyrodinium instriatum* live cells at concentrations ranging from 1,000 – 10,000 cells/ml.



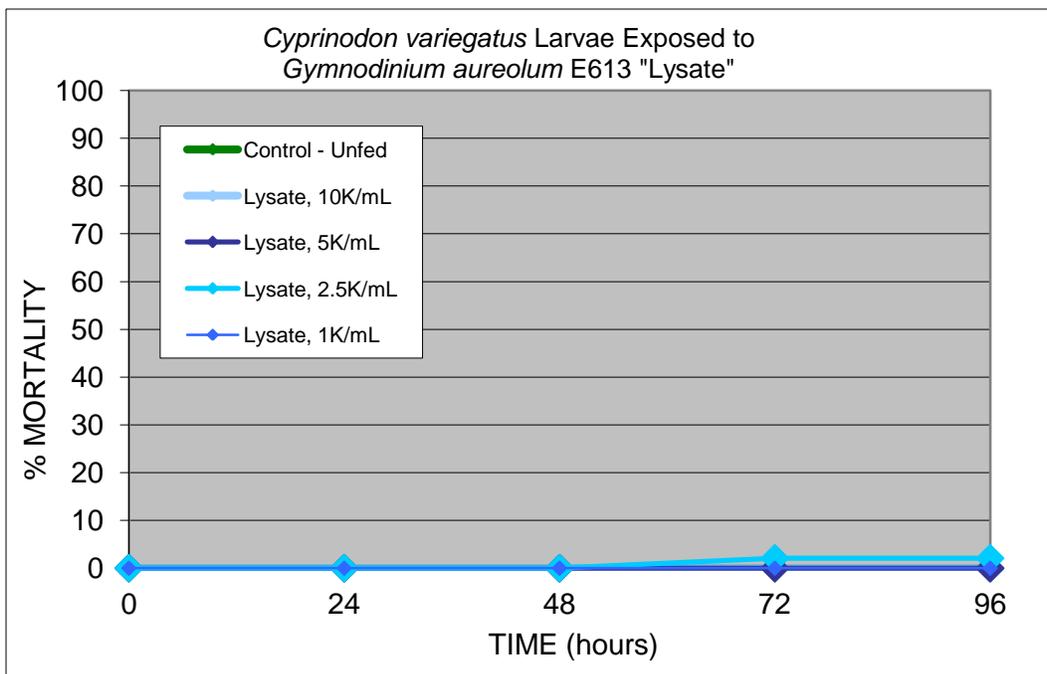
**Figure 21:** Percent cumulative mortality observed in a dose response bioassay using *C. variegatus* larvae exposed to *Gyrodinium instriatum* lysates at concentrations ranging from 1,000 – 10,000 cells/ml.



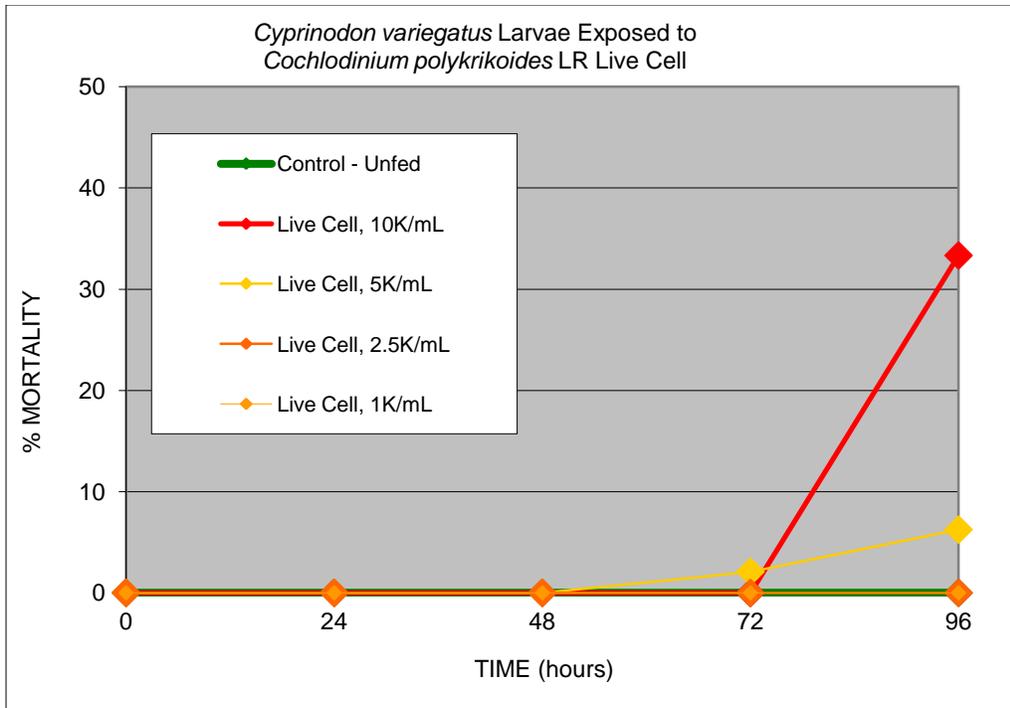
**Figure 22:** Percent cumulative mortality observed in a dose response bioassay using *C. virginica* veligers exposed to *Gymnodinium aureolum* live cells and lysates at concentrations ranging from 1,000 – 10,000 cells/ml.



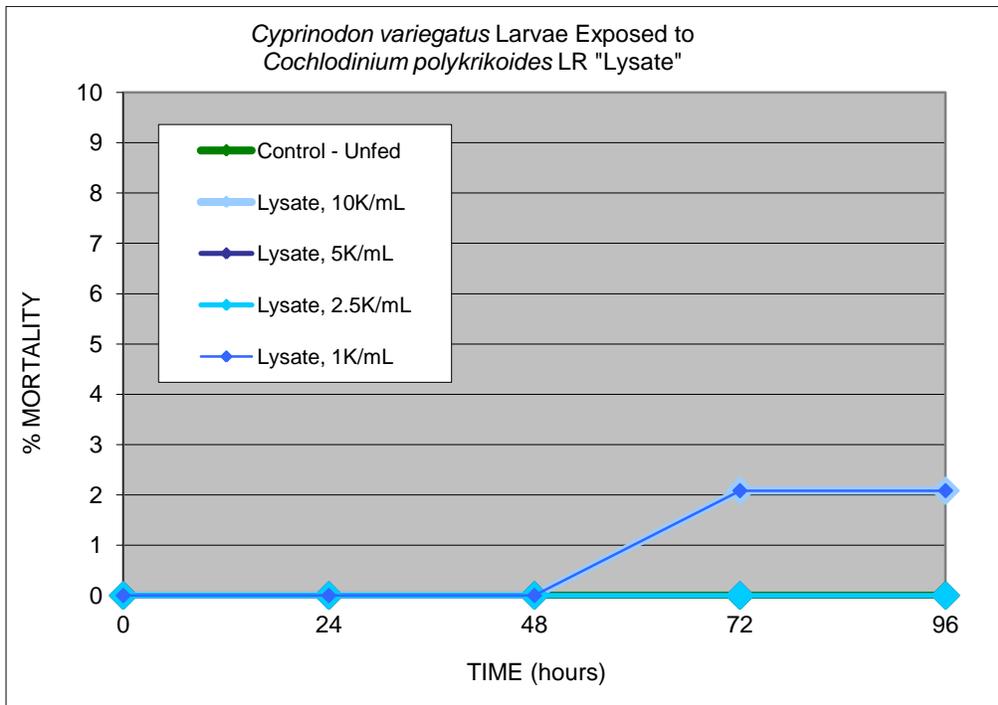
**Figure 23:** Percent cumulative mortality observed in a dose response bioassay using *C. variegatus* larvae exposed to *Gymnodinium aureolum* live cells at concentrations ranging from 1,000 – 10,000 cells/ml.



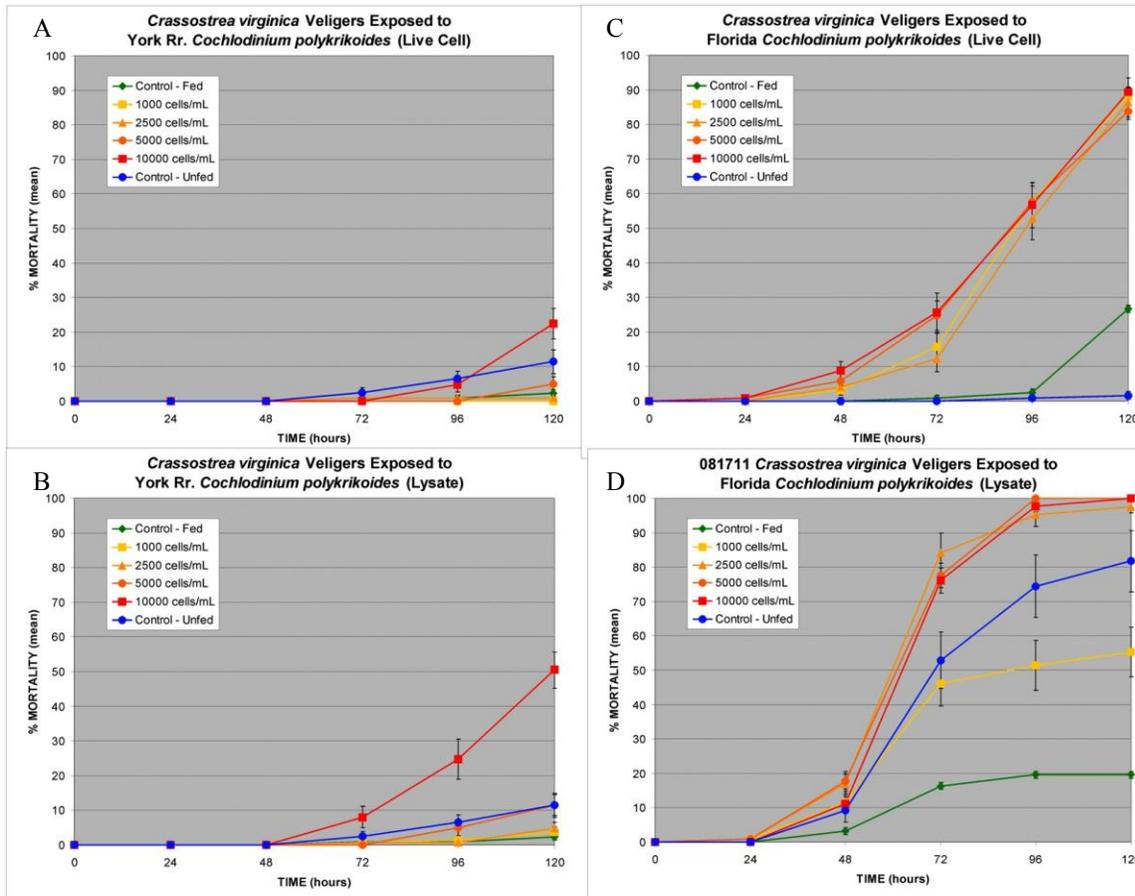
**Figure 24:** Percent cumulative mortality observed in a dose response bioassay using *C. variegatus* larvae exposed to *Gymnodinium aureolum* lysates at concentrations ranging from 1,000 – 10,000 cells/ml.



**Figure 25:** Percent cumulative mortality observed in a dose response bioassay using *C. variegatus* larvae exposed to *Cochlodinium polykrikoides* live cells at concentrations ranging from 1,000 – 10,000 cells/ml.



**Figure 26:** Percent cumulative mortality observed in a dose response bioassay using *C. variegatus* larvae exposed to *Cochlodinium polykrikoides* lysates at concentrations ranging from 1,000 – 10,000 cells/ml.



**Figure 27:** Percent cumulative mortality observed in a dose response bioassay using *C. virginica* veligers exposed to *Cochlodinium polykrikoides* live cells and lysates at concentrations ranging from 1,000 – 10,000 cells/ml. A. Live cell exposure using a York River isolate culture. B. Lysate exposure using a York River isolate culture. C. Live cell exposure using a Florida isolate culture. D. Lysate exposure using a Florida isolate culture.