Final 2014-15 Report

TO:

Department of Environmental Quality

Watersheds Program

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Project Name: **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** Contract Number: **15427**		
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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) and raphidophytes including *Chattonella verculosa* and *Heterosigma akashiwo* (Keppler et al. 2005, 2006, Zhang et al. 2006), and cyanobacteria (Codd et al. 2003, Wiegand & Pflugmacher 2005) species found primarily in freshwater and the lower salinity portions of estuaries including *Microcystis aeruginosa, Anabaena* spp. and *Oscillatoria* spp. Blooms of these species could represent a significant emerging threat to the Bay ecosystem.

The overall goal of the project is to provide information that is vital to evaluate existing numeric criteria for the tidal James River system. During the course of this multi-year study at VIMS we have undertaken a three-tiered framework for assessing the risk of adverse effects on aquatic life due to harmful algae in the lower James River. Within this framework, during 2012 and 2013 CHLa was routinely monitored by VIMS scientists at a continuous monitoring (ConMon i.e. fixed station) in the mesohaline region of the James River (Site #9 Table 1 and Figure 1:37.04892/-76.504404). In addition, during 2012 and 2013 comprehensive on-board and underway monitoring (DATAFLOW; see www.VECOS.org and Moore et al. 2014) was done by VIMS scientists in the mesohaline region of the James. The Reece laboratory determined phytoplankton community composition and cell density via microscopy and/or molecular-genetic approaches for samples collected at the mesohaline ConMon station and during dataflow cruises. In addition, Reece and Vogelbein have assessed risk of adverse impacts to aquatic life using both field and laboratory studies. During 2012 and 2013 oysters were used as a sentinel species for the analysis of the harmful effects of algal blooms (for results see Reece and Vogelbein reports to DEQ from 2012 and 2013). 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 (Hudson and Murray 2015). In addition, numerous oyster restoration projects are underway throughout the state. Laboratory and field studies indicate that shellfish aquaculture and fisheries, as well as restoration efforts, could be hindered by blooms of species such as *Cochlodinium polykrikoides* (Mulholland et al. 2009, Friedland et al. 2011, Reece et al. 2012), which commonly blooms in the lower James River. Studies have shown lethal impacts of *C. polykrikoides* on bivalves (Gobler et al. 2008, Mulholland et al. 2009, Tang & Gobler 2009a) and particularly on bivalve larvae (Ho & Zubkoff 1979, Tang & Gobler 2009b).

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 the aquatic life designated use in the lower James River. The work conducted in the Reece and Vogelbein laboratories focused specifically on two objectives: "Characterization of Algal Blooms" and "Characterization of Impairments Associated with Algal Blooms". Laboratory toxicity bioassays were done with both bloom samples collected from the field and some laboratory cultures from 2012 through early 2015 to evaluate potential adverse health impacts on aquatic life 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 2013-2015 were done with *Crassostrea virginica* veligers, *Cyprinodon variegatus* larvae and *Ceriodaphnia dubia* neonates. During 2014 through early 2015, the period covered by this report, the focus was on examining the biological impacts of the specific HAB species that may threaten the lower James River system by doing laboratory bioassays using both *in vitro* isolate cultures established previously using field samples from the James River system and directly with bloom samples collected from the field in 2014. This work involved performing two of the subtasks identified by the scientific advisory panel for the lower James River (Bell et al. 2011): 1) "Subtask 1.2—CHLa, diagnostic pigments and the occurrence of harmful algae" and "Subtask 2.1—Determining linkages between algal blooms and impairments". Subtask 1.2 during this year was done almost exclusively on bloom samples and Subtask 2.1 was addressed by doing larval fish and shellfish bioassays with field samples and in vitro isolate cultures.

Table 1 lists the bioassays that were proposed for 2014 and those that were conducted. We expected to collect at least four James River *Cochlodinium polykrikoides* bloom samples during late July or through August of 2014 with which to conduct laboratory bioassays on larval fish and oysters. The 2014 *C. polykrikoides* bloom(s), however, in the lower James River were sporadic, as well as of short duration and restricted spatial coverage compared with previous years. Therefore, we obtained only 2 bloom samples with which to conduct bioassays.

In addition to the field-collected bloom samples, we did larval fish and oyster bioassays with *in vitro* isolate cultures of *Karlodinium veneficum* and *Alexandrium monilatum*. Additional

bioassays were done with early-, mid- and late-log and stationary phases of the Virginia isolate culture of *C. polykrikoides* with *Cyprinodon variegatus* as the test organism because the early-mid and late- bloom field samples were not available for assays that had been planned for this year. Several *Microcystis aeruginosa* bioassays were done with *Ceriodaphnia dubia* neonates, as we were optimizing the assay conditions in order to minimize mortality in the control animals.

Table 1: Bioassays conducted during 2014 through early 2015 in order to examine the biological impacts of key harmful algal bloom organisms that have been found in the James River, VA.

Bioassay	**Test Organism**		
	# bioassays with *Crassostrea virginica*	# bioassays with *Cyprinodon variegatus*	# bioassays with *Ceriodaphnia dubia*
Cochlodinium polykrikoides Bloom-early 50-75 ug/L CHLa- dilution series (100%, 50%, 25%, 12.5%, 6.25%)	1	ND	-
C. polykrikoides Bloom mid 50-75 ug/L CHLa - dilution series (100%, 50%, 25%, 12.5%, 6.25%)	1	ND	-
C. polykrikoides Bloom mid 50-75 ug/L CHLa - dilution series (100%, 50%, 25%, 12.5%, 6.25%)	ND	ND	-
C. polykrikoides Bloom late 50-75 ug/L CHLa - dilution series (100%, 50%, 25%, 12.5%, 6.25%)	ND	ND	-
C. polykrikoides-culture-VA isolate whole cell early-log	ND*	1	-
C. polykrikoides-culture-VA isolate whole cell mid-log	ND*	1	-
C. polykrikoides-culture-VA isolate whole cell late-log	ND*	1	-
C. polykrikoides-culture-VA isolate whole cell stationary	ND*	1	-
Karlodinium veneficum-culture-VA isolate whole cell (50 -100 to 10,000 cells/ml)	2	-	-
K. veneficum-culture-MD isolate whole cell (50 -100 to 10,000 cells/ml)	1	-	-
Alexandrium monilatum-York River isolate whole cell (50 -100 to 10,000 cells/ml)	1	2	-
Microcystis aeruginosa whole cell and lysate	-	1	3

*Assays planned for spring 2015 when veligers are available from hatcheries.

Materials and Methods:

Details regarding the materials and methods can be found in the SOP (Appendix 7) at http://www.deq.virginia.gov/Portals/0/DEQ/Water/WaterQualityStandards/James%20River%20Chl%20A%20Study/SAP_Reports/QAPP_JR_CHLa_Study_with_signatures.pdf

Collection of water samples

Two replicate 100 ml water samples were collected by HRSD, CBF, VA-VDH and VIMS personnel, often during bloom events, as regular monitoring samples were not collected in 2014. Samples were transported to VIMS in a cooler with insulating material between the water sample and blue ice. Sampling sites from 2013 (yellow and red pins) and 2014 (green and red pins) are listed in Table 2 and indicated on the map (Fig. 1). 2014 sites are indicated by asterisks in the Table and green and red pins on the map.

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 (Appendix 7) at (http://www.deq.virginia.gov/Portals/0/DEQ/Water/WaterQualityStandards/James%20River%20Chl%20A%20Study/SAP_Reports/QAPP_JR_CHLa_Study_with_signatures.pdf).

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.

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 (i.e. visually counted) 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.

Laboratory toxicity bioassays

Toxicity bioassays were conducted using clonal cultures that were established from bloom samples and are being maintained long-term (Table 2) as outlined in the SOP with the addition of a lysate treatment. 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 reduce 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. Dose response studies with *M. aeruginosa* were conducted with both clonal isolate live whole cell and cell lysate material, while assays with *A. monilatum*, *K. veneficum* and *C. polykrikoides* were only done with live whole cells.

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. The live cell *C. polykrikoides* bioassays conducted to examine the effects of early-, mid- and late-log, and

stationary phase cultures on *C. variegatus* were done using the culture material directly without filtering and concentrating the cells. For previous *C. polykrikoides* assays the cultured cells were filtered and concentrated in order to achieve a high dose cell concentration of at least 10,000 cells/ml.

For the duration of each bioassay (i.e. up to ~96hr or 120hr for oyster veliger assays) both mortality and animal activity (swimming, feeding) versus lack of activity were noted in order to determine the condition during the exposures. Morbidity/mortality of the oyster veligers was assessed by activity (swimming, feeding) versus lack of activity (closed and not feeding) 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, heartbeat, 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. For *Ceriodaphnia* the criterion for judging morbidity/mortality also was primarily associated with motility. *Ceriodaphnia* were used in assays with *M. aeruginosa*. The *M. aeruginosa* culture material, particularly at high concentrations, clouded the water in the wells and contained flocculates. If the animals were actively swimming in the water column, even if they were entrapped in flocculates of live cells or cellular debris but still moving, they were considered viable. Once the swimming behavior became weaker, they were considered moribund. Mortality was based on the cessation of movement/swimming and obvious tissue degradation.

Results

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

A total of 26 samples were collected for microscopic and molecular analyses from the lower James River system from May to September 2014 comprised of 5 samples from the mesohaline region of the James, 1 Elizabeth River sample and 20 Lafayette River samples (sites indicated in Table 2). 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. We received a sample from a bloom of *Akashiwo sanguinea* (1,400 cells/ml) in mid-June and only five samples with bloom levels of *Cochlodinium polykrikoides* from July 1 through mid-August (highest cell count 6,271 cells/ml). The bloom that was noted in the Lafayette River on July 1 was a very small patch near the Haven Creek boat ramp. The only other *C. polykrikoides* bloom that was noted was patchy and started around July 30 and was reported intermittently until mid-August in the mesohaline region of the James River. We conducted bioassays (see below) with two samples collected during this period using *Crassostrea virginica* veligers as the test organism. Results of the microscopic examinations and molecular assays are given in Appendix 1 (spreadsheet).

Determining linkages between blooms and adverse effects on aquatic life.

Field sample (*C. polykrikoides*) bioassays: Laboratory bioassays were conducted with two field bloom samples during the summer of 2014. A bioassay with oyster veligers was done with a sample collected when the *C. polykrikoides* sample was first noted in the mesohaline region of the James River on July 30, 2014. We considered this sample an "early bloom" sample. This sample contained 1,756 cells/ml based on the qPCR assay and had a (uncorrected, i.e. field determined) CHLa concentration of 30.51 µg/L. No mortality was observed within the first 24 hrs, however, at 48 hrs ~33% mortality was observed in the full dose (undiluted sample) treatment and at 96 hr (and 120 hr) there was close to 100% mortality (Fig. 2). At 96 hr 25-30% mortality was observed in the treatments with the bloom sample at concentrations of 50%, 25% and 12.5%. Less than 5% mortality was observed in the fed and unfed controls.

Another oyster veliger bioassay was done with a field sample collected on August 13 from what we considered to be a mid-bloom sample, however, we were unable to obtain additional bloom samples because HRSD and VIMS personnel did not see *C. polykrikoides* blooms in the James,

Elizabeth or Lafayette Rivers after this time. The August 13 sample had 184 cells/ml as determined by qPCR and a CLHa concentration of 29.67 µg/L as determined in the field (i.e. uncorrected). We noted that lysis of the *C. polykrikoides* cells had occurred during transport and during the bioassay using this field sample an unknown dinoflagellate multiplied and was observed swarming the oyster veligers. The cumulative mortality in all treatments with cell concentrations ranging from 12.5-100% was around 35-40% at 120 hr (Fig. 3). The mortality at 96 hr in the 100% and 50% treatments was 25% and 18%, respectively, however, there was also high mortality in the fed control veligers. 17% and 25% mortality was observed in the fed control animals at 96 and 120 hr, respectively, suggesting that there may have been a contaminant in the food that was harmful. The cumulative mortality in the unfed controls, however, was only ~7% at 96 hr and ~10% by the end of the assay at 120 hr.

<u>*M. aeruginosa* culture bioassays</u>: In an attempt to optimize the assay conditions, three assays were conducted with *Ceriodaphnia dubia* as the test organism challenged with the *M. aeruginosa* culture material. Mortality for the controls was very high during the first two assays and we became concerned about the quality of food and appropriate feeding regime for these animals. Patrice Mason had several discussions regarding food quality problems with Paul Sachs at Sachs Systems Aquaculture, the vendor in Florida from whom we ordered *C. dubia*. In addition, she discussed this problem with Pete DeLisle of Coastal Bioanalysts Inc., Gloucester, VA. He suggested that there is likely a vitamin deficiency problem. We obtained his recipe for YCT (yeast, cereal, tetramin), which uses digested fish chow in place of tetramin. We used this YCT as food augmented with the alga *Selenastrum capricornutum* as the source for vitamins. Figures 4-6 illustrate the results of these assays. As mentioned above, the control mortality for the first two assays was high >50%. In fact the unfed controls in the first assay had cumulative mortality of ~85%, comparable to what was observed for the whole cell 100% treatment. Therefore, only fed controls were used for the subsequent assays. The 100% concentrations were 157,000 cells/ml (CHLa 484 µg/L) for assay #1 and 242,000 cells/ml as determined by qPCR (CHLa 774.6 µg/L) for assay #2. It appears that the *C. dubia* possibly used the *M. aeruginosa* as food source, even though it was toxic. After the food was changed for the third assay, the cumulative control mortality was 20%. For the third assay mortality of both the whole cell and lysate 100% (1.60×10^6 cells/ml by qPCR, CHLa 1290 µg/L) treatments was >90%. The cumulative

mortality in the whole cell and lysate 50% (8 X 10^5 cells/ml by qPCR) treatments was 73% and 62%, respectively. For all other treatments (except the controls) the cumulative mortality ranged between 50 – 60%.

For the *M. aeruginosa* assay with *C. variegatus* as the test organism relatively high mortality (44%) was observed only for the 100% (1.57 X 10^6 cells/ml by qPCR, CHLa = 1,094 μg/L) lysate treatment. No mortality was observed for either the fed or unfed controls, while mortality in the 100% whole cell treatment was only ~10% (Fig. 7).

<u>*K. veneficum* culture bioassays</u>: Three bioassays were done with *K. veneficum* cultures with *C. virginica* as the test organism. Two different *K. veneficum* isolates were tested, one with an isolate culture established from a Maryland bloom sample (V1974) and the other two with the isolate established from a James River bloom sample (E613). Results are shown in Figures 8-10. The qPCR cell counts (See appendix) were about twice the visual cell counts suggesting that the cultures were in log phase when these assays were conducted. Live cells exhibited consistently faster and higher mortality than lysate treatments. With the V1974 culture a cumulative mortality in the high dose live cell treatment (205,000 cells/ml by qPCR, CHLa 386 μg/L) was 85% (Fig.8), while a cumulative mortality of only ~15% was observed with the live cell treatment at ~ 20,000 cells/ml (CHLa ~39 μg/L). For all other treatments mortality was < 10% and there was no mortality in the controls. In the first E613 (James River Isolate) culture bioassay cumulative mortality was <10% (Fig. 9) for all treatments including the high dose treatment (104,000 cells/ml, CHLa = 125 μg/L). The next bioassay with E613 was only done with live cells. The high dose treatment (520,000 cells/ml, CHLa = 200 μg/L) exhibited a cumulative mortality of >90% by 96 hr and 100% by 120 hr (Fig. 10). A cumulative mortality of ~15% was observed in the treatment with ~18,000 cells/ml (CHLa ~6.7 μg/L). Less than 10% mortality was observed in all of the lower doses and there was <1 % mortality in the controls.

<u>*A. monilatum* culture bioassays</u>: Three bioassays were done with the York River *A. monilatum* culture. One utilized *C. virginica* as the test organism and the other two used *C. variegatus*. The two assays with *C. variegatus* were done to test the culture grown under replete and depleted phosphorous conditions to test the effects of nutrient limitation on toxicity.

The assay with *C. virginica* as the test organism was done with live cells and the treatments consisted of 61, 307, 613, 1,533, 3,065 and 6,130 cells/ml based on qPCR results. These cell concentrations would be equivalent to CHLa concentrations of ~3, 15, 30, 76, 152 and 304 μg/L, respectively. 28% cumulative mortality was observed in the high dose treatment (6,130 cells/ml) after 96hr, while mortality reached ~80% after 120 hr (Fig. 11). Interestingly, high mortality (~50%) was also observed for the 307 cells/ml treatment after 120hr. In fact, it was slightly higher than the ~43% mortality observed for the 3,065 cells/ml treatment. In addition, ~28% mortality was observed in the 61 cells/ml treatment. We did a qPCR assay on the material in the 307 cells/ml wells at the end of the assay and determined that *A. monilatum* had been multiplying in the wells during the assay because the concentration was determined to be ~2,750 cells/ml. At the low doses apparently the cells were able to continue their log phase growth following dilution in the media for the assay.

The two assays with *C. variegatus* were done to test the culture grown under replete and depleted phosphate conditions to test the effects of nutrient limitation on toxicity. The cell concentrations were visually determined for these assays. With the culture grown under both replete and deplete PO_4 conditions the *C. variegatus* mortality was extremely low (Figs 12,13). The highest mortality was 2% (Fig. 13). The CHLa concentrations for the 100% cell concentrations in the *A. monilatum* replete and deplete phosphate condition bioassays were 421 and 367 μg/L (3,240 cells/ml and 5,350 cells/ml), respectively.

<u>*C. polykrikoides* culture bioassays</u>: Four bioassays were done with *C. polykrikoides* whole cell culture material. These were done to compare the impacts of different stages for a population of *C. polykrikoides* from early log phase through mid- and late log phase into stationary phase in order to simulate the early, mid, late and decline stages of a bloom. They were done using the *Cochlodinium polykrikoides* clonal isolate that was established from a York River bloom sample with *Cyprinodon variegatus* as the test organism. For these assays the cells were not concentrated, as they have been for many of the previous assays with culture, but rather the cell number (i.e. cells/ml) in the culture at the time that it was collected for these assays was used as the highest dose for the assays. Dilutions of 50%, 25% and 10% were also tested. The early log phase culture had a qPCR concentration of 1,410 cells/ml (CHLa = 174.2 μg/L) with dilution

treatments having 705, 353 and 141 cells/ml (Fig. 14). There was rapid mortality at the highest dose with 100% of the animals dead after 24 hr. At a concentration of only 705 cells/ml 50% of the animals were dead after 48hr and almost 100% mortality was observed by 96 hr. In the 353 cells/ml treatment ~67% cumulative mortality occurred over 96hr and there was no mortality in the 10% or 141 cell/ml treatment or in the controls.

The mid-log phase culture material had a qPCR concentration of 1,890 cells/ml (CHLa = 174.6 μg/L). Very high and rapid mortality was observed for this assay (Fig. 15). Results from the early log phase assay prompted us to test the toxicity of the cell-free filtrate and to put freshly washed cells into fresh media to determine whether they would demonstrate toxicity and, if they did, when mortality would be observed (i.e. how long before fish would die exposed to the cells at a concentration of ~2,000 cells/ml in fresh media). 100% mortality was observed after 2.5 hr for the undiluted (i.e. ~1,890 cells/ml) mid-log phase cells in the "spent" media. At a concentration of 945 cells/ml 88% mortality was observed after 24 hr with 100% mortality by 48hr. The filtrate alone resulted in 73% mortality after 24 hr and 100% by 48 hr. For all other treatments (473 cells/ml, 189 cells/ml and the cells in fresh media), no mortality was observed until after 48 hr. At the end of 96 hr the cells in fresh media and the 473 cells/ml treatments resulted in ~90% mortality, while less than 10% mortality was observed for the controls and the 189 cells/ml treatment.

The late-log phase culture had a qPCR concentration of 2,980cells/ml (CHLa = 504 μg/L) at the beginning of the assay. As with the mid-log phase culture, we tested the filtrate and cells in fresh media, as well as culture material at 100%, 50%, 25% and 10% concentrations. Rapid and 100% mortality was observed for the 100% or 2,980 cells/ml (within 1 hr), the filtrate (within 4 hr) and the 50% or 1,490 cells/ml (within 5 hr) treatments (Figs. 16 and 17). After 24 hr ~70% mortality was observed in the 25% or 745 cells/ml treatment and there was 100% mortality after 48 hr in this treatment. In addition, 100% mortality was observed after 48 hr for the cells put in fresh media at the beginning of the assay at an initial concentration of 2,980 cells/ml. At the end of the assay (96 hr) there was ~10% mortality in the 10% or 298 cells/ml treatment and no mortality was observed in the controls.

The stationary phase culture had a qPCR concentration of 4,670 cells/ml (CHLa =103 μg/L) at the beginning of the assay. We tested the filtrate and 100% lysate material, as well as whole cell culture material at 100%, 50%, 25% and 10% concentrations. All of the animals in the 100% (i.e. 4,670 cells/ml) treatment wells were moribund within 2hr and were collected for histopathology. 81% of the animals in the 50% (i.e. 2,335 cells/ml) treatment wells were moribund within 4hrs with 100% mortality by 24 hr. (Fig. 18). By 48 hr 88% mortality was observed for the filtrate treatment with 100% mortality by 72 hr. At 96 hr there was 96% mortality for the 25% (1,168 cells/ml) treatment and 25% mortality for the 10% treatment (467 cells/ml). No mortality was observed in the controls or in the lysate treatment wells.

Discussion

The human and animal health impacts of most of the organisms that are found to bloom in the James River system have not been adequately assessed. Many of the James River bloom species, including *C. polykrikoides, K. veneficum* and *M. aeruginosa,* are reported to produce harmful toxins under certain conditions and have demonstrated effects on marine life based on studies conducted in other estuarine systems (Deeds et al. 2002, Dorantes-Aranda et al. 2009, Mulholland et al. 2009, Tang & Gobler 2009 a, Place et al. 2012, Acuna et al. 2012 a,b). 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 and cell concentrations of specific phytoplankton species and biological impacts.

We had the CHLa levels determined for each of the cultures at the beginning of each bioassay. Unfortunately, for unknown reasons the CHLa levels did not track the cell numbers very well for any of the isolates. Although the accepted EPA fluorometric method (EPA/600/R-97/072) is used for determining CHLa levels and they are corrected for pheophytin levels, it is possible that isolate cultures grown in the laboratory have a different pigment profile that confounds CHLa determinations.

The *Microcystis aeruginosa* bioassay results in this study indicate that both the live cell and lysate material are toxic with the invertebrate water flea, *Ceriodaphnia dubia,* more sensitive than the larval fish, *C. variegatus*. *M. aeruginosa* produces a variety of microcystin toxins. Previous bioassay studies have shown delayed development and significantly lower survival for *Daphnia* and *Ceriodaphnia* spp. exposed to *M. aeruginosa* at concentrations of ~1-2 X 10^6 cells/ml (Nandini and Rao 1998, Liping et al. 2011). In addition, several studies have demonstrated that populations of *Daphnia* and *Ceriodaphnia* (as well as other organisms) previously exposed to *M. aeruginosa* are more tolerant of microcystins indicating a selection mechanism (Nandini and Rao 1998, Gustafsson and Hansson 2004, Gustafsson et al. 2005, Rodgers et al. 2008, Lemaire et al. 2012). In the current study, after the assay conditions were optimized to minimize mortality in the controls, >70% mortality (adjusted for control) in both the whole cell and lysate treatments was observed for *C. dubia* exposed to 1.6 X 10^6 cells/ml and >20% at 1.6 X 10^5 cells/ml. These *Ceriodaphnia* were purchased from a company that grew the animals in laboratory culture suggesting that they did not come from a population previously exposed to and selected for tolerance to *M. aeruginosa*. Few other studies have examined the effect of microcystins on finfish. Studies in San Francisco Estuary on the cyprinid, Sacramento splittail, and the pelagic threadfish shad indicated sublethal effects on nutritional status and liver toxicity (Acuna et al. 2012a,b). Results of the larval fish assays conducted here suggest that lysing the cells increased the amount of toxin in the isolate samples because the full dose lysate exposure, which was equivalent to 1.56 X 10^6 cells/ml, resulted in an overall 44% mortality, while exposure of larval fish to the 100% concentration of whole cells resulted in only 8% mortality overall.

Karlodinium veneficum produces karlotoxin, which is toxic to both finfish and shellfish. As with many other HAB species, previous work has demonstrated that there is substantial variation in toxicity among isolates from different geographic regions (Bachvaroff et al. 2009, reviewed in Place et al. 2012). Therefore, we tested both an isolate from Maryland that we obtained from a culture collection

and one that we established at VIMS from a James River water sample using oyster veligers as the test organism. Results of these bioassays with both the MD and VA isolates suggest that the karlotoxin was rapidly inactivated in the lysate material. Results with the highest dose (>200,000 cells/ml) of MD isolate whole cell material resulted in >60% mortality at 96 hr and >80% overall mortality of oyster veligers at the end of the assay (i.e. 120hr), while low mortality (i.e. 5% or less) was observed with the lysate material. At a concentration of ~20,000 cells/ml 18% mortality occurred with the whole cell MD isolate. However, with the VA isolate at a concentration of >100,000 cells/ml ~5% mortality of oyster veligers occurred by 96hr. In another assay with the VA isolate at a concentration of 520,000 cells/ml >90% mortality was observed at 96hr while <10% at 96 hr for a concentration of 52,000 cells/ml. In previous studies with the MD isolate significant mortality of *C. virginica* early embryos (<2 days old) occurred at concentrations as low as 10,000 cells/ml (Stoecker et al. 2008) and in the shallow Maryland tributaries fish kills have been reported when cell counts are >10,000 cells/ml (Place et al. 2012).

Results of the *A. monilatum* bioassays suggested that it is more toxic to oyster veligers (*C. virginica*) than to larval fish. May et al. (2010) demonstrated that *A. monilatum* lysate and stationary (senescent) phase culture at a concentration of 550 cells/ml isolated from a Gulf of Mexico (GOM) bloom sample would kill *C. variegatus* within 90 minutes while whole cell log phase culture at the same concentration did not cause mortality. In contrast, no mortality was observed in *C. virginica* larvae exposed to the GOM isolate at 550 cells/ml and only 10% mortality was observed in those exposed to the lysate. During the current study with whole cells of a York River isolate culture 40% overall mortality during the course of the 120hr assay was observed with oyster veligers at a concentration of 6,130 cells/ml, while no mortality was observed for *C. variegatus* with whole cell *A. monilatum* at concentrations of ~3,200 cells/ml and 5,350 cells/ml grown under either standard conditions or under phosphate-depleted conditions, respectively. At 96hr in the oyster veliger assay 20-30% mortality was observed in both the 3,065 cells/ml and 6,130 cells/ml treatments. It is likely that *A. monilatum* produces an endotoxin that is released only when the cells are lysed or die. This hypothesis is consistent with the results of May et al. (2010) and previous results from our laboratory (Reece et al. 2012), which demonstrate higher toxicity in lysates.

Results of the oyster veligers assays (7-day old) conducted as part of this study with field-collected *C. polykrikoides* bloom samples 2014 indicate that this organism is toxic. Both samples had relatively low cell numbers (<2,000 cells/ml) and CHLa levels (~30 µg/L), however, the sample where cells had not lysed caused 99% overall mortality at the highest dose (i.e. undiluted field samples) during the course of the assay. In the sample where cells had lysed during transport ~40% mortality was observed. These results are comparable to what Tang and Gobler (2009a) observed using *C. polykrikoides* isolates established from samples collected off the coast of Long Island, New York. They observed 60-80% mortality with 4-day old oyster larvae in 72hr bioassays at cell concentrations between 1,000 -2,000 cells/ml.

With *Cyprinodon variegatus* as the test organism Mulholland et al. (2009) observed 100% mortality within 15hr at with bloom samples at cell concentrations of ~10,000 cells/ml, while only ~20% mortality occurred in juvenile oysters. Results from the early, mid- and late log, and stationary phase bioassays reported here with the York River *C. polykrikoides* clonal isolate revealed interesting information regarding the biological impacts of this HAB species. These particular assays were done to examine the effect of different life cycle stages of the culture, i.e. early-, mid- and late-log phase, in lieu of using early, mid- and late blooms samples from the field. To conduct these assays the cells were not concentrated, as they have been for many of the previous assays with culture material, but rather the cell number (i.e. cells/ml) in the culture at the time that it was collected for these assays was used as the highest dose. We found that for all of the logarithmic growth phases, as well as for the stationary phase culture, >20% mortality occurred at ~500 cells/ml. Apparently during the process of concentrating the cells by filtering and washing and resuspending the cells in new media, we were filtering out the 'old' media that contained a lethal (toxic?) compound. These most recent assays generally demonstrated higher and more rapid acute mortality at even lower doses than earlier bioassays. As with the highest dose treatments, the filtrate material that we tested for the mid-, late and stationary phase assays also demonstrated rapid and high mortality. This suggests that *C. polykrikoides* cells are releasing a toxic compound during the logarithmic growth phase. During the process of preparing lysate material in some cases we seem to have begun to inactivate the toxin. The lysate material was generally less toxic than whole cell material during the course of 96 hr assays. In many of the earlier assays we often saw delayed mortality. Mortality would begin to occur ~48 hr after assay initiation suggesting that as the cells recovered in the new media during the course of the

assay they were releasing the toxic compound. Future studies are planned to conduct similar life cycle bioassays with oyster veligers as the test organism, as well as to better characterize this compound and determine its stability.

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Table 2: Sampling site information for samples analyzed at VIMS during 2013 and 2014.

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	37.18978	-76.63282
JR mesohaline				
8	Meso1	HRSD	36.93730	-76.46060
9	JMS017.96	VECOS CMON	37.04892	-76.50440
10	Meso2	HRSD	37.00277	-76.52387
13 #	Huntington Beach	VIMS/VDH	37.01697	-76.45664
30	Idle Fleet	VECOS	37.11975	-76.64627
36	Cypress Creek	VIMS	36.98297	-76.62029
37	JMS-16AM	HRSD/VECOS	36.92970	-76.41670
38	JMS-1AM (LE.5.3)	HRSD/VECOS	36.99000	-76.46000
39	JMS-20AM (LE.5.2)	HRSD/VECOS	37.05600	-76.59310
47 *	Meso 130136	HRSD	37.04042	-76.50383
48 *	Meso 2048	HRSD	37.00270	-76.44430
49 *	JMSMH	HRSD	37.06633	-76.54995
50 *	Hilton area	VDH	37.03103	-76.47108

JR polyhaline				
21 #	Lafayette River	CBF	36.90537	-76. 30676
33	Hampton River	CBF	37.02177	-76.34206
34	Elizabeth River	VIMS	36.80756	-76.29307
35	Elizabeth River	VIMS	36.80911	-76.28711
40 *	Lafayette River, Granby St. Bridge	CBF	36.88731	-76.28040
41 *	Elizabeth River, Anna St., Norfolk	VIMS	36.85241	-76.22710
42 *	Lafayette River BT site 1	VIMS	36.87981	-76.27070
43 *	Lafayette River BT site 2	VIMS	36.90046	-76.29440
44 *	Lafayette River BT site 3	VIMS	36.90883	-76.30083
45 *	Lafayette River BT site 4	VIMS	36.90995	-76.30964
46 *	Lafayette River	HRSD	36.88040	-76.27287

* New sites in 2014

Sites where samples were collected in both 2013 and 2014

Table 3: Summary of bioassay data from April 2014 – January 2015 including CHLa (µg/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	Salinty (PSU)	CHLa (µg /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
4-30-14	*Microcystis aeruginosa*	*Cyprinodon variegatus*	Isolate: Rooty Branch Lake	0	1094	1.57×10^6	8	44	-	-	0	-
5-26-14	*Microcystis aeruginosa*	*Ceriodaphnia dubia*	Isolate: Rooty Branch Lake	0	484	1.57×10^5	-	-	91	80	99	61
6-25-14	*Microcystis aeruginosa*	*Ceriodaphnia dubia*	Isolate: Rooty Branch Lake	0	775	2.42×10^5	-	-	95	66	-	81
8-27-14	*Microcystis aeruginosa*	*Ceriodaphnia dubia*	Isolate: Rooty Branch Lake	0	1290	1.60×10^6	-	-	98	93	-	21
5-28-14	*Karlodinium veneficum* (MD isolate)	*Crassostrea virginica*	Isolate: MD Potomac River	20	386	2.05×10^5	-	-	84	2	0	0
5-28-14	*Karlodinium veneficum* (VA isolate)	*Crassostrea virginica*	Isolate: James River	20	125	1.04×10^5	-	-	2	5	0	0
7-8-14	*Karlodinium veneficum* (VA isolate)	*Crassostrea virginica*	Isolate: James River	20	200	5.2×10^5	-	-	99	-	0	1

Table 3: Summary of bioassay data from April 2014 – January 2015 including CHLa (µg/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	Salinty (PSU)	CHLa (µg /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
7-8-14	*Alexandrium monilatum*	*Crassostrea virginica*	Isolate: York River	20	380	6.13×10^3	-	-	80	-	0	1
8-6-14	*Alexandrium monilatum*	*Cyprinodon variegatus*	Isolate: York River Replete	20	421	3.24×10^3	0	-	-	-	0	-
8-6-14	*Alexandrium monilatum*	*Cyprinodon variegatus*	Isolate: York River Deplete	20	367	5.35×10^3	0	-	-	-	0	-
7-30-14	*Cochlodinium polykrikoides*	*Crassostrea virginica*	Field Sample: James River, MESO	20	31	1.76×10^3	-	-	99	-	3	2
8-13-14	*Cochlodinium polykrikoides*	*Crassostrea virginica*	Field Sample: James River, MESO	20	30	1.84×10^2 (lysed)	-	-	38	-	11	24
11-4-14	*Cochlodinium polykrikoides*	*Cyprinodon variegatus*	Isolate: York River (early log)	20	174	1.41×10^3	100	-	-	-	0	-
11-11-14	*Cochlodinium polykrikoides*	*Cyprinodon variegatus*	Isolate: York River (mid-log)	20	175	1.89×10^3	100	100 (filtrate)	-	-	0	-

Table 3: Summary of bioassay data from April 2014 – January 2015 including CHLa (µg/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	Salinty (PSU)	CHLa (µg /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
11-18-14	*Cochlodinium polykrikoides*	*Cyprinodon variegatus*	Isolate: York River (late log)	20	504	2.98×10^3	100	100 (filtrate)	-	-	0	-
2-3-15	*Cochlodinium polykrikoides*	*Cyprinodon variegatus*	Isolate: York River (stationary)	20	103	4.67×10^3	100	0 (lysate) 100 (filtrate)	-	-	0	-

BIOASSAY DATE	HAB SPECIES	TEST ORGANISM	ISOLATE SOURCE or FIELD LOCATION	Salinty (PSU)	CHLa (µg /L) High Dose	Cell Count (cells / mL) High dose	Min. Live Cell Conc. w/ ≥20% mortality at 96 hr-adjusted based on control	Min. Lysate Conc. w/ ≥20% mortality at 96 hr-adjusted based on control
4-30-14	*Microcystis aeruginosa*	*Cyprinodon variegatus*	Isolate: Rooty Branch Lake	0	1094	1.57×10^6	NA	1.57×10^6
5-26-14	*Microcystis aeruginosa*	*Ceriodaphnia dubia*	Isolate: Rooty Branch Lake	0	484	1.57×10^5	7.86×10^4	1.57×10^5
6-25-14	*Microcystis aeruginosa*	*Ceriodaphnia dubia*	Isolate: Rooty Branch Lake	0	775	2.42×10^5	NA	NA
8-27-14	*Microcystis aeruginosa*	*Ceriodaphnia dubia*	Isolate: Rooty Branch Lake	0	1290	1.60×10^6	1.60×10^5	1.60×10^5
5-28-14	*Karlodinium veneficum* (MD isolate)	*Crassostrea virginica*	Isolate: MD Potomac River	20	386	2.05×10^5	2.05×10^5	NA
5-28-14	*Karlodinium veneficum* (VA isolate)	*Crassostrea virginica*	Isolate: James River	20	125	1.04×10^5	NA	NA
7-8-14	*Karlodinium veneficum (VA isolate)*	*Crassostrea virginica*	Isolate: James River	20	200	5.2×10^5	5.2×10^5	-
7-8-14	*Alexandrium monilatum*	*Crassostrea virginica*	Isolate: York River	20	380	6.13×10^3	3.07×10^3 6.3×10^1*	-
8-6-14	*Alexandrium monilatum*	*Cyprinodon variegatus*	Isolate: York River-Replete	20	421	3.24×10^3	NA	-

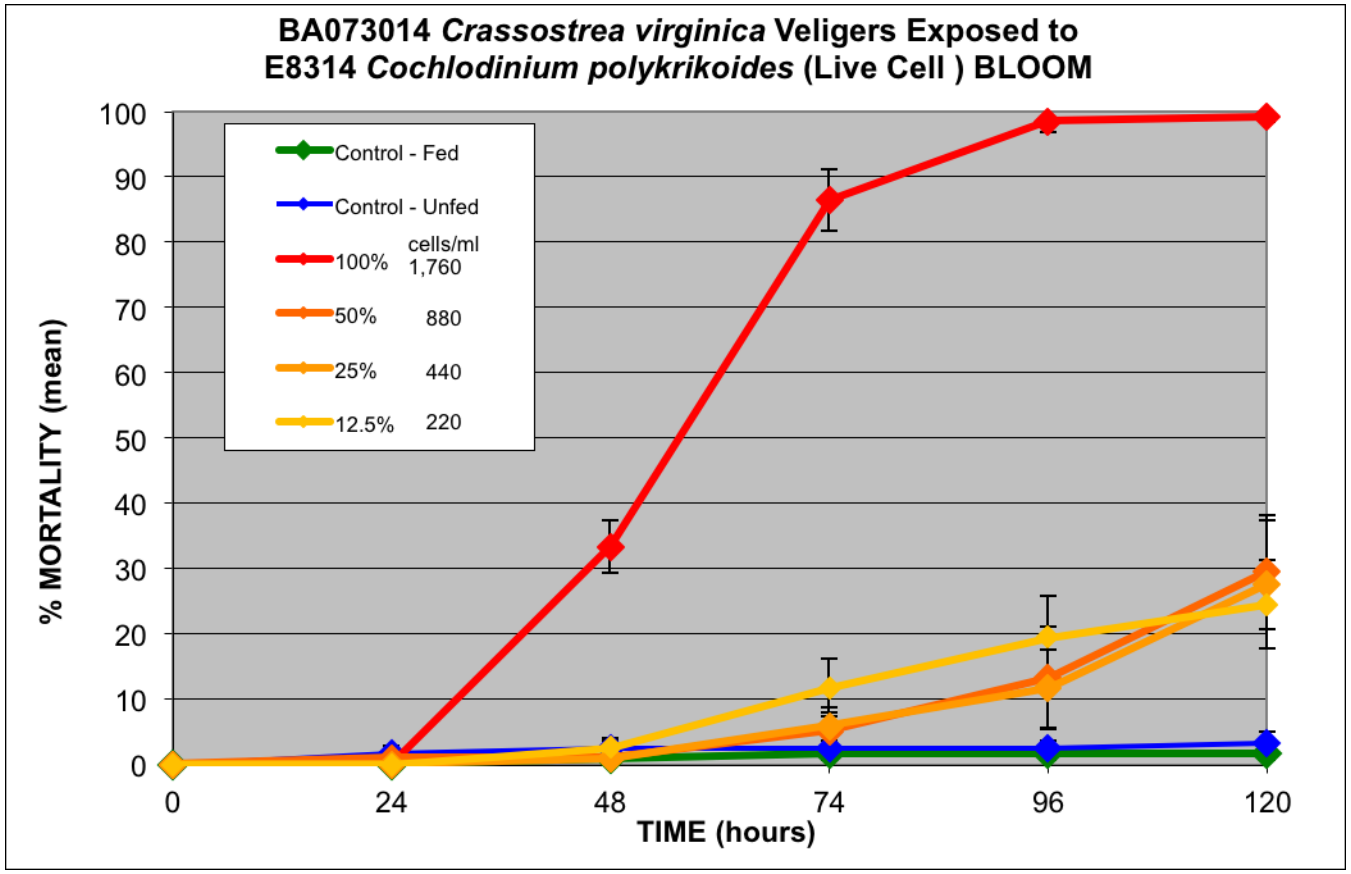
BIOASSAY DATE	HAB SPECIES	TEST ORGANISM	ISOLATE SOURCE or FIELD LOCATION	Salinty (PSU)	CHLa (µg /L) High Dose	Cell Count (cells / mL) High dose	Min. Live Cell Conc. w/ ≥20% mortality at 96 hr-adjusted based on control	Min. Lysate Conc. w/ ≥20% mortality at 96 hr-adjusted based on control
8-6-14	Alexandrium monilatum	*Cyprinodon variegatus*	Isolate: York River-Deplete	20	367	5.35×10^3	NA	-
7-30-14	*Cochlodinium polykrikoides*	*Crassostrea virginica*	Field Sample: James River, MESO	20	31	1.76×10^3	2.2×10^2	-
8-13-14	*Cochlodinium polykrikoides*	*Crassostrea virginica*	Field Sample: James River, MESO	20	89	1.84×10^2 (lysed)	NA	-
11-4-14	*Cochlodinium polykrikoides*	*Cyprinodon variegatus*	Isolate: York River (early log)	20	174	1.41×10^3	3.53×10^2	-
11-11-14	Cochlodinium polykrikoides	*Cyprinodon variegatus*	Isolate: York River (mid log)	20	175	1.89×10^3	4.73×10^2	-
11-18-14	*Cochlodinium polykrikoides*	*Cyprinodon variegatus*	Isolate: York River (late log)	20	504	2.98×10^3	7.45×10^2	-
2-3-15	*Cochlodinium polykrikoides*	*Cyprinodon variegatus*	Isolate: York River (stationary)	20	103	4.67×10^3	4.67×10^2	-

* The culture grew within treatment. Starting cell concentration is given, however, ending cell concentration was higher, but the exact count was not determined.

Figures

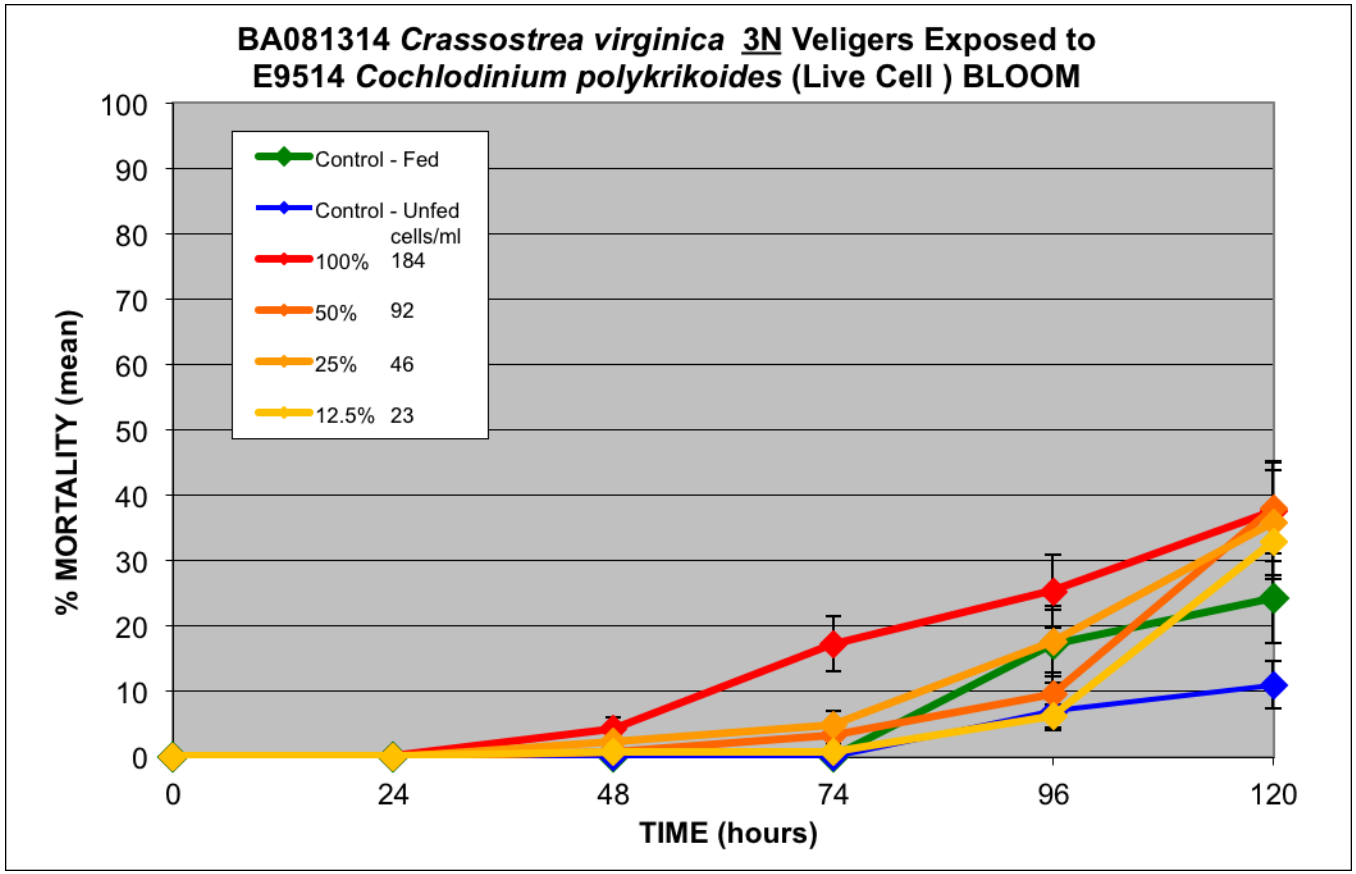
Figure 1: Map of sampling locations for VIMS samples in 2013 and 2014. Red pins indicate sites sampled in both years. Yellow pins indicate sites sampled in 2013 and green pins indicate pins sampled in 2014.

Figure 2.



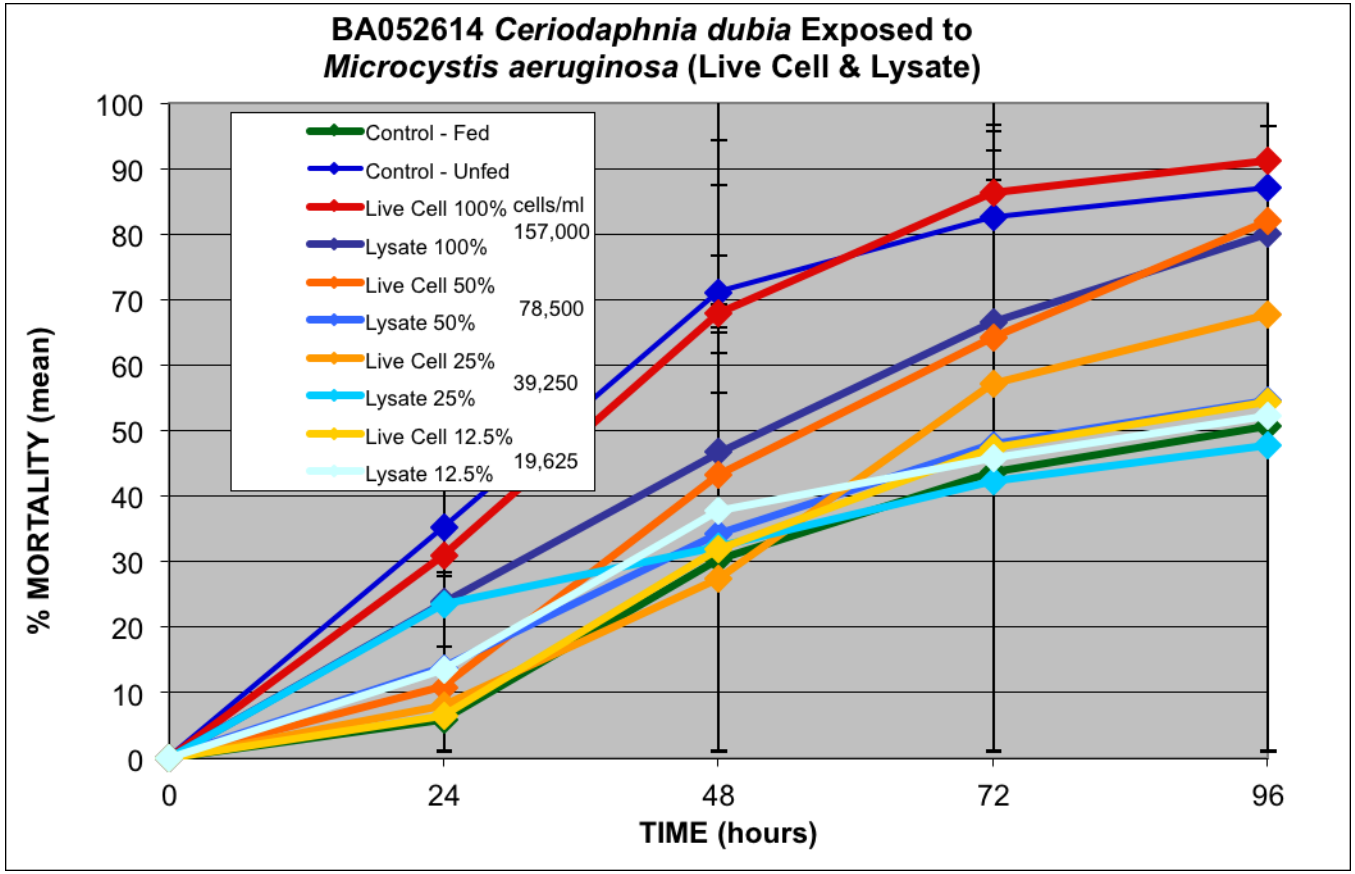
100% = 1,760 cells/mL

Figure 3.



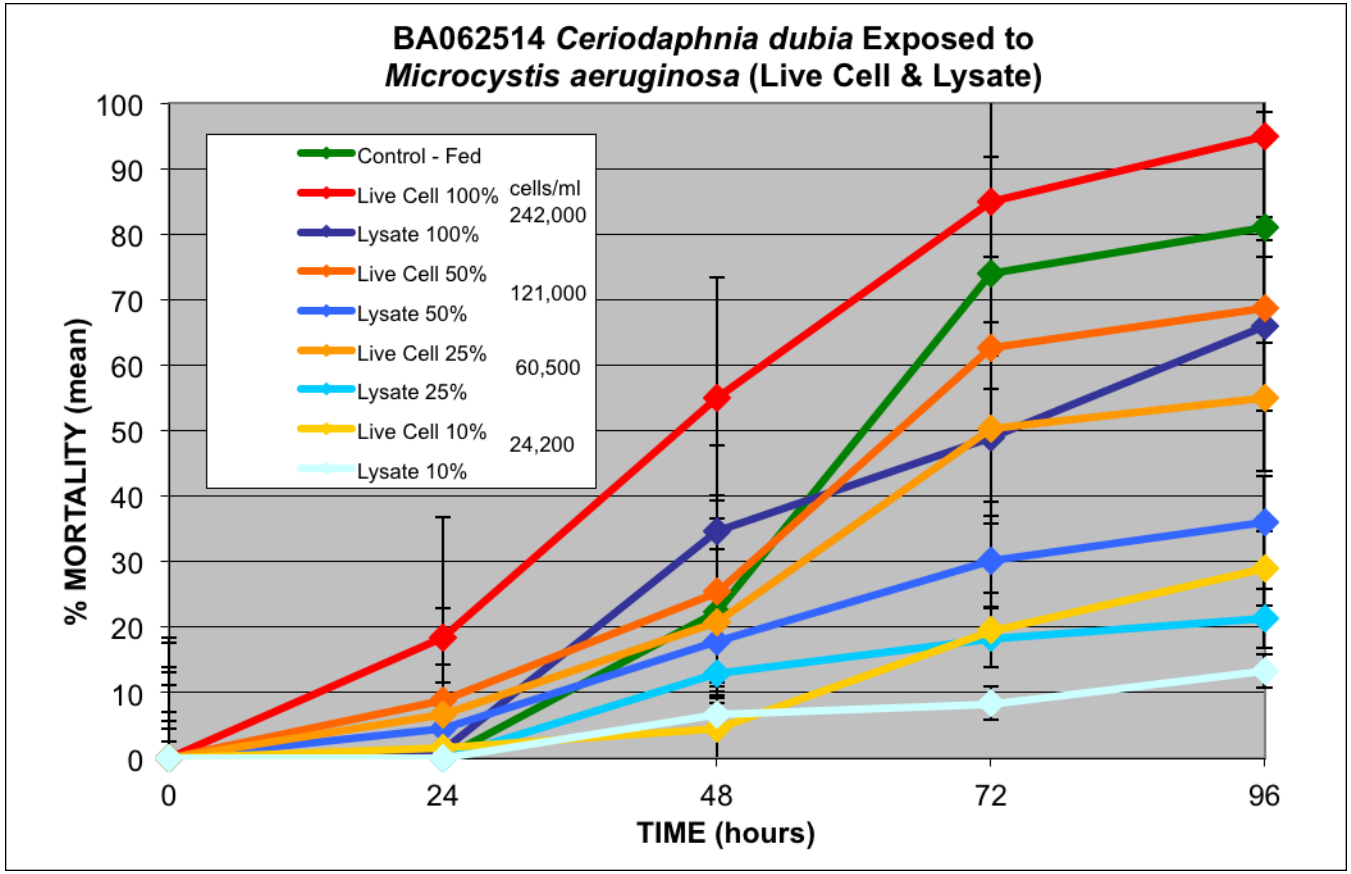
100% = 184 cells/mL (lysed)

Figure 4.



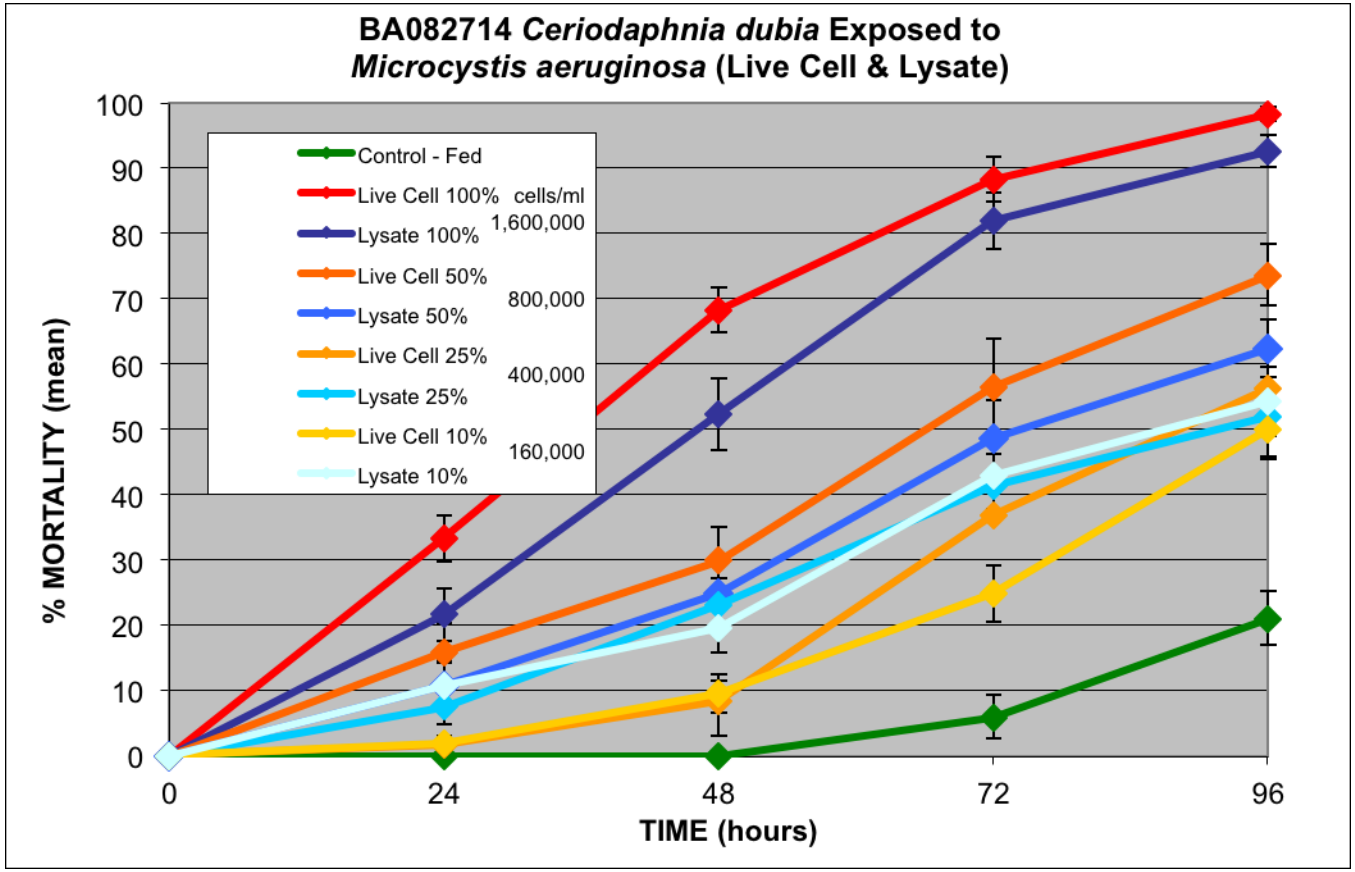
100% = 157,000 cells/mL

Figure 5.



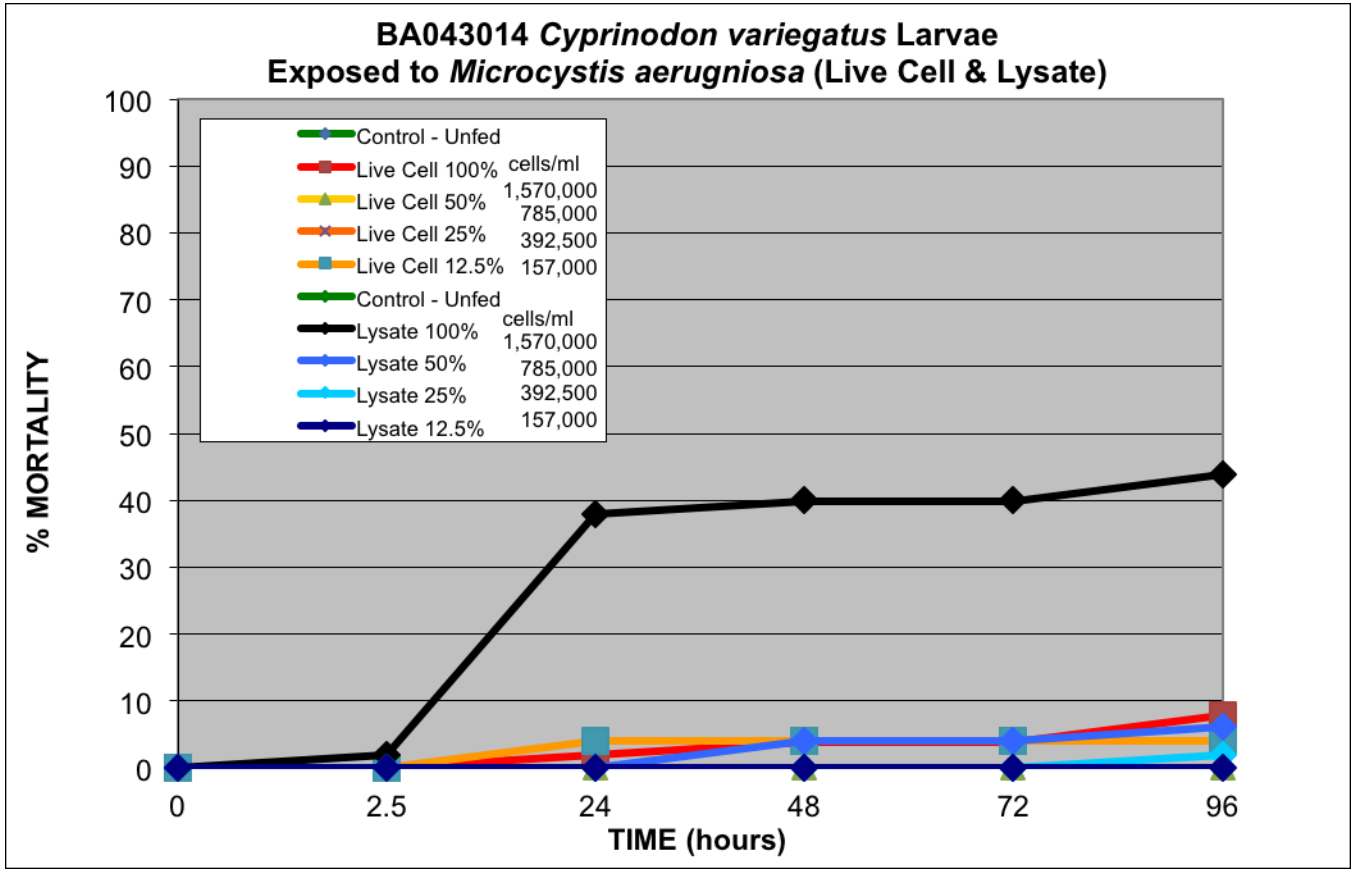
100% = 242,000 cells/mL

Figure 6.



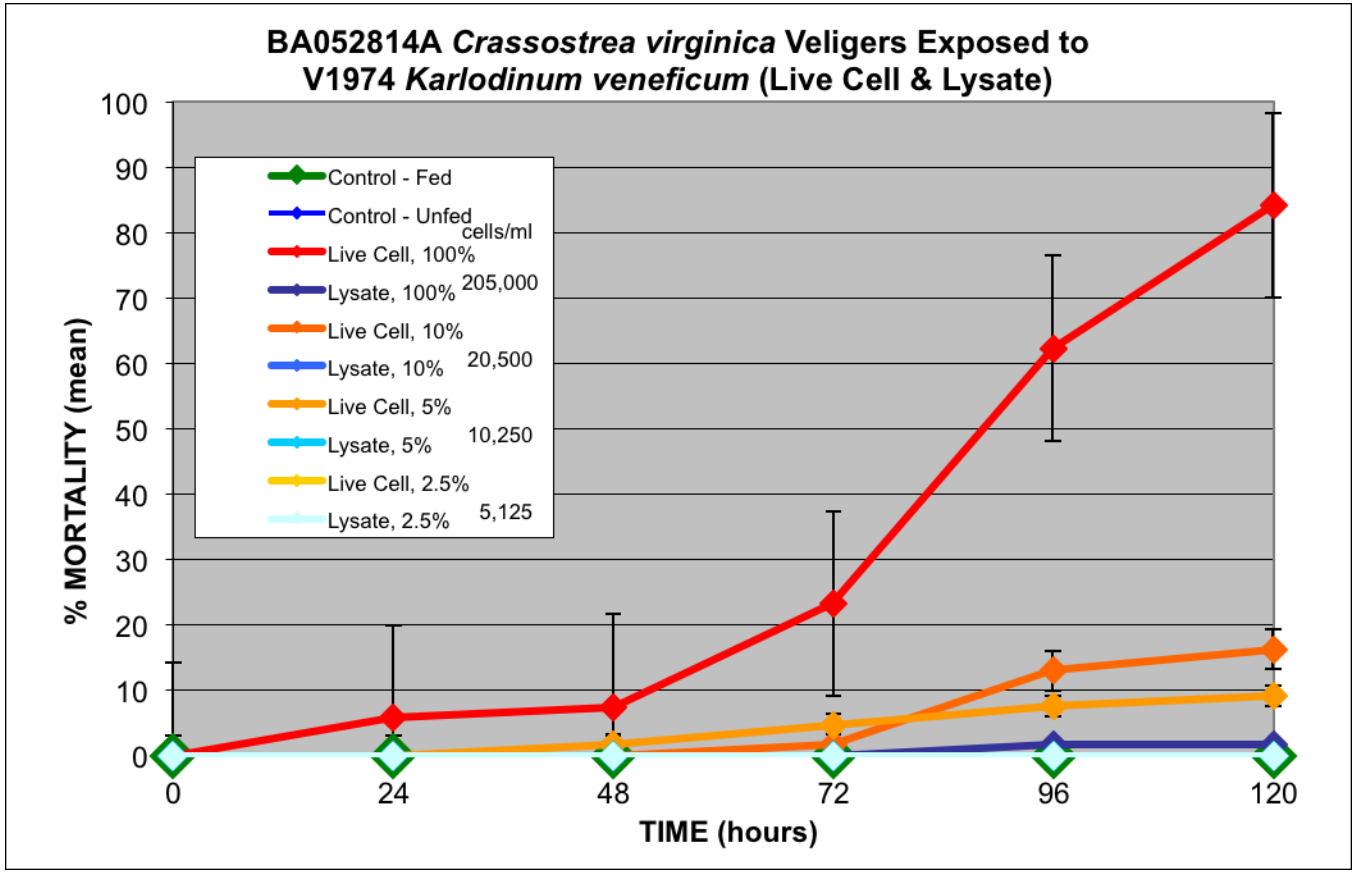
100% = 1,600,000 cells/mL

Figure 7.



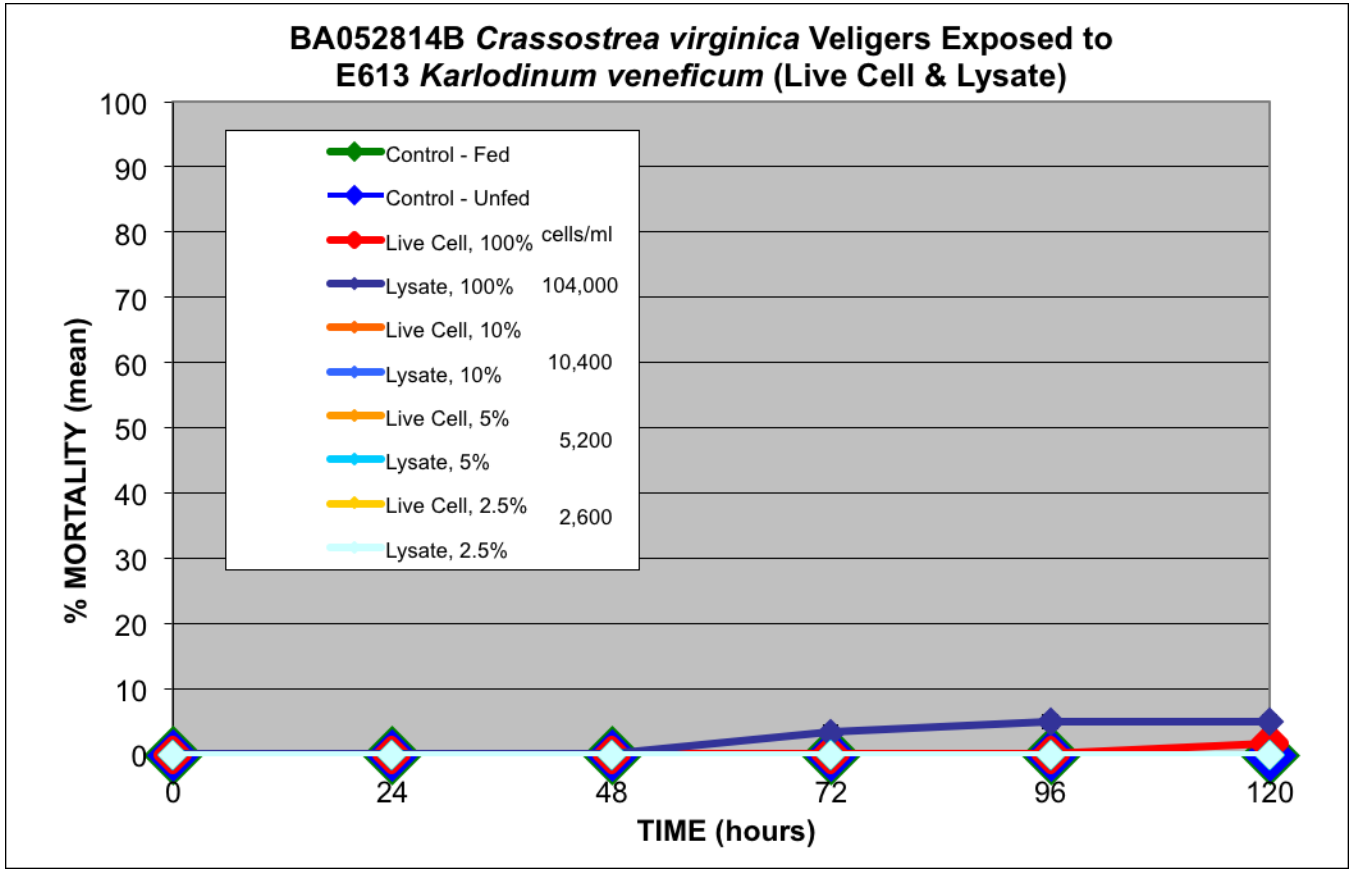
100% = 1,570,000 cells/mL

Figure 8.



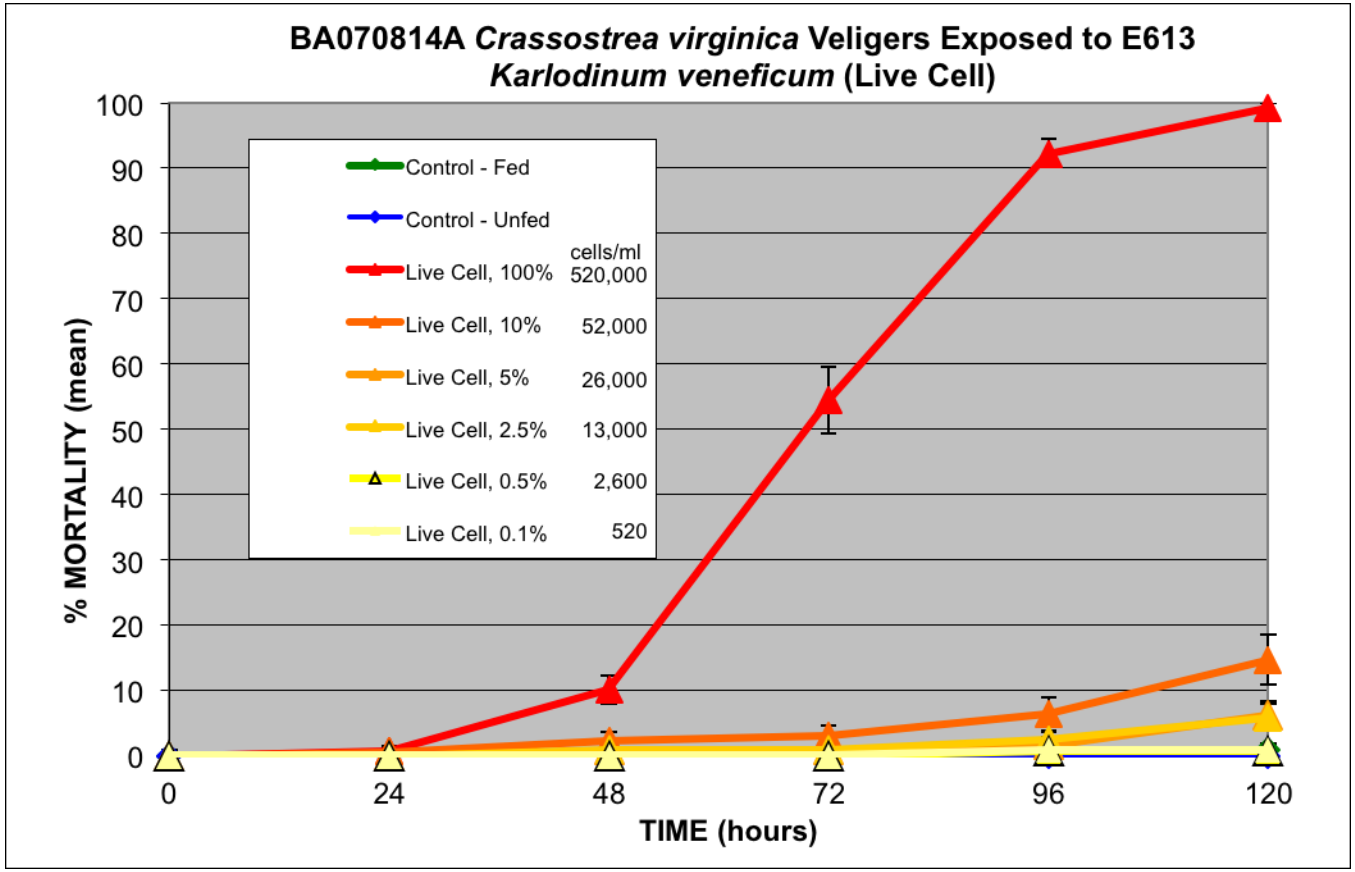
100% = 205,000 cells/mL

Figure 9.



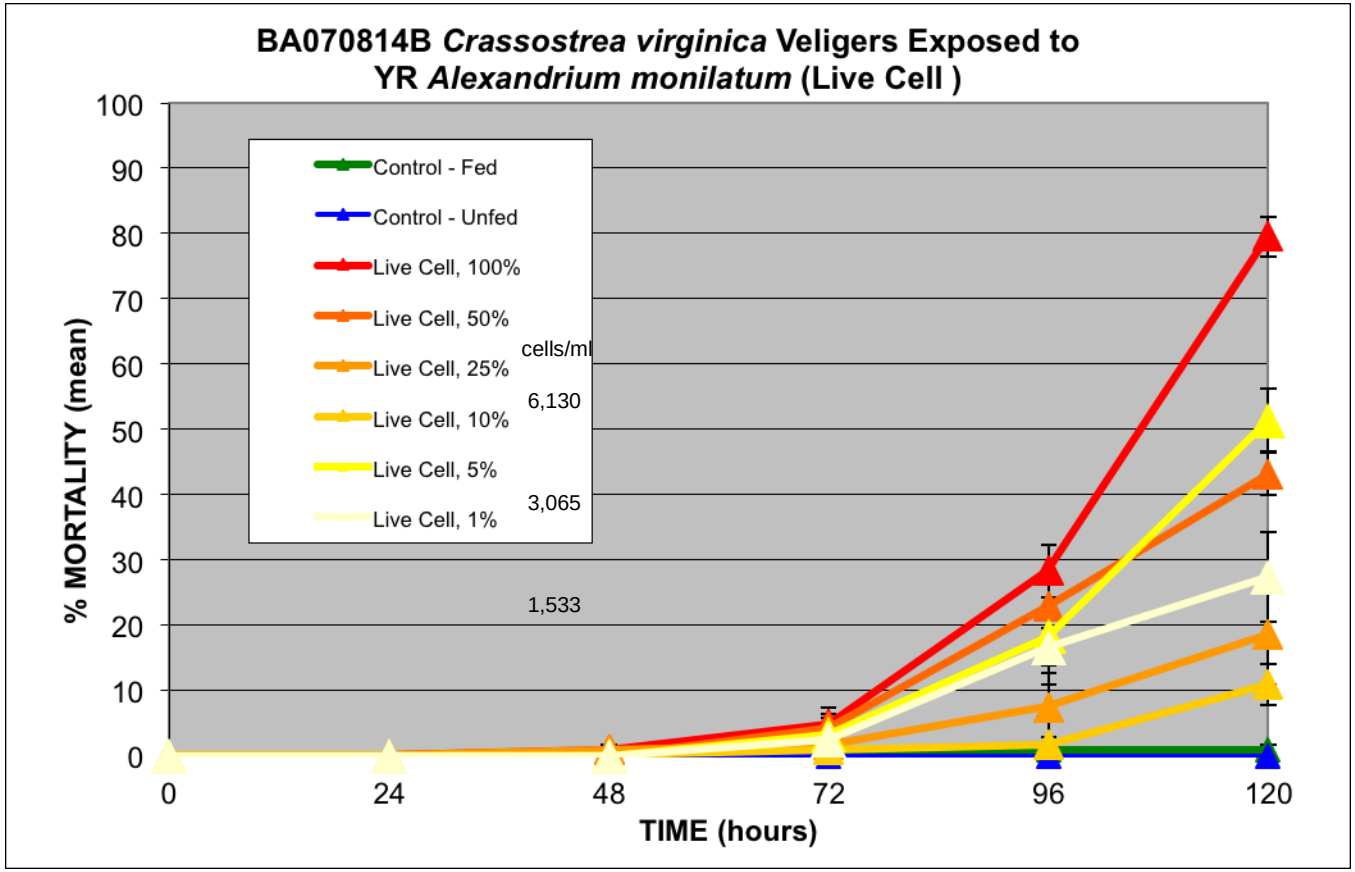
100% = 104,000 cells/mL

Figure 10.



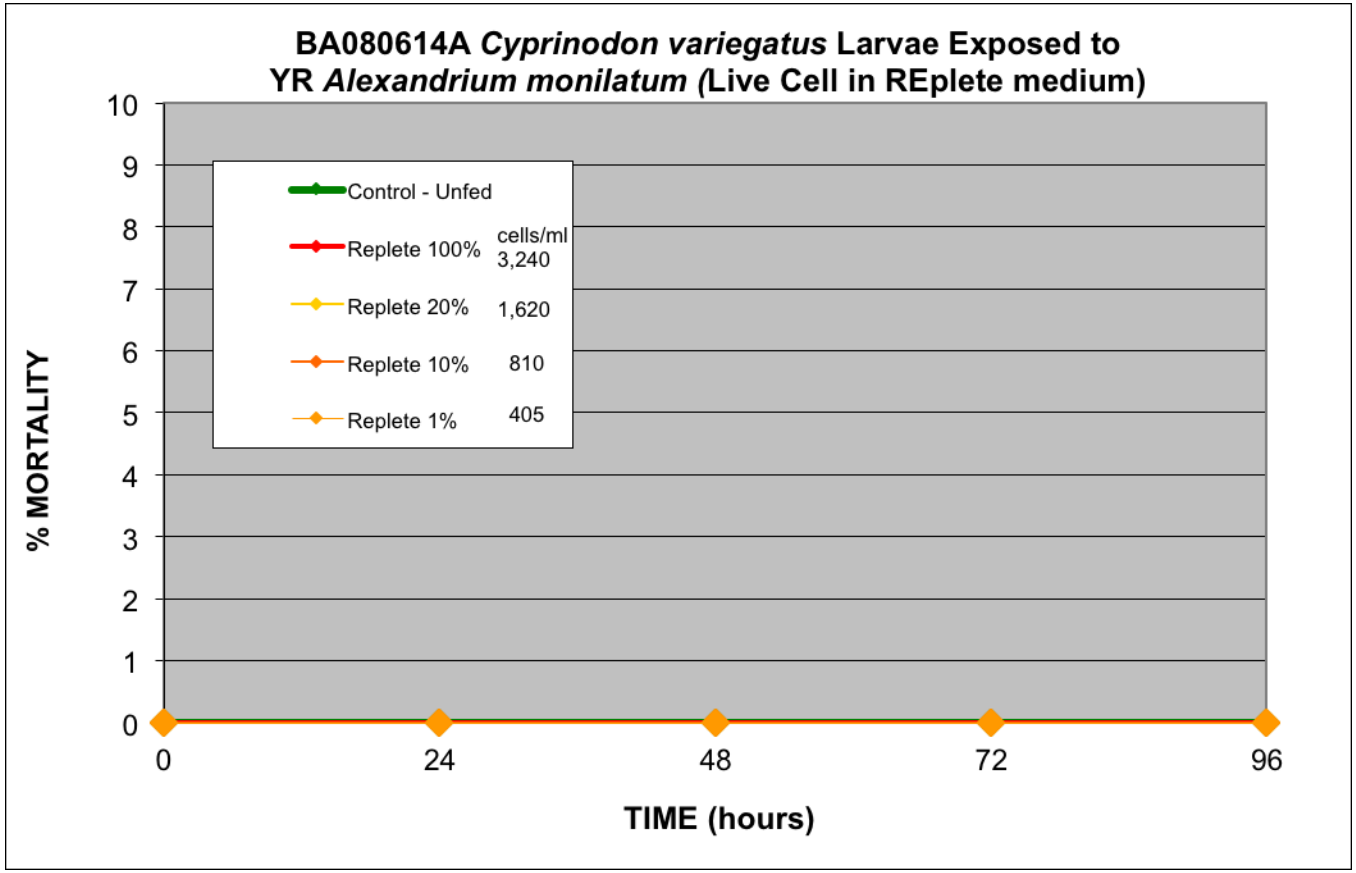
100% = 520,000 cells/mL

Figure 11.



100% = 6,130 cells/mL. Note that the *A. monilatum* grew in the 5% (307 cells/ml) and likely in the 1% wells during the course of the assay. The 5% wells had with a final concentration of 2,750 cells/ml at the end of the assay.

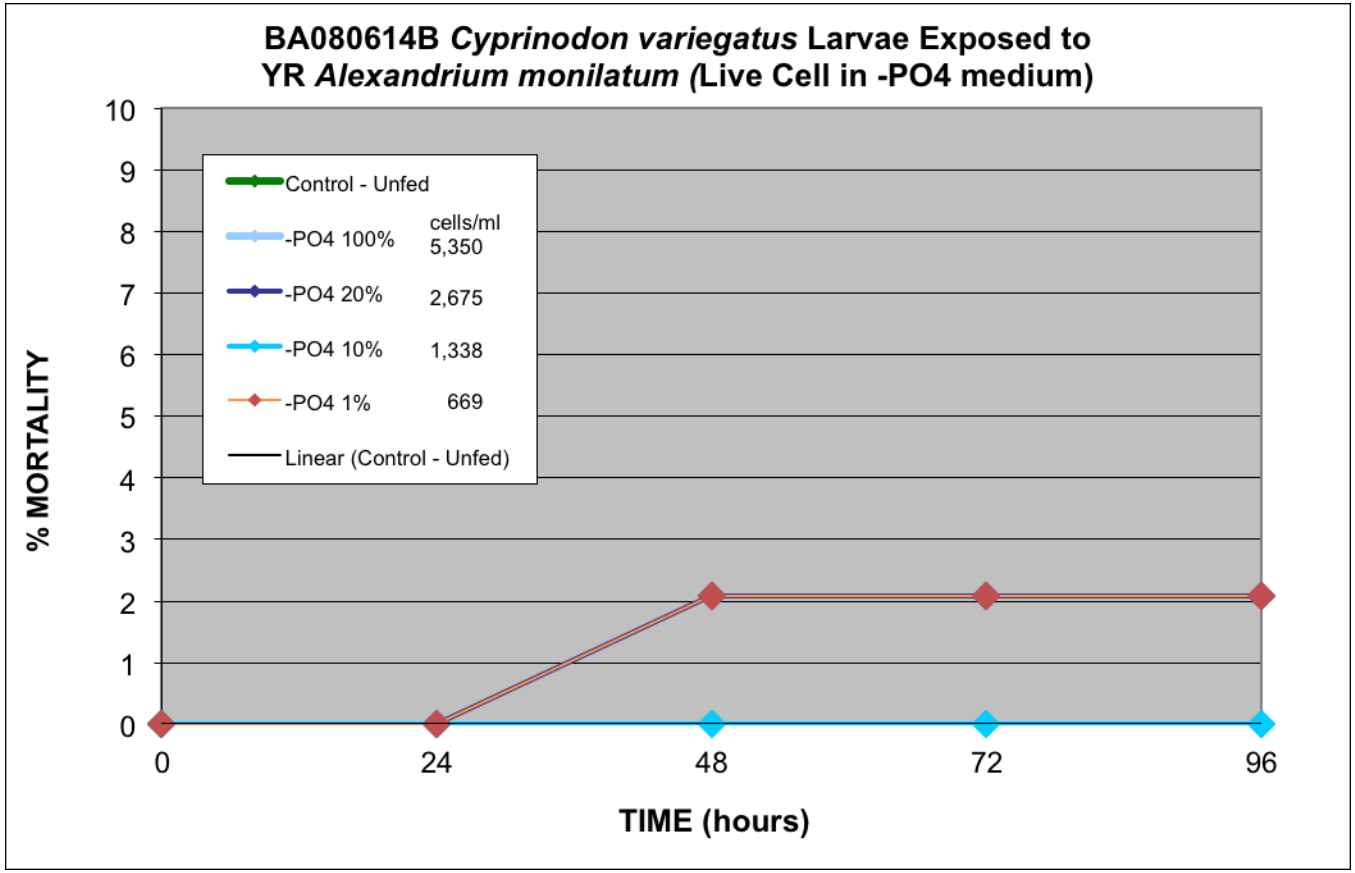
Figure 12.



100% = 3,240 cells/mL

Note that the scale for the % mortality is 1-10%.

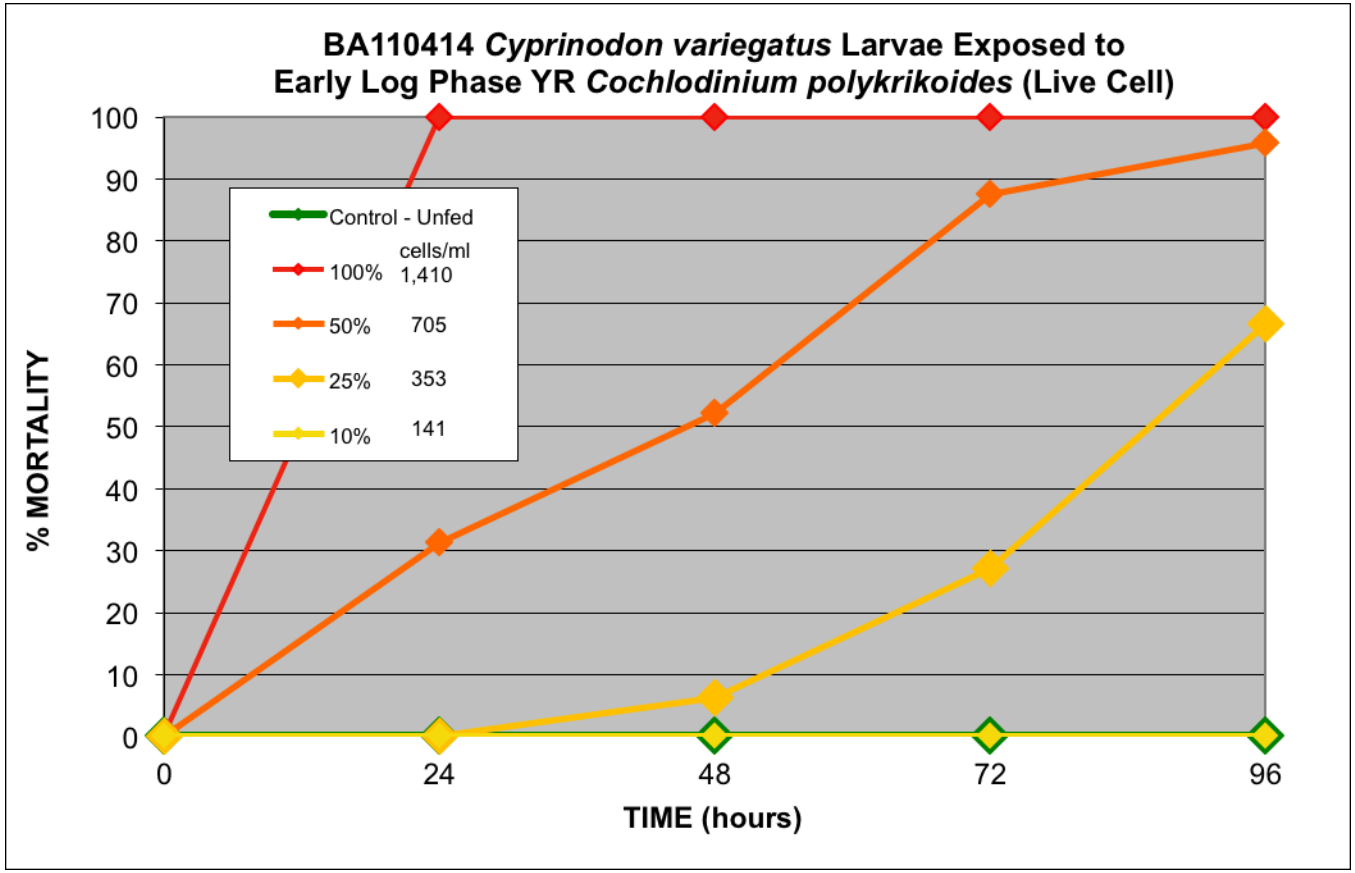
Figure 13.



100% = 5,350 cells/mL

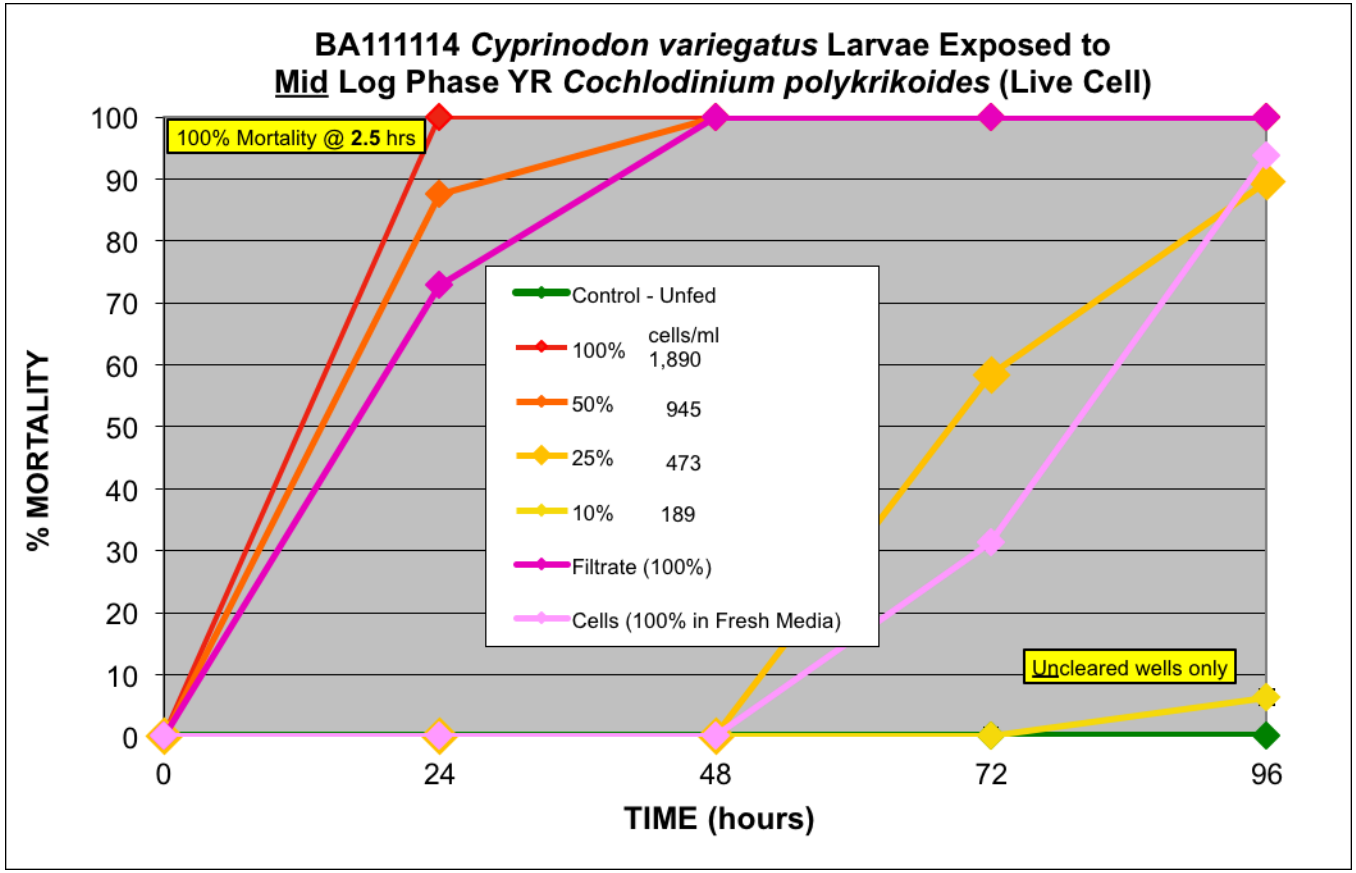
Note that the scale for the % mortality is 1-10%.

Figure 14.



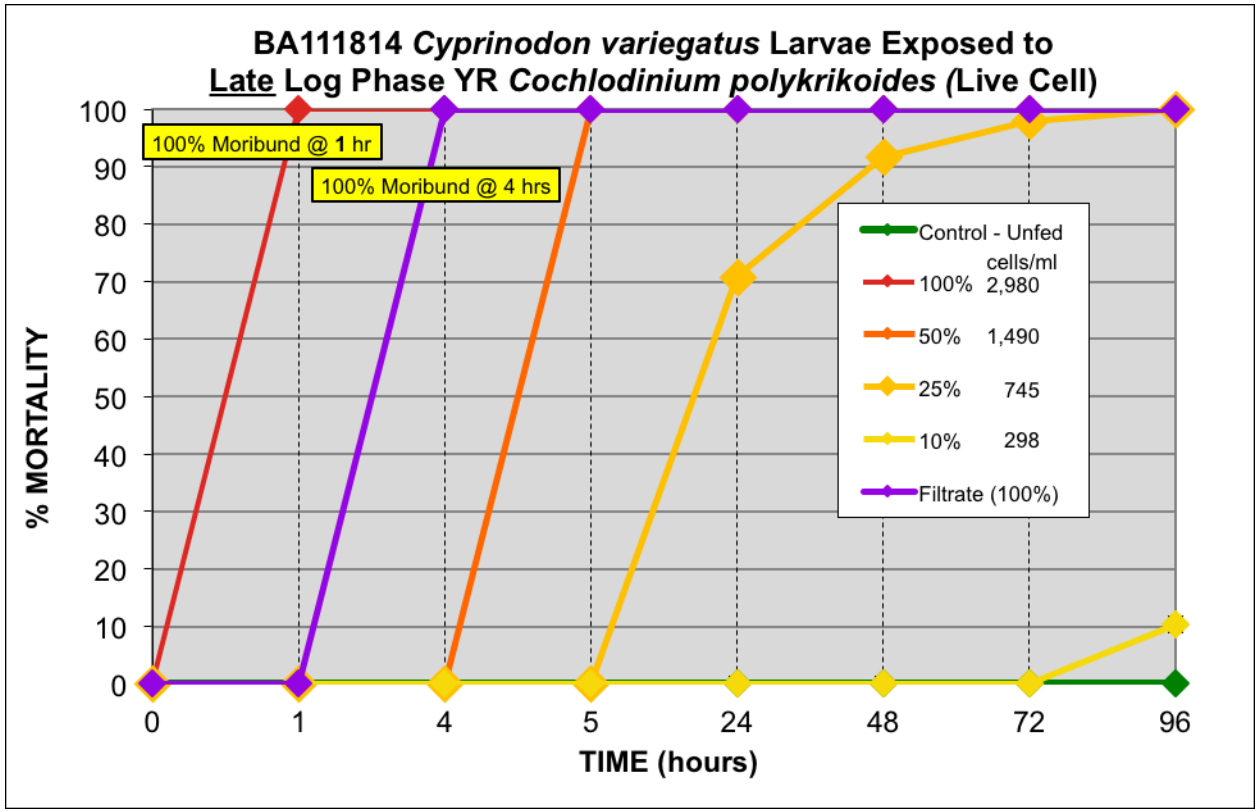
100% = 1,410 cells/mL

Figure 15.



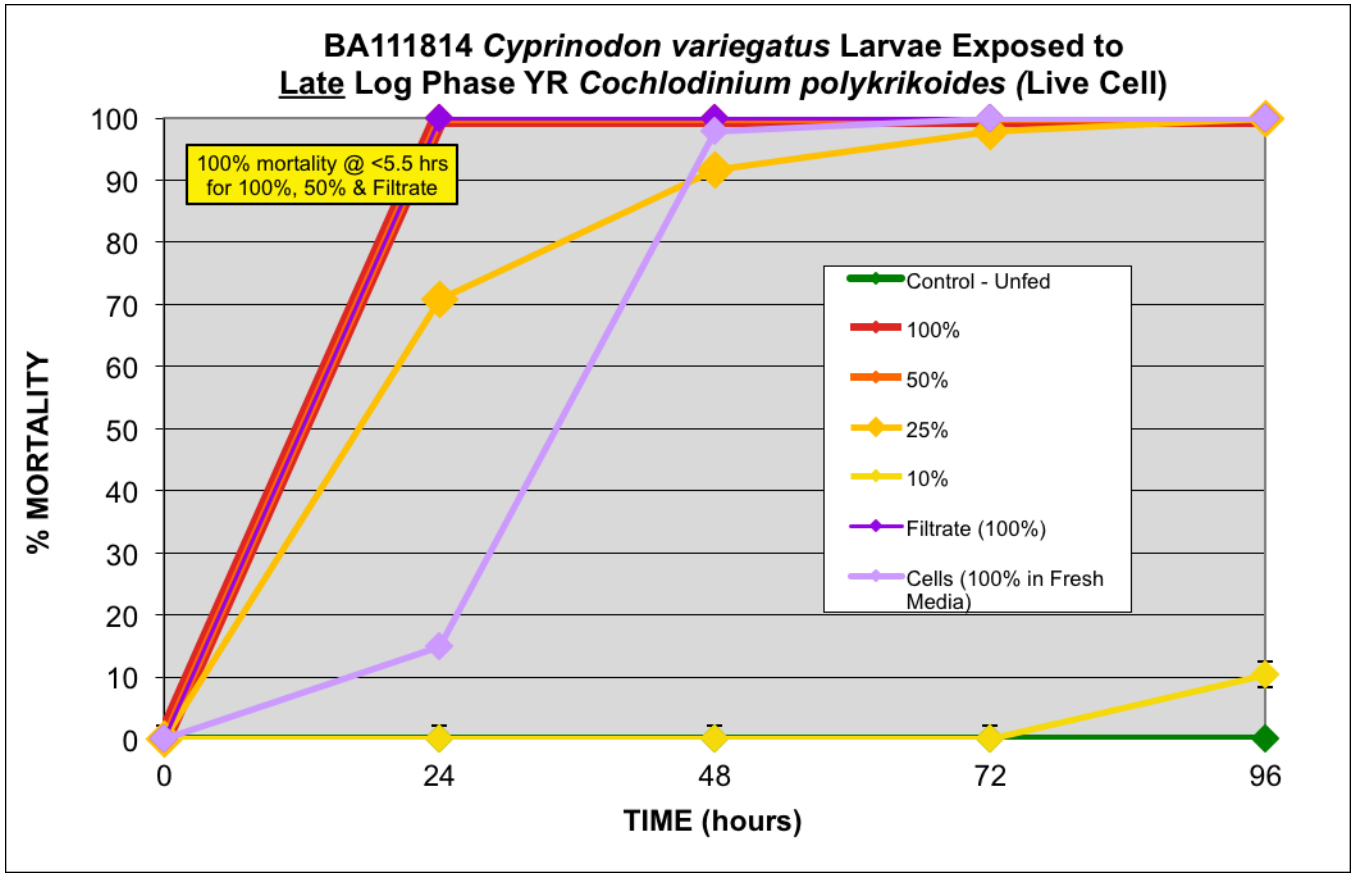
100% = 1,890 cells/mL

Figure 16.



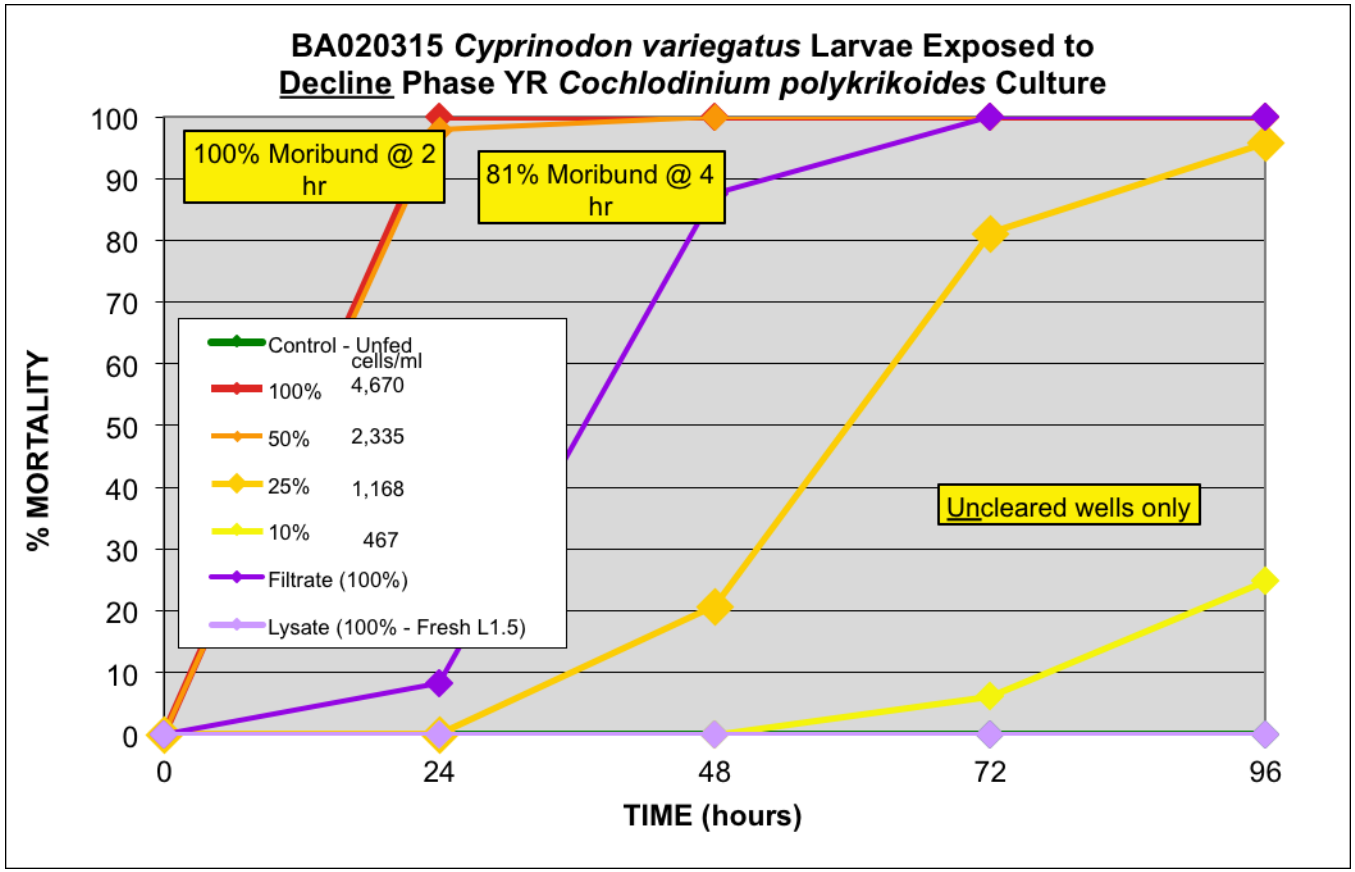
100% = 2,980 cells/mL

Figure 17.



100% = 2,980 cells/mL

Figure 18.



100% = 4,670 cells/mL