

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 fish or shellfish mortalities and have impacted recreational water usage in either the Bay or 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: 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 impacts on aquatic life. 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 that we began to address in 2012 are:

1. What were the timing, intensity, duration, and spatial extent of algal blooms in the lower James, Elizabeth and Lafayette Rivers during 2012?
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?

A three-tiered framework was proposed 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 and Moore et al. 2013). Additional sample collection for toxicity bioassays was triggered when CHLa concentrations exceeded screening criteria. At the onset of the study the CHLa level selected to trigger sample collection was 50 ug/L, however this was adjusted to 150 ug/L when too many samples were being received for efficient delivery and analysis. Samples were analyzed to determine phytoplankton community composition and cell density via microscopy and/or molecular-genetic approaches, and we 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 on both bloom samples and 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. Brine shrimp, *Artemia salina* nauplii were exposed to whole cells or lysates of HAB organisms.

Materials and Methods:

Collection of water samples

Two replicate 100 ml water samples were collected from the VIMS ConMon station and during DATAFLOW cruises (for additional details on the VIMS ConMon and DATAFLOW cruises see Moore et al. 2013). VIMS and Hampton Roads Sanitation District (HRSD) personnel collected additional samples for toxicity bioassays when CHLa levels exceeded 50 ug/L until July 18, 2012 when this level was adjusted to 100 ug/L for VIMS samples and 150 ug/L for samples from HRSD. Samples were either transported in a cooler wrapped in wet towels (no ice) by HRSD and by 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 identification of dominant dinoflagellate, raphidophyte and cyanobacteria species from one of the replicate water samples was 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 and/or used for conducting bioassays as described below. 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. 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 except for the *Microcystis aeruginosa* assay developed during this study. *Microcystis aeruginosa* specific primers MICRO4_for (5'-ACGCCCCGAAGTCAGTTACCTCAAC-3') and MICRO182_rev (5'-TTTGCCTCGCTCCGACCTTTTG-3') were used for both the standard PCR assay and the SYBR[®] Green quantitative real-time PCR assay. The standard PCR mix (25 µl) contained 1× PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% w/v gelatin), 1mM MgCl₂, 0.4 mg/ml bovine serum albumin, 0.8 mM of each dNTP, 1 µM of each primer, 0.025 U of

Ampli*Taq* polymerase (Life Technologies), and 1 µl of DNA. The thermal cycling parameters included an initial denaturation of 94°C for 4 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 1-½ minutes. The final extension was at 72°C for 5 minutes. Products were electrophoresed on a 3% gel (1.5% low-melt and 1.5% standard agarose), stained with ethidium bromide and the visualized UV image was digitally recorded. The Fast SYBR[®] Green Master Mix (Life Technologies) was used for the quantitative assays following the manufacturer's protocol with 0.9 µM primer concentration using the default 7500Fast Real-time PCR System cycling parameters except that a melting analysis step was added as a control for specificity.

All bloom samples were immediately processed to determine the cell counts using the specific quantitative real-time PCR assay. All 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[®]4-TOPO[®] and transformed into TOP10 *Escherichia coli* using a TOPO[®]TA Cloning[®] 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 MgCl₂, 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. 2013) in the mesohaline region of the James River on May 30, 2012. Five oysters were processed for histopathological analysis before deployment and 15 oysters were collected and processed on June 13 as baseline (i.e. before elevated chlorophyll levels). 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 natural James River phytoplankton bloom samples or clonal cultures that we established and maintain long-term. Clonal cultures were originally established from bloom samples. No water samples were collected for analysis at VIMS from fish kills in the James River region during 2012. Larval brine shrimp (*Artemia salina*) were the test species for the natural James River samples and for dose response studies during 2012 conducted with culture whole cell and lysate material. Typically bioassays were conducted when bloom events exhibited cell densities ranging from ~100 cells/ml to ~25,000 cells/ml at salinities dependent upon the field conditions where samples were originally collected. A dilution of the bloom samples in some instances, or a dilution series of bloom-derived culture was conducted to achieve desired exposure concentrations.

Toxicity bioassays were generally conducted as outlined in the SOP protocols for the Reece and Vogelbein laboratories. Briefly, brine shrimp nauplii (10 animals per well with twelve replicates) were placed in 24-well plastic tissue culture plates with 2mL of bloom or culture material per well. For dose response assays, 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 whole 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. Brine shrimp nauplii morbidity or mortality was assessed by animals failing to continue swimming in the water column and discontinuing movement of their appendages. 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.

Results

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

A total of 157 samples were collected from the lower James River system including the Elizabeth and Lafayette Rivers for microscopic and molecular analyses during the 2012 sampling season. Sixteen samples were collected by HRSD from the Lafayette, Elizabeth or Lower James (poly- and mesohaline segments) Rivers. 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. Results of the microscopic examinations and molecular assays are given in Appendix 1 (spreadsheet). There were elevated levels of several potentially toxic species in the meso and oligohaline regions including *Heterocapsa rotundata*, *Gyrodinium instriatum*, *Scrippsiella trochoidea* and *Cochlodinium polykrikoides*. Blooms of *S. trochoidea*, *Akashiwo sanguinea* and *C. polykrikoides* were observed in the poly and mesohaline regions July through September with *C. polykrikoides* being the dominant species in the mesohaline region with cells counts up to 27,500 cells/ml (by visual count) in samples that VIMS received from HRSD. A bloom of *G. instriatum* was observed in mid-August in the oligohaline region near the Surry power plant. Bioassays (see below) were conducted on six of the bloom samples. Additionally, bioassays were conducted using in vitro isolate cultures of *Microcystis aeruginosa* and *H. akashiwo* that were established at VIMS (see below). Molecular assays indicated that *M. aeruginosa* cells were present in many of the samples collected at the site furthest upriver (lowest salinity) during the DATAFLOW cruises (Moore et al. 2013) in the oligohaline region of the James. We developed a standard PCR assay during 2012 to detect *M. aeruginosa* and converted it to a quantitative PCR (qPCR) assay to use during the 2013 season. The quantitative assay indicated <1 cell/ml in all samples, however, because we did not use a proper filtration protocol for cyanobacteria, we do not think that the cell densities were accurately determined by qPCR.

Cultures were established for several of the James River bloom species including *Akashiwo sanguinea*, *Heterosigma akashiwo*, *Scrippsiella trochoidea* and genetic analysis including DNA sequencing has confirmed the species identification of these cultures. Most of these cultures were not used in bioassays during 2012, as they had not been cloned and grown to a sufficient cell density. Fifteen *M. aeruginosa* clonal cultures were established from a bloom sample collected in 2011 from Lake Rooty, and whole 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 the pre-deployment or baseline samples of sentinel oysters, *Crassostrea virginica*, deployed at the VIMS ConMon station in the mesohaline region of the James River. Between July 22 and Aug. 10 there were several spikes in chlorophyll levels observed (>30 µg/L). This is the summary of results from the deployed sentinel oysters.

1. Fifteen oysters were collected on July 25. This followed a ten-day period with the CHLa average and maximum levels slightly elevated ranging from 5.21 – 16.27 µg/L and 10.69 – 24.30, respectively. Four days of those ten days the CHLa concentrations spiked above 20 µg/L. Twelve oysters had increased hemocytosis (elevated numbers of circulating cells in the hemolymph) in the gills and some gill erosion was observed in one individual compared to the baseline sample.
2. Fifteen oysters were collected on Aug. 8. This followed a 24 day period with CHLa levels slightly elevated. Eleven of those days the CHLa concentrations spiking above 20 µg/L and during three of the days the concentrations spiked above 30 µg/L. The average levels again ranged from 4.10 – 16.27 µg/L. Two of the 15 animals had minor gut epithelial disruption, with metaplasia (a change in epithelial cell form, often observed in oysters healing from earlier *Perkinsus marinus* challenge) observed in one of those cases. In addition, there were two animals with increased hemocytosis in the gills for a total of four animals exhibiting some minor, but non-specific, pathology.
3. Thirteen live and two dead oysters were collected on Aug. 23. One oyster exhibited gill erosion. The CHLa levels during the previous three weeks had mean levels ranging from

- 1.14 – 11.55 µg/L. On two days the levels spiked above 30 µg/L (Aug. 2, and Aug. 6), however, they did not spike above 20 µg/L on any other day from Aug. 2-23.
4. Eleven live and four dead oysters were collected on September 13. One of the oysters in this sample was moribund and exhibited extensive gill erosion. This was during a period of relatively high CHLa levels. The maximum levels were spiking between 13.39 and 103.93 µg/L during the prior three weeks. Mean levels ranged from 6.79 – 25.77 µg/L.
 5. A final sample of thirteen live and two dead oysters was collected on November 12. At that time the average CHLa levels had been below 5 µg/L for almost eight weeks except for two days in late October when the levels spiked at ~25 µg/L with mean levels of 7.8 and 9.0 µg/L on those days. These oysters exhibited no pathology.

We expect the oysters to be able to recover from some of the pathological impacts incurred by exposure to blooms during extended periods of low or no bloom activity.

A summary of the laboratory bioassays conducted for this study using *Artemia salina* nauplii as the test species and *C. virginica* larvae during a previous Sea Grant funded project is provided in Table 3.

Bioassays with *A. salina* as the test species were conducted on the following six *C. polykrikoides* bloom samples collected from the James, Lafayette or Elizabeth Rivers during 2012:

1. Collected on June 26, 2012 (assay initiated on June 27, 2012) from the Lafayette River with ~5,300 cells/ml as determined by the molecular assay, 4,400 cells/ml by a visual count and a CHLa level 94.61 µg/L. (Fig. 2a). Note that many other potential HAB species were in the sample including *C. subsalsa*, which grew during the bioassay and may have impacted this assay.
2. Collected on July 2, 2012 from the Elizabeth River with ~17,000 cells/ml as determined by the molecular assay (15,750 visual) and a CHLa level of 153.66 µg/L (Fig. 2b).
3. Collected from Hampton Roads on July 10, 2012 with ~6,000 cells/ml as determined by the molecular assay and 3,950 by a visual count and a CHLa level of 115.86 µg/L (Fig. 2c).

4. Collected on July 17, 2012 from the James River near the Warwick River with ~27,500 cells/ml by a visual count and 15,800 by the molecular assay and a CHLa level of 351.90 µg/L. This assay was conducted with the full strength bloom sample and a 1/10 dilution of the sample (Fig. 2d).
5. Collected on July 31, 2012 from Hampton Roads with a CHLa level of 451.02 µg/L. This sample lysed during transport to VIMS so that a visual cell count could not be determined. The count by the molecular assay was 2,929 cells/ml, however this was likely low due to loss of material during lysis (Fig. 2e).
6. Collected on Sept. 13, 2012 from a visible red patch in the James River at the pier where the ConMon station is located. The real-time ConMon CHLa level was 8.0 µg/L at that time. Therefore, the CHLa measurement is from a nearby location and not directly from the sample collection site. The bioassay cell concentration was 12,800/ml as determined by the molecular assay (Fig. 2f)

Mortality results for the bioassays with bloom samples are summarized in Table 4. Substantial mortalities (80-100%) were observed in the *C. polykrikoides* bloom sample bioassays with the Elizabeth River (sample #2 above, Fig. 2b) and James River (sample #4 above, Fig. 2d) field samples, while low mortality <20% was observed with the Lafayette (sample #1 above, Fig. 2a) and the July 10 Hampton Roads (sample #3 above, Fig. 2c) samples. The James River sample (#4) was used at two concentrations; undiluted and at a 1/10 dilution. 65-100% mortality was observed in the unfed *Artemia* for both concentrations after 96 hrs. Less than 10% mortality was observed in the unfed controls and for the fed animals exposed to the 1/10 dilution during this bioassay. For the lysed Hampton Roads sample that was collected on July 31 (sample #5 above, Fig. 2e), about 60% mortality was observed after 100 hours in the fed animals and about 85% mortality in the unfed animals exposed to the lysate. Substantial mortality was also observed with the *C. polykrikoides* bloom sample collected from the ConMon station in the mesohaline region of the James River relatively late in the season (i.e. Sept. 13, 2012). During this study, high mortality (~95%) was observed after 75 hrs in the unfed control animals (sample #6 above, Fig. 2f), whereas very low mortality (~10%) was observed in the fed control animals. On the other hand, ~70% mortality was observed for the fed animals exposed to the bloom sample. Overall, bioassays with *C. polykrikoides* bloom samples where CHLa levels measured over

150 µg/L and cell counts >12,000 cells/ml resulted in 80-100% mortality of *A. salina* within 96 hr.

An *Artemia salina* bioassay was done with a *Gyrodinium instriatum* bloom sample collected from the James River near the Surry Power Plant in low salinity (~5 ppt) (Fig. 3) (~2,400 cells/ml-visual count, CHLa 154µg/L). Low mortality (~10%) was observed in the unfed animals exposed to the sample after 114 hrs. The fed exposed and control animals exhibited less than 5% mortality.

Two dose response bioassays were conducted with a *Heterosigma akashiwo* *in vitro* culture that was isolated from a Lafayette River bloom sample in 2012 (Fig. 4a). The test organism was *A. salina*. A preliminary assay was terminated at 48 hrs because even the control organisms being fed *Chaetoceros muelleri* were experiencing mortality and we suspected a contaminant in the food source. This assay was repeated with a fresh food source. Extremely low mortality was observed even after 96 hr exposure (5%), even at moderately high cell concentrations (e.g., 8000 cells/ml: Fig. 4a). Slightly higher mortality (~8%) was observed in the unfed control animals.

A culture of *Microcystis aeruginosa* was previously established from a bloom sample collected from Lake Rooty in 2011. A dose response bioassay (Fig. 4b) was done with this culture and the molecular (i.e. qPCR) assay indicated that the cell concentration for both whole cell and cell lysate assays were low, ranging from 5-50 cells/ml. These cell counts were likely inaccurate because a 3 µm filter was used to collect material for the DNA extraction and the PCR/molecular assay and the size of individual *M. aeruginosa* cells is ~1-2 µm. The highest mortality (~32%) was observed in the unfed control animals after 120 hrs. The animals exposed to the 50 cell/ml lysate material experienced ~10% mortality, while <5% mortality was observed in all of the other treatments (Fig. 4b).

Discussion

There were substantial blooms (as assessed by discoloration of water or CHLa levels above 50 µg/L) of several HAB species during 2012 in the lower James River system, most notably *Cochlodinium*

polykrikoides, *Scrippsiella trochoidea*, *Microcystis aeruginosa*, *Prorocentrum micans*, *Heterocapsa triquetra*, *Heterocapsa rotundata*, *Akashiwo sanguinea* and *Gyrodinium instriatum*. The *C. polykrikoides* bloom was extensive starting in the Lafayette and Elizabeth River systems in late June-early July and spreading into the lower James by mid-July. This bloom continued into August and a sample collected as late as Sept. 13 near the James River ConMon in the mesohaline region had almost 13,000 cells/ml. While the human and animal health impacts of most of these organisms, including *C. polykrikoides*, have not been adequately assessed, many of the organisms that bloomed in the James during 2012 are known to produce harmful toxins 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).

While some pathology was observed in adult oysters deployed at the VIMS James River ConMon station site during the course of this study, its cause cannot be determined. 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. The VIMS Shellfish Pathology Laboratory investigated reports of mortality of triploid *C. virginica* that were widespread in 2012 in the Virginia portion of Chesapeake Bay. Typically, oysters surviving these events displayed similar disease signs as those expressed by oysters in this study: primarily hemocytosis at the gills, with some erosion. The 2012 mortality event waned by August, with

oysters recovering in late summer and fall, which is precisely the pattern observed in the presentation of gill pathology in this study. While we cannot rule out an event localized in the James River, it seems more likely that the oysters in this study were responding to the same general stressor, whatever it was, that was affecting oysters as far north as Milford Haven and along the bayside Eastern Shore. The level of oyster mortality was on a par with that reported by oyster growers during this period, and may have been partly due to the phenomenon described above, at least through August, after which impacts of *P. marinus* increased.

It is important to note from the results of bioassays with *C. polykrikoides* bloom samples that the level of *A. salina* mortality seen in these assays was directly related to *C. polykrikoides* cell concentrations and correlated with high CHLa levels in the original field sample. Thus, bioassays exposing *A. salina* nauplii to comparably “low” (i.e. relative to what was seen in some very dense bloom samples) cell concentrations (e.g., 2,500 - 4,000 cells/ml) (Fig. 2a, 2c, 2d 10-fold dilution treatment) and CHLa concentrations <120 µg/L exhibited final % mortality levels of < 20%. In contrast, exposure to field samples with *C. polykrikoides* concentrations > 10,000 cells/ml and CHLa concentrations >150 µg/L resulted in significantly higher mortality in the range of 60-100%. This can be seen by the results presented in Figure 2b, 2d and 2f. Figure 2e shows high mortality in a lysate exposure in which the cell concentration was unknown due to sample lysis, however, the exceedingly high chlorophyll level (i.e. 451.02 µg/L) suggests that the cell count was in excess of 20,000 cells/ml. Bloom samples of *C. polykrikoides* and the other species from the lower James River were found to be comprised of complex mixtures of phytoplankton, some of which can serve as food to zooplankton and filter-feeding metazoans. Therefore, the bioassays during which a food source is added (i.e. fed) likely most accurately reflect the impact of the bloom organisms from this study. The generally higher mortalities observed in the unfed treatments are likely a starvation effect. Overall, results highlight the critical need to conduct more detailed dose response studies on most if not all of the phytoplankton species of interest in the James River.

The results to date suggest that the *G. instriatum* bloom sample, at least at the concentrations observed during this study (i.e. ~2,400 cells/ml), does not negatively impact *A. salina*. The CHLa level from this sample, however, was 154µg/L, which is comparable to that observed for a *C. polykrikoides* bloom sample that resulted in high bioassay mortality. Again, a cautionary note

is that in the case of this *G. instriatum* bloom, the high CHLa concentration in the field was not indicative of adverse biological effect based on the bioassay.

During this sampling season we discovered several samples from the oligohaline region of the river collected during the DATAFLOW cruises that were positive in the standard PCR assay for *M. aeruginosa*. The filtration protocol that we currently use for water samples and cultures, however, did not adequately capture the cyanobacteria because the average single cell size is smaller than that of the other HAB organisms in the study. Therefore, for the 2013 samples we plan to use 0.45 μm or 0.22 μm filters, rather than 3 μm . *M. aeruginosa* forms large globular colonies that would likely be caught on filters with the larger pore size, although the single cells and smaller colonies may not have been caught on the filter resulting in us being unable to obtain an accurate cell count with the molecular assay.

Our bioassays to date have focused on organisms that bloom in the lower James River, however, there is a critical need to better understand the biological impacts of cyanobacterial blooms that are common in the upper James River and may be impacting lower regions, as well, when the organisms and associated toxins are transported downstream. Therefore, we have established cultures of *Microcystis aeruginosa* and have done some preliminary bioassays with *Artemia salina*. *M. aeruginosa* is known 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). Therefore, in our development of these assays for 2013, we plan to conduct a series of bioassays including ones that use finfish and oyster larvae. We additionally propose to explore the use of additional assay endpoints (e.g., fish liver histopathology, tissue toxin analyses, etc.) in order to develop a sensitive and rapid bioassay system to measure adverse impacts of these blooms at different cell concentrations.

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Table 1: Sampling site information for samples analyzed at VIMS

Region site #	Site	Collector	Latitude	Longitude
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
4	Jamestown Marina	VECOS	37.226692	-76.775869
5	JMS042.92	2-VECOS	37.20294	-76.78219
6	Surry Power Discharge	VECOS	37.171931	-76.702867
7	JMS032.59	3-VECOS	37.20297	-76.64833
JR mesohaline				
8	Meso1	HRSD	36.9373	-76.4606
8a	Meso1 (event)	HRSD	37.03645	-76.53607
9	JMS017.96	VECOS ConMon	37.04892	-76.504404
10	Meso2	HRSD	37.002770	-76.52387
11	Meso3	HRSD	36.99515	-76.49538
12	Meso4	HRSD	37.01115	-76.47417
13	Huntington Beach *	VIMS	37.016969	-76.456644
14	James River Bridge *	VIMS	37.013781	-76.456057
JR polyhaline				
15	13P Hampton Roads	HRSD	36.9548667	-76.39275
16	Poly5	HRSD	36.95710	-76.39030
17	JMPH2	HRSD	36.93988	-76.36980
18	Poly3	HRSD	36.94992	-76.34572
19	Poly1	HRSD	36.9258	-76.34088
19a	Poly1	HRSD	36.91658	-76.41480
19b	Poly1	HRSD	36.950400	-76.345120
20	Poly2	HRSD	36.9641	-76.33553
20a	Poly2	HRSD	36.930850	-76.409830
20b	Poly2	HRSD	36.956750	-76.3894

21	Lafayette River #	CBF	36.905373	-76.306761
22	Lafayette River – LAF1	HRSD	36.902570	-76.28990
23	Lafayette River La164.3	HRSD	36.88968	-76.28003
24	Lafayette Haven Creek #	CBF	36.885823	-76.283037
25	Elizabeth River West	HRSD	36.8448	-76.3594
26	Elizabeth River East	HRSD	36.85018	-76.30847
27	Poly4	HRSD	36.97288	-76.34945
28	21P Hampton Roads	HRSD	36.998833	-76.3135
29	HRSD1439 #	HRSD	36.987835	-76.308579

* Lat/Long is approximate. Replicate samples were collected from these sites.

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

Table 2: HAB clonal cultures currently maintained at VIMS.

Organism	Origin	Identifier
<i>Alexandrium monilatum</i>	York River isolate	YK-Am
<i>Alexandrium monilatum</i>	Mississippi isolate	AM01
<i>Akashiwo sanguinea</i>	Lafayette River isolate	Laf-As
<i>Chattonella subsalsa</i>	Delaware isolate	CCMP 2191
<i>Cochlodinium polykrikoides</i>	York River isolate	YK-Cp
<i>Cochlodinium polykrikoides</i>	Florida isolate	FL-Cp
<i>Cochlodinium polykrikoides</i>	Chincoteague isolate 2B	Ch-Cp-2b
<i>Cochlodinium polykrikoides</i>	Chincoteague isolate 3D	Ch-Cp-3d
<i>Cochlodinium polykrikoides</i>	Chincoteague isolate 6D	Ch-Cp-6d
<i>Heterosigma akashiwo</i>	Delaware isolate	CCMP 2393
<i>Heterosigma akashiwo</i>	Lafayette River isolate	Laf-Ha
<i>Karlodinium veneficum</i>	Chesapeake Bay isolate	CCMP 1974
<i>Karlodinium veneficum</i>	Chesapeake Bay isolate	CCMP 1975
<i>Karlodinium veneficum</i>	Poquoson River isolate	VIMS 2006
<i>Microcystis aeruginosa</i>	Lake Rooty, VA isolate	LR-Ma
<i>Prorocentrum minimum</i>	Choptank River isolate	JA98.10
<i>Prorocentrum minimum</i>	Colonial Beach isolate	CB-Pm
<i>Scrippsiella trochoidea</i>	Lafayette River isolate	Laf-St-1
<i>Scrippsiella trochoidea</i>	Lafayette River isolate	Laf-St-2

Table 3: Dose response bioassays using clonal *in vitro* isolate culture material.

Test Organism	Approximate Salinity range	<i>Artemia salina</i>	<i>Crassostrea virginica</i>
<i>Microcystis aeruginosa</i>	< 2 ppt	X ¹	
<i>Microcystis aeruginosa</i> lysate		X ¹	
<i>Karlodinium veneficum</i>	6 ppt - >20 ppt		X ²
<i>Karlodinium veneficum</i> lysate			X ²
<i>Gyrodinium instriatum</i>	>12 ppt	X	
<i>Gyrodinium instriatum</i> lysate			
<i>Heterosigma akashiwo</i>	>12 ppt	X	
<i>Heterosigma akashiwo</i> lysate			
<i>Prorocentrum</i> spp.	>15 ppt		
<i>Prorocentrum</i> spp. lysate			
<i>Alexandrium monilatum</i>	>18 ppt	X	X ²
<i>Alexandrium monilatum</i> lysate		X	X ²
<i>Cochlodinium polykrikoides</i>	>20 ppt	X	X ²
<i>Cochlodinium polykrikoides</i> lysate		X	X ²

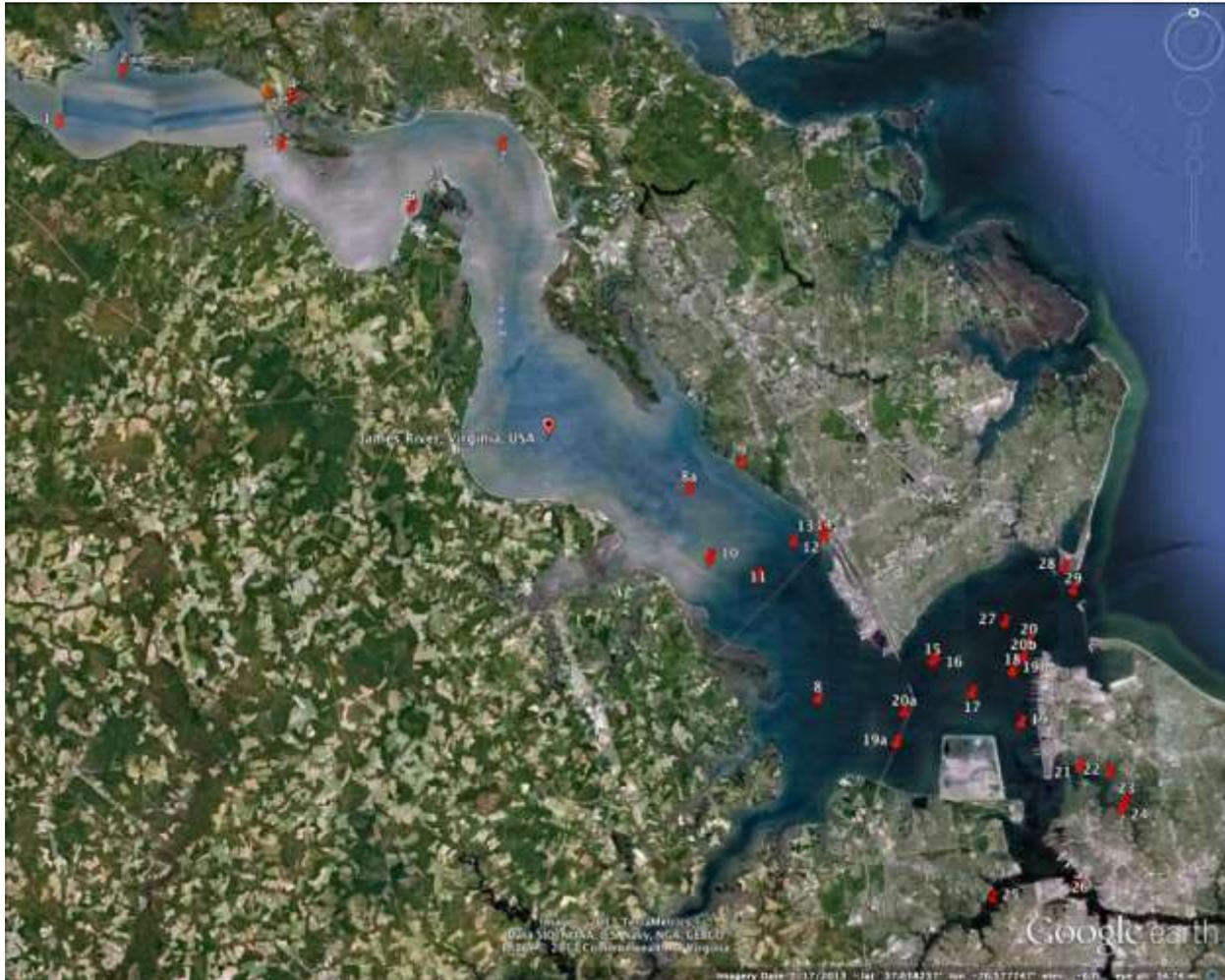
¹Additional assays will be done during 2013.

²Assays were conducted previously during VA Sea Grant funded project (see Reece et al. 2012)

Table 4: Summary of sample data and results of *A. salina* bioassays with select bloom samples

Date	Dominant Bloom Species	Location	CHLa	Cell Counts (Visual)	Cell Count (Molecular)	% Mortality (Unfed Treatments)	% Mortality (Fed Treatments)	
26-Jun	<i>C. polykrikoides</i>	Lafayette	94.61	4,400	5,300	<2	0	
July 2	<i>C. polykrikoides</i>	Elizabeth	153.66	15,740	16,981	80.15	86.36	
10-Jul	<i>C. polykrikoides</i>	Hampton Roads	115.86	3,950	5,897	19.85	4.92	
17-Jul	<i>C. polykrikoides</i>	James-Warwick	351.9	27,500	15,832	63.85, 98.41*	7.66, 90.56*	
31-Jul	<i>C. polykrikoides</i>	Hampton Roads	451.02	not determined	>2,929 (Lysed)	85.38	62.88	
13-Sep	<i>C. polykrikoides</i>	James	8.0*	not determined	12,813	82.95	72.05	
15-Aug	<i>G. instriatum</i>	James	154	2,400	not determined	11.22	4.1	

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



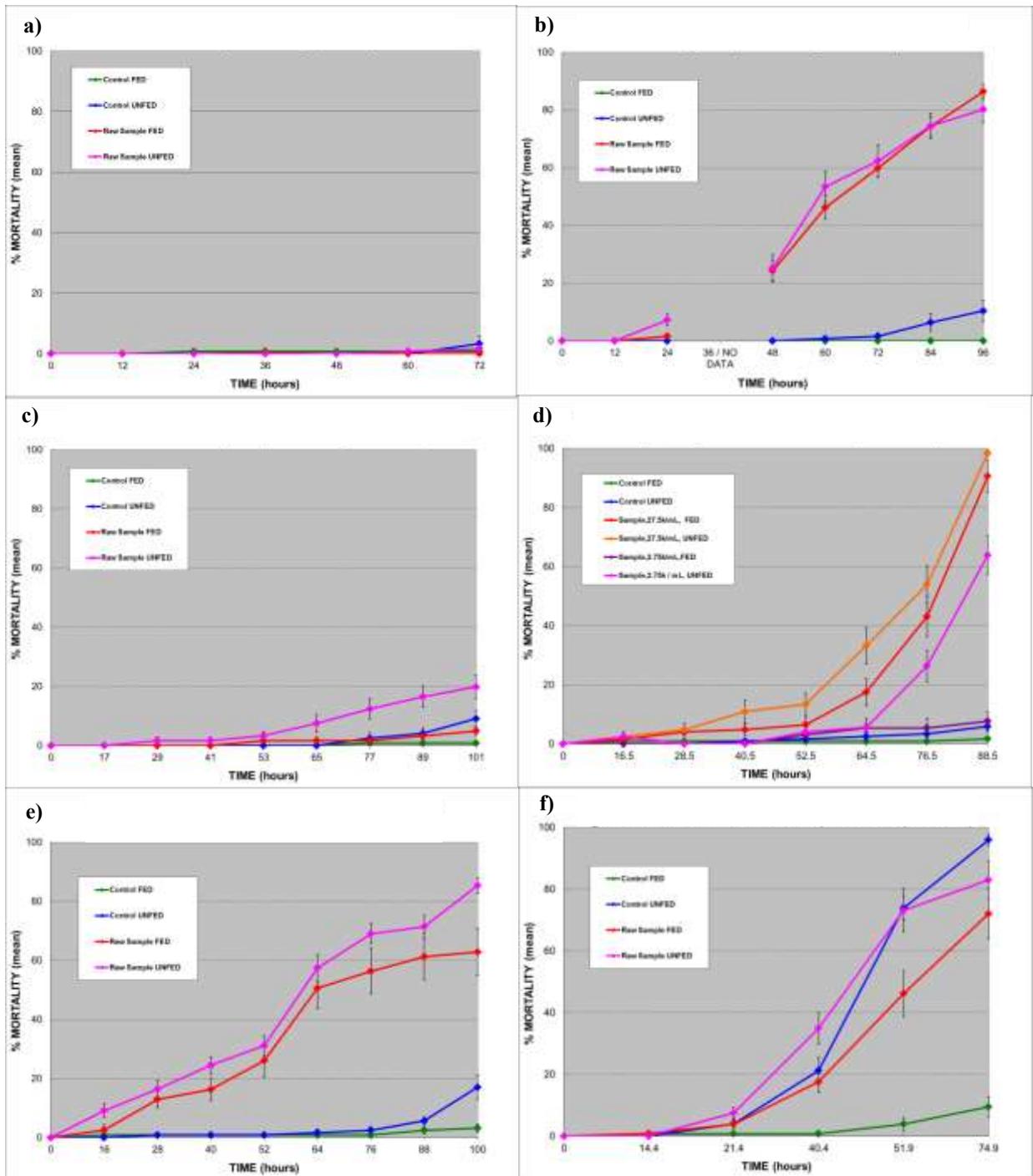


Figure 2. Percent mortality in toxicity bioassays of *Cochlodinium polykrikoides* bloom natural samples collected in the James River, Virginia during 2012 using 12-24 hr old *Artemia salina* nauplii as bioassay organism. a) Lafayette River *C. polykrikoides* bloom 26 June, 2012. Cell counts: 4400 cells/ml by microscopic visual count; 5300 cells/ml by molecular assay; CHLa of 94.61 $\mu\text{L/L}$. b) Elizabeth River *C. polykrikoides* bloom, 2 July, 2013. Cell counts: 15,750 cells/ml by microscopic visual count; 16,981 cells/ml by molecular assay; CHLa 153.66 $\mu\text{L/L}$. c) Hampton Roads *C. polykrikoides* bloom, 10 July, 2013. Cell counts: 3,950 cells/ml by microscopic visual count; 5,897 cells/ml by molecular assay; CHLa 115.86 $\mu\text{L/L}$. d) James River (near Warwick River) *C. polykrikoides* bloom, 17 July, 2013. Cell counts: 27,500 cells/ml by microscopic visual count (1:10 dilution also used);

15,832 cells/ml by molecular assay; CHLa 351.90 µl/L. e) Lysate assay (all other assays were conducted using live *C. polykrikoides* cells), Hampton Roads *C. polykrikoides* bloom, 31 July, 2013. Cell counts: no microscopic visual count; 2929 cells/ml by molecular assay (probably low due to cell lysis); CHLa 451.02 µl/L. f) Live cell assay, James River COMMON station, 13 Sept., 2013. Cell counts: no microscopic visual count taken; 12,813 cells/ml by molecular assay; CHLa 8.0 µl/L.

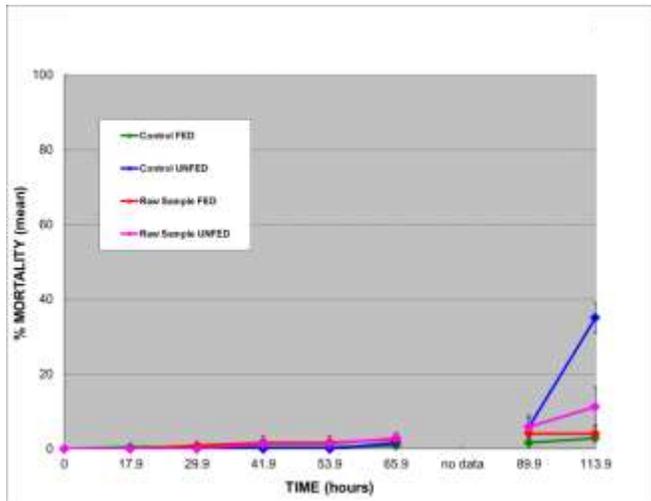


Figure 3. Percent mortality in toxicity bioassays using *Artemia salina* nauplii exposed to a natural James River phytoplankton bloom sample (Live Cells) of *Gyrodinium instriatum* collected in 2012. James River *G. instriatum* bloom, 15 Aug., 2013. Cell counts: 2400 cells/ml by microscopic visual count; no molecular assay conducted; CHLa 154 µl/L.

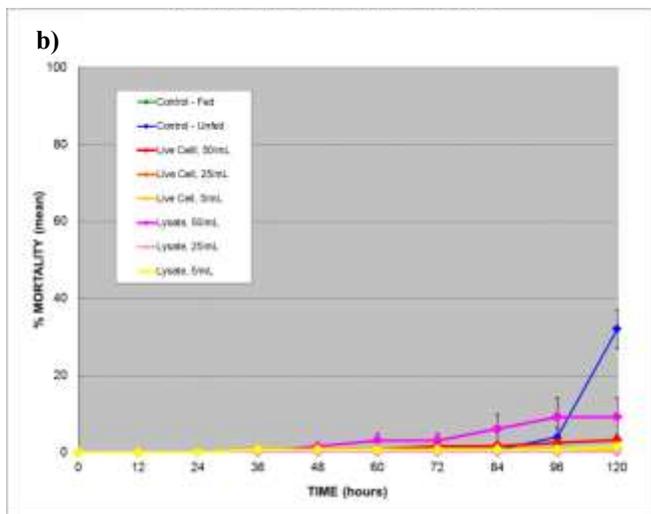
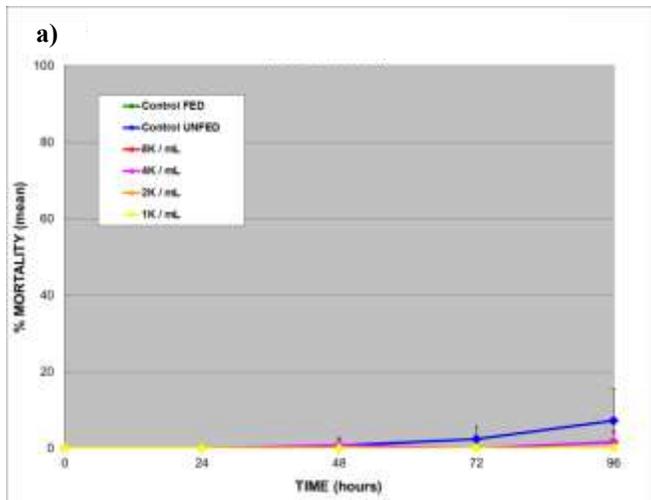


Figure 4. Dose response curves using *Artemia salina* nauplii exposed to isolate cultures of *Heterosigma akashiwo* (whole cell) and *Microcystis aeruginosa* (whole cell and lysate). a) *H. akashiwo* assay on culture established from a Lafayette River bloom sample in 2012. b) *M. aeruginosa* assay on culture established from Lake Rooty bloom sample in 2011.

