FINAL REPORT

Effects of *Microcystis aeruginosa* in Threadfin Shad (*Dorosoma petenense*)

Submitted to:

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*Note: An extension to continue with the project was requested beyond October 2010 to conduct the *Microcystis*/microcystin (MC) exposures of embryonic and larval stages of Threadfin shad (TFS). Difficulties were encountered with culturing and finding other sources of early life stages of TFS hence the medaka *Oryzias latipes* was used as a surrogate species for the exposure trials.

Background

Threadfin shad (TFS, *Dorosoma petenense*), a member of the family Clupeidae, is one of the two clupeid species found in California's freshwater. The Clupeid family also includes many valuable species for fisheries such as herring and sardines. The TFS is a small fresh water pelagic species which inhabits open waters of reservoirs, lakes, and shallow water habitats of the upper San Francisco Estuary (SFE). It feeds on small zooplankton, phytoplankton, and detritus particles via filter feeding but will also feed on larger zooplanktons when available. Optimum spawning occurs in July-August when water temperatures exceed 20°C (Moyle 2002).

Because TFS spawning coincides with the highest incidence of *Microcystis aeruginosa* (cyanobacterium) blooms, the embryos and larvae are the most vulnerable life stages. These early forms are very likely exposed to algal toxins affecting their development, behavior, physiology, and reproductive performance. Their indiscriminate feeding behavior and the timing of their reproduction and larval growth during peak of cyanobacterial blooms make them an ideal model species for this targeted study. Due to the difficult nature of obtaining embryos and larval threadfin shad we decided to perform the exposure experiments using Medaka (*Oryzias latipes*). Medaka is a standard fish for toxicity testing. Medaka have embryos that bind to a substratum just like threadfin shad and have larvae that form a functioning mouth shortly after hatching. They are both temperate to warm water species. Medaka is a sensitive species that can be used to characterize the effects of *Microcystis* and MC-LR exposure. Medaka has been used to model the toxic effects of cyanobacteria (Jacquet et al., 2004; Huynh-Delerme et al., 2005; Escoffiera et al., 2007; Mezhoud et al., 2008). Part one of this study is to determine the dietary and ambient effects of *Microcystis* on medaka. We hypothesized that 1) Larvae are significantly affected by exposure to *Microcystis* and MC-LR. 2) Embryos will hatch normally after exposure to *Microcystis* and MC-LR due to the impermeability of the chorion.

Recurring blooms in the San Francisco Estuary (SFE) are composed primarily of colonial forms and single cells of *Microcystis aeruginosa* that are distributed across varying salinities (0.1–18 ppt) from low-flow waters of the central delta, seaward to marine and brackish water habitats to the western fresh water reaches of the Sacramento River (Lehman and Waller 2003, Lehman et al. 2005). Bloom season in the SFE occurs from July to November and peaks in September. Bloom toxicity in the SFE is associated with the production of microcystins (MCs, hepatotoxic heptapeptides) that are released into water during or upon senescence of cyanobacterial blooms. The dominant MC congeners present in the SFE include the MC-LR, MC-LA and MC-WR that vary spatially and temporally (Lehman et al. 2005, 2008). Although MC toxin concentrations have been commonly below the World Health Organization (WHO) recommended limit of 1 µg/L for drinking water (WHO 1999) in the first half meter of the water column (Lehman et al. 2005, 2008), MC levels are relatively higher (0.007 – 10.81 µg/L) in plankton tissues as observed from blooms in 2007 (Baxa et al. 2010). As such, fish can be exposed to MCs directly during feeding (especially for filter feeders such as TFS) or passively when the toxins pass through gills during breathing.

Microcystin-LR is one of the most toxic variants of the more than 80 congeners of the protein (Zurawell et al. 2005). Due to the vertical migration of blooms to the surface during daylight hours and to the benthic region at twilight, benthic and pelagic food web organisms have a

greater risk of exposure to the toxins. For this reason, *M. aeruginosa* may have a widespread impact in the SFE prompting the Interagency Ecological Program Management Team to list *M. aeruginosa* as a potential factor contributing to fish population declines in the estuary since 2000 (Sommer et al. 2007).

In our studies, establishing a link between the adverse effects of *M. aeruginosa* to the general health of threadfin shad is necessary to demonstrate the threat of the cyanobacterium. The impacts of MC exposure using medaka as the surrogate species may be potentially used to devise control measures designed to reduce toxicity in TFS and in other pelagic and benthic organisms susceptible to MC toxicity.

Fish are hypothetically exposed to MCs through: 1) direct consumption of *Microcystis* and other cyanobacteria containing MCs, 2) interaction of organisms in the food web by grazing on organisms consuming *Microcystis* and other cyanobacteria producing MCs, and 3) exposure to dissolved MCs during or at the senescence of *Microcystis* and other cyanobacteria producing MCs. While the toxicity of the *Microcystis* blooms is associated with the production of MCs, the actual mechanism linking *Microcystis* with the decline of fish populations in the SFE has not been determined (Sommer et al. 2005, Feyrer et al. 2009). MC toxicity has been established in laboratory conditions in different fish species including rainbow trout (*Oncorhynchus mykiss*) (Tencalla et al. 1994), carp (*Cyprinus carpio*) (Carbis et al. 1996) and medaka (*Oryzias latipes*) (Mezhoud et al. 2008, Deng et al. 2009b).

To date, there have been no studies determining the toxicity of MCs produced by *Microcystis* blooms on resident fish species in the SFE. As MCs have been detected in the benthic and pelagic food webs of the estuary (Lehman et al. 2005), it is important to evaluate the biological fate and potential risk of the toxins to fish and other aquatic organisms in these food webs. The second part of this study is to determine whether *Microcystis* is a potential threat to fish in the estuary by utilizing the threadfin shad, *Dorosoma petenense* as an indicator species for the pelagic food web.

Task 1: Laboratory Culture of Threadfin Shad

Fish collection, transport, and maintenance

2008

Groups of threadfin shad (TFS) were collected for broodstock development from two locations in the San Francisco Estuary (SFE): Toe Drain (N=16, mean wt = 8 ± 0.5 g) on August 28, 2008 and from Brannon Island (N=106, mean wt = 10 ± 0.5g) on September 3, 2008; water temperatures were 20 and 22ºC, respectively from the sites. The fish were transported to the Center of Aquatic Biology and Aquaculture (CABA) at UC Davis using tanks supplied with CABA well water. During transport, the fish were mildly sedated with MS-222 (10 ppm) in aerated transport water maintained at 19ºC. Following arrival at CABA, the fish were immediately transferred to a 200 L flow through system supplied with 18ºC well water. The fish were fed once a day at 2% body weight with a Silver Cup #1 diet. The flow-through system was checked daily for any impairment, uneaten food were removed by siphoning, automatic feeders

were refilled, fish were observed for any abnormalities, and mortalities were recorded and discarded. All tank equipments were disinfected in Argentyne, rinsed and dried after each use.

2009

Delta: Wild broodstock was collected on several occasions in the Delta with the help of Dr. Ted Sommer, Kevin Reece, and DWR staff. On May 5, 2009 Sherman and Brannon Islands and Stockton were sampled. No fish were found in Sherman or Brannon Island. Among the 40 fish collected at Stockton, 10 fish were preserved in 10% buffered formalin for histopathology, 20 fish were held on dry ice for nutrition (N=10) and disease (N=10) analyses, and the remaining 10 fish were transported to CABA for broodstock development as described below. On May 15, 2009 at Stockton, only 32 fish were collected to determine if there were any ripe females and males. The male spermiated but none of the females were mature. The remaining 15 fish were brought to CABA and added to the broodstock. Toe drain was sampled in June at multiple locations but no fish were found as in the previous year. Kevin Reece made inquiries to the Department of Fish and Wildlife (DFW) and other labs at UC Davis (Drs. Sih, Moyle and Cech) but did not receive any information on TFS distribution.

Reservoir: Additional sites for obtaining broodstock for larvae were recommended by Teejay O'Rear from Dr. Peter Moyle's laboratory. With Teejay's support for gear and collection permit, Lakes Hennessey and Berryessa were beach seined at several sites on the shore. No viable broodstock were detected at these sites.

Tracy Fish Collection Facility: The 400 fish acquired from August to November from Brent Bridges at the Tracy Fish Collection Facility (Tracy, CA) were maintained at CABA for broodstock development. Transport proved more successful (mortalities reduced from 60 to 4%) by holding the TFS using a mixture of CABA well water and Tracy and adding salt to reduce osmotic stress. The tank was oxygenated during transport and the water was chilled several degrees below the Tracy water temperature. Upon arrival at CABA the fish were acclimated to the well water temperature for over 2 hrs. Using this modified transport technique, mortalities associated with stress during transport were at 4% (August), 8% (September), and 20% (November).

2010

Delta: Threadfin shad were collected in the field by electrofishing with the aid of Sih Laboratory from UC Davis and equipment from the DFW. On June 17, 2010, threadfin shad (N=3) were collected in Sugar Slough and from Grant Line Slough (N=3). The threadfin from Sugar Slough included one gravid female but was ingested by a largemouth bass. The bass was pumped, the female shad was recovered but did not survive the process, and eggs were stripped. The remaining two shads from Sugar Slough were males with only one responding to stripping. The sperm was collected from the responsive male and added to the eggs from the female using dry fertilization. River water was used to activate the sperm. The resulting embryos became adhesive and stuck to the sides of the Nalgene container and to each other. The embryos were transported in a Coleman cooler to Haring Hall at UC Davis.

Broodstock development

2008-2009

When ambient water temperatures began to drop below 18ºC in October, gonad maturation was enhanced by decreasing to 1ºC/week beginning in October 30, 2008 until 12ºC was reached by December 31, 2008. This was followed by increases in water temperature by 1ºC increments/day from 12 to 18ºC from March 17 to April 20, 2009. The TFS diet was shifted to Silver Cup #2 at 2% body weight/day and then increased to 3% when water temperature remained stable at 18ºC.

Sexual maturation was determined on April 13, 2009 by histological examination of gonad development in TFS (N=10, total length: 9.5- 13.3 cm) randomly collected from the broodstock pool. Of the 10 fish collected, 8 were females and 2 were males. Except for one female with mature stage IV oocytes, all females (stage I-III) and males (no spermatozoa observed) were in immature stage. Histological evaluation also confirms that TFS is a multiple spawner.

2009-2010

Adult TFS (N=400) from the Tracy Fish Collection Facility were maintained at 12°C in 200 and 300 L flow-through circular tanks in CABA, UC Davis since November 2009. This TFS population was used for broodstock development for the 2010 spawning season.

2010

When ambient water temperatures began to drop below 18ºC in October, gonad maturation was enhanced by decreasing to 1ºC/1-2weeks depending on water temperature levels in the SFE. Temperatures were decreased beginning in October 30, 2009 until 12ºC was reached in January 9, 2010. This was followed by gradual increases in water temperature from 12 to 22ºC by no more than 1ºC each week from March 8 to May 10, 2010. The TFS diet was shifted to Silver Cup #2 at 2% body weight/day and then increased to 3% when water temperature remained stable at 22ºC.

Sexual maturation was determined by daily examination of gonad development in TFS beginning on March 30, 2010 until May 10th, 2010. Of the fish collected, 22 were females and 12 were males. All females (stage I-III) and males (no spermatozoa observed) were in immature stage. Following injection of LnRHa on May 25, maturation of females and males progressed. Females were found to be in Stage III and IV and males exhibited spermatozoa.

Natural spawning

Natural spawning of TFS was attempted in May 2009. Adult TFS (N=26) collected in the delta in 2008 and held for broodstock were transferred to a 100 L flow-through system at CABA, UC Davis. Water temperature was increased from 18 to 22ºC by using heated well water. Spawn Tek was placed in the tank for embryonic attachment, water flow was decreased, and the temperature was maintained with temperature-regulated heaters to allow natural spawning of the broodstock. The Spawn Tek was checked daily for presence of embryos but none were observed hence induced spawning was initiated one week following unsuccessful natural spawning.

Induced spawning

2009

On June 24, 2009, induced spawning was initiated with the help of Joel Van Eenennaam from Dr. Sergei Doroshov's laboratory at UC Davis. The 26 TFS (mean wt = 15±3 g/fish) that failed to spawn naturally were held in a 100 L circular tank in CABA supplied with 22^{o}C flow-through well water. Spawn Tek was used to line the tanks to capture the embryos. After acclimating the fish to 22ºC, daily feeding was stopped on June 30th in preparation for induced spawning. At 8:30 pm on June 30, 2009, 20 ppb GnRH (gonadotropin releasing hormone, Bachem, Torrance, CA) was prepared in 1 ml syringes with 29½ G ultra fine needle (Becton Dickinson, Franklin Lakes, NJ). The fish were anesthetized in 50 mg/L MS-222 water bath prior to injection. About 3-4 sedated TFS were injected intraperitoneally at a time with 0.01-0.02 ml GnRH/fish and then placed in a second tank with the same water temperature to recover for 12 hrs following hormone injection. On July 1th at 8:30 am, 3-4 TFS were netted each time and placed in a 50 mg/L MS-222 bath. The fish were stripped to determine if they were susceptible to GnRH. Several males were stripped of sperm but no females were successfully stripped of eggs. Previous sampling suggested that the male to female ratio was 50:50. While the males responded positively to the GnRH, the females failed to develop mature eggs. A potential explanation for this result is that the females may have been at the end of their reproductive cycle and have already released their eggs precluding any positive response to GnRH. Induced spawning was therefore planned in March 2010 spawning season. No mortalities were observed due to handling and hormone injection.

2010

On May 25, 2010, induced spawning was initiated on the broodstock housed in CABA at UC Davis. A total of 16 TFS (mean wt = 10±3 g/fish) were held in a 100 L circular tank in CABA supplied with 22^{o}C flow-through well water. Spawn Tek was used to line the tanks to capture the embryos. After acclimating the fish to 22ºC, daily feeding was stopped on May 24th in preparation for induced spawning. At 7:00 pm on May 25, 2010, 6 ppb LnRHa (luteinizing-releasing hormone, Bachem, Torrance, CA) was prepared in 1 ml syringes with 30½ G ultra fine needle (Becton Dickinson, Franklin Lakes, NJ). The fish were anesthetized in 50 mg/L (ppt = g/L; ppm= mg/L) MS-222 water bath prior to injection. About 3-4 sedated TFS were injected intraperitoneally at a time with 4.0 µl of 6 ppb LnRHa/fish and then placed in a second tank with the same water temperature to recover for 12 hrs following hormone injection. Injecting with 4.0 µl hormone assured that 2.4-3.0 µl/g is introduced to the fish (10±3 g/fish). On May 26th at 8:00 am, 3-4 TFS were netted each time and placed in a 50 ppt MS-222 bath. The fish were stripped to determine if they responded to LnRHa. Several males were stripped of sperm but no females were successfully stripped of eggs. Previous sampling suggested that the male to female ratio was 50:50. While the males responded positively to the LnRHa, the females did not respond consistently to the treatment. The oocytes of the female threadfin shad matured but did not exhibit asynchronous egg maturation. No mortalities were observed due to handling and hormone injection.

On May 28, 2010 the 16 fish injected previously were injected again with 4.0 µl of 6 ppb LnRHa/fish. In addition, 20 untreated fish were also injected with the same hormone concentration. The fish were stripped to determine if they responded to LnRHa. Several males

were stripped of sperm but no females were successfully stripped of eggs. Additional injections on June 1 had similar results.

On June 7, 2010 a new batch of threadfin shad were treated with LnRHa. A total of 98 fish were injected with an increased dose of 6.0 µl of 6 ppb LnRHa/fish. Males could be stripped of sperm but the females were still unresponsive. Subsequent injections on June 9th and 15th had similar results. Examination of the gonads showed maturation of the oocytes following injections but no final stage oocytes were present.

Some potential explanations for the lack of spawning may be due to the treatment technique and culture conditions of the broodstock. Maturation occurred in the threadfin shad relative to untreated threadfin shad and males could be stripped but the females would never progress to the final stage of oocyte maturation. The threadfin shad appeared to respond to the injections of LnRHa but optimizing the protocol may be necessary such as injections with gradual increased doses of the hormone or by using implants. The culturing conditions of the broodstock may have been inadequate to promote consistent maturation of the oocytes for spawning. The Silver Cup #2 diet, which has been used in previous literature for threadfin shad culture and salmonid broodstock, may not have been suitable for broodstock development in threadfin shad. Spawn Tek was added to the water to promote spawning behavior but the threadfin shad tended to avoid the material. Acclimating the broodstock to the Spawn Tek or utilizing another form of spawning aid may be necessary.

Culture system development

One static culture system equipped with water bath (200 L) and beakers (2-4 L) were prepared for raising larval stages. In addition, 3 recirculating systems comprising 4 tanks (70 L each with a total of 450 L for each system) were built for growing juvenile to adult stage. Growth and survival of larval TFS collected in the wild were to be compared to larvae spawned from laboratory-maintained broodstock.

Task 2: Lethal and sublethal effects of *Microcystis* on TFS

2.1. Water exposure of TFS embryo and larvae to microcystin-LR (MC-LR)

Materials and Methods

Due to the unavailability of TFS embryos and larvae, medaka (*Ozyrias latipes*) was used as a surrogate species. Medaka is a standard toxicity testing species (Jacquet et al. 2004). Microcystin-LR (MC-LR) was obtained from a manufacturer (Abraxis, CA) and diluted to 10ppt stock solution with reconstituted water.

2.1.1 Embryo exposure

Embryos (n=250) were collected for the exposure trials. The 250 embryos were separated into scintillation vials at 5 treatments and five replicates. The treatments were prepared by serial dilution of the MC-LR stock solution with reconstituted water to a final concentration of 0, 0.01, 0.1, 1.0, and 10 ppm of MC-LR. Each scintillation vial contained 10 ml of the treatment solution and was kept at 25^{o}C in a water bath. The 50% of the treatment solution was renewed daily with fresh solution using the stock solution. Mortalities, hatching time, and heart rate were recorded daily. The embryos were exposed at day 1 and the exposure ran until all embryos were hatched

or perished. Hatched larvae were euthanized by MS-222 and preserved in 10% buffered formalin.

2.1.2 Larval exposure

Medaka larvae (n=240) were selected for the exposure trials. The larvae were separated into 250 ml glass jars at 5 treatments and three replicates. The treatments were prepared by serial dilution of the MC-LR stock solution using reconstituted water to a final concentration of 0 (control), 0.01, 0.1, 1.0, and 10 ppm of MC-LR. Each glass jar contained 200 ml of the treatment solution and was kept at 25^{o}C in a water bath. 50% of the treatment solution was renewed daily with fresh solution made from the stock solution. Mortalities and swimming behavior were recorded daily. The study lasted for 7 days. All mortalities were discarded. At the end of 7 days all remaining larvae were euthanized with MS-222 and fixed in 10% buffered formalin.

Results and Discussion

2.1.1 Embryo exposure

Mortalities, hatching time and heart rate were recorded and summarized in Table 2.1.l. Hatching occurred first on day 6 in the 0.1 ppm treatment with 1 hatch. Multiple larvae hatched on day 7 for the 0 (9 hatch), 0.01 (11 hatch), 0.1 (7 hatch), 1.0 (12 hatch), and 10 (3 hatch) ppm MC-LR. Mortalities began on the 7th day for 0.01 (1 mortality) and 1.0 (3 mortality) ppm MC-LR. Heart rates averaged 125 beats per minute by day 5, 155 beats per minute by day 6, 180 beats per minute by day 7, and 200 beats per minute by day 9. The last hatch occurred on the 15th day for the 1.0 ppm MC-LR treatment.

There was no significant difference between the treatments and the control. Heart rate was similar between all treatments and the control. Mortalities of embryos were not significantly different between treatments and the control. Hatching time occurred earlier in the controls and at lower MC-LR concentrations (0.01 and 0.1 ppm) but did not statistically differ from the other treatments of 1.0 and 10 ppm. The results are consistent with previous exposure studies on loach (*Misguruns mizolepis)* (Liu et al 2002) and zebrafish (*Danio rerio*) (Wang et al 2003) using embryonic stages. Our results suggest that the chorion of the embryos is an effective barrier to microcystin exposures.

Table 2.1.1. Medaka embryos exposed to 0.00 (Control), 0.01, 0.1, 1.0, and 10 µg/L microcystin-LR on day 0. Heart rate (HR), hatch day start and end, total days to hatch all embryo, % hatch, and mortalities (% Morts) were recorded.

Treatment	n	Ave HR	Hatch day start	Hatch day end	Total days	% Hatch	% Morts
Control-1	10	169.38 ± 5.1	8.0 ± 1.0	10.2 ± 1.1	3.2 ± 1.3	96.0 ± 8.9	4 ± 8.9
0.01-1	10	160.4 ± 2.2	7.6 ± 0.5	9.0 ± 0.0	2.4 ± 0.5	98.0 ± 4.5	2 ± 4.5
0.1-1	10	163.7 ± 6.2	7.4 ± 0.9	9.4 ± 0.9	3.0 ± 1.0	100 ± 0.0	0 ± 0.0
1.0-1	10	171.0 ± 18.9	7.6 ± 0.5	10.6 ± 2.6	4.0 ± 2.2	80 ± 0.0	20 ± 0.0
10-1	10	168.4 ± 3.9	8.0 ± 0.7	10.4 ± 0.5	3.4 ± 1.1	98 ± 0.0	2 ± 4.5

2.1.2. Larval exposure

Mortalities and swimming behavior were recorded and summarized in Table 2.1.2. Poor swimming behavior began on day 3. Poor swimming behavior was characterized by slow to lethargic swimming compared to the active swimming of normal larvae. Poor swimming occurred in 0.01 (4 poor swimmers), 0.1 (1 poor swimmer), 1.0 (3 poor swimmers), and 10 (2 poor swimmers). Moribund behavior was characterized by a complete lack of movement in the larvae but they still have a heart rate. Moribund fish began to occur on day 6 for Control (1 moribund), 0.01 (1 moribund), and 0.1 (2 moribund). The poor swimming and moribund fish for the Control and 0.01 ppm treatment were found to be contaminated with an unknown algae that may have contributed to the change in behavior.

There was a significant difference between the treatments and the control. There was a significant relationship with increasing MC-LR and increasing incidence of slow swimming behavior by day 5. The 1.0 and 10 ppm fish were exhibiting significantly higher incidences of poor swimming (day 6) and moribidity (day 7). The results are consistent with previous studies on larvae exposure of zebra fish (*D. rerio*) and *Leucaspius delineates* (Baganz et al 2004). Exposure to microcystins may result in severe nutritional stress due to energy expenditure for detoxifying the MC-LR and/or an impaired liver due to damage from the microcystin that may render impaired swimming and growth.

Table 2.1.2. Medaka larvae exposed to 0.00 (Control), 0.01, 0.1, 1.0, and 10 µg/L microcystin-LR after 7 days. The number of poor swimmers and mortalities are recorded.

Treatment	n	# of Poor swimmers	Mortalities
Control-1	20	0	0
Control-2	20	0	0
Control-3	20	3	0
0.01-1	20	3	0
0.01-2	20	7	0
0.01-3	20	6	0
0.1-1	20	5	1
0.1-2	20	4	1
0.1-3	20	5	1
1.0-1	20	6	3
1.0-2	20	5	1
1.0-3	20	6	3
10-1	20	5	2
10-2	20	8	1
10-3	20	7	1

In the absence of adult TFS in the SFE for broodstock development, we were compelled to obtain embryos from alternative sources including J & J Aquafarms (Sanger, CA) and from broodstock maintained at CABA. Unfortunately, the broodstocks in J & J Aquafarms have been impaired by birds over the winter rendering the absence of embryos. The broodstock at CABA did not produce any embryos despite repeated injections of LnRHa. Embryos that were stripped

and fertilized in the field were obtained on June 17th 2010 from the SFE. The embryos were held in an 18°C water bath. After 6 days, over 1100 embryos hatched. Because there were few larvae with the potential for significant mortality, the embryos were reserved for Task 2.2.

2.2. Water exposure of embryo and larval TFS to environmentally-relevant concentrations of algal toxins (i.e., microcystins and its metabolites)
2.2A and 2.2B Effects of algal toxins on embryo and larval TFS

Materials and Methods
Due to the unavailability of TFS embryos and larvae, medaka (*Ozyrias latipes*) was used as a surrogate species. Medaka is a standard toxicity testing species. Microcystin-LR (MC-LR) was obtained from a manufacturer (Abraxis, CA) and diluted to 10ppt stock solution with reconstituted water.

Embryo exposure
Medaka embryos (n=182) were selected for the exposure trials. The embryos were separated into 250 ml glass jars at 3 treatments and three replicates. The treatments were exposure to Nanochloropsis (Nano) from Reed Mariculture (Campbell, CA), *Microcystis* (Micro) collected from the SFE, and a Control (no phytoplankton). The Nano and Micro treatments contained 40,000 cells per ml. Each glass jar was kept at 25°C in an incubator with aeration. Each day 50% of the water was replaced with fresh water containing 40,000 cells of the appropriate phytoplankton. Mortalities, heart rate, twitch rate and hatching embryos were recorded. The study lasted for 14 days. Hatching rate, twitch rate (tail twitch/min), and heart rate (heart beat/min) were recorded. Hatched larvae were euthanized by MS-222 and fixed in 10% buffered formalin.

Larval exposure
Medaka larvae (n=270) were selected for the exposure trials. The larvae were separated into 250 ml glass jars at 3 treatments and three replicates. The treatments were exposure to Nanochloropsis (Nano), *Microcystis* (Micro), and a Control (no phytoplankton). The Nano and Micro treatments contained 40,000 cells per ml.. Each glass jar was kept at 25°C in a water bath. Each day 50% of the water was replaced with fresh water containing 40,000 cells of the appropriate phytoplankton.. The study lasted for 96 hrs. At the end of 96 hrs, mortalities, and swimming behavior were recorded and the remaining larvae were euthanized by MS-222 and fixed in 10% buffered formalin.

Results and Discussion

Embryo exposure
Heart rate, Twitch rate, % Mortalities, and % Hatch were recorded and summarized in Table 2.2.1. Hatching occurred first on day 7 in all treatments. A majority of the larval hatch occurred on day 7 and 8 for Control and Nano while the majority hatch for Micro was on day 10. Control (7.08 beats/min/day), Nano (8.53 beats/min/day) and Micro (5.93 beats/min/day) (Fig. 2.2.2). There was no discernible pattern to twitch rates in each treatment.

There was a significant difference between the treatments and the control. Heart rate was significantly greater in Nano. Mortalities of embryos were significantly greater in the Micro treatment (Fig. 2.2.3). *Microcystis* appeared to cling and smother the embryos. This result may be due to the reduced permeability of dissolved oxygen for the embryos. Peak hatching time occurred earlier in the Controls and Nano (Fig. 2.2.1). Micro's peak hatching time occurred after 10d of exposure and was very poor compared to Control and Nano (Fig. 2.2.1). The results are consistent with a previous study on Baltic herring (*Clupea harengus*) embryos following exposure to *Microcystis*. The herring embryos exhibited significantly greater mortalities and increased maturation time (Ojaveer et al. 2003).

Table 2.2.1 Medaka embryos exposed to no phytoplankton (Control), *Nanochloropsis* (Nano), and microcystin producing *Microcystis* (Micro) from the San Francisco Estuary. Heart rate (beats/min) and Twitch rate (tail twitch/ min) were averaged over length of the experiment. The % larvae that hatched or perished were totaled after 14 days of exposure.

Treatment	n	No. of fish	Heart rate	Twitch rate	% Mortalities	% Hatch
Control	3	182	128.54 ± 11.38	0.24 ± 0.08	8.24 ± 1.90	90.29 ± 2.22
Nanochloropsis	3	182	138.58 ± 4.98	0.58 ± 0.08	8.24 ± 1.98	90.66 ± 2.52
Microcystis	3	182	114.67 ± 5.06	0.11 ± 0.05	69.41 ± 16.30	20.33 ± 3.85

Figure 2.2.1 Medaka embryos exposed to no phytoplankton (Control), *Nanochloropsis* (Nano), and microcystin producing *Microcystis* (Micro) from the San Francisco Estuary. The average hatch totals is reported for each treatment.

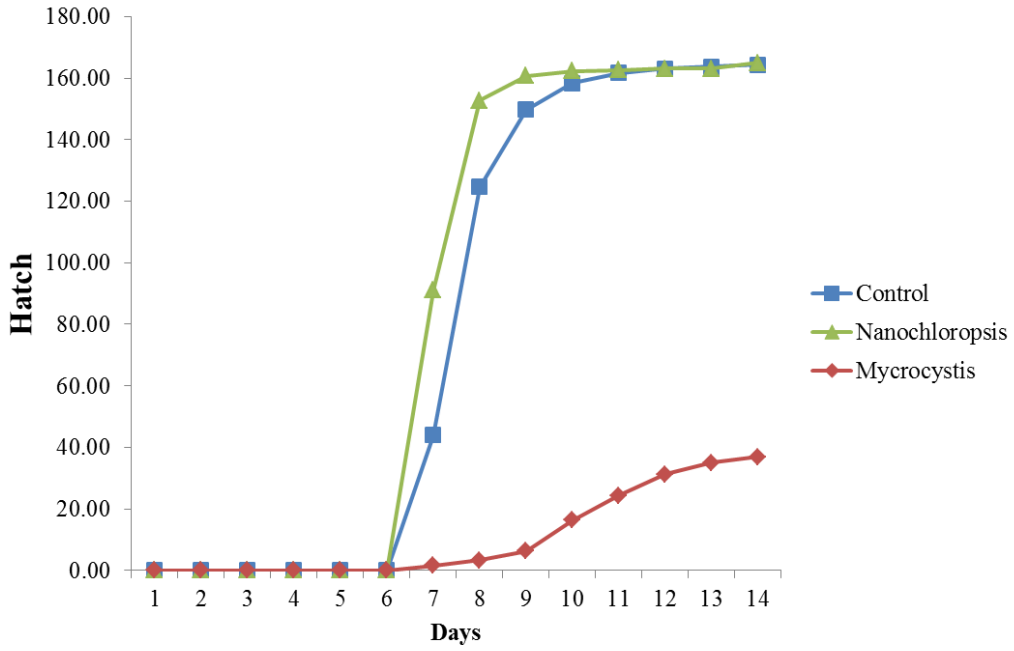


Figure 2.2.2 Medaka embryos exposed to no phytoplankton (Control), *Nanochloropsis* (Nano), and microcystin producing *Microcystis* (Micro) from the San Francisco Estuary. The average heart rate (heart beats per min) is reported for each treatment.

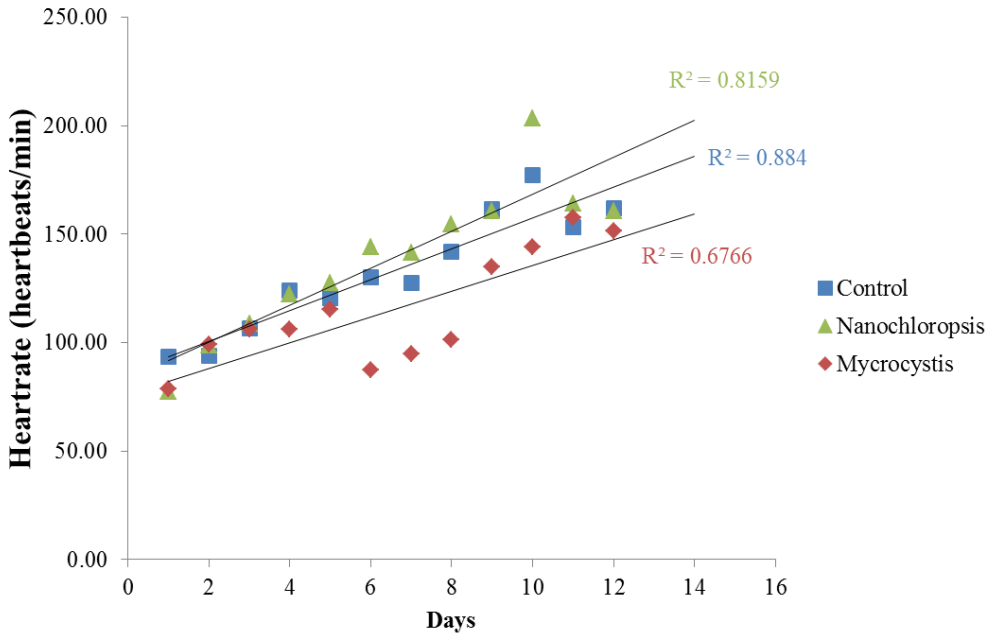
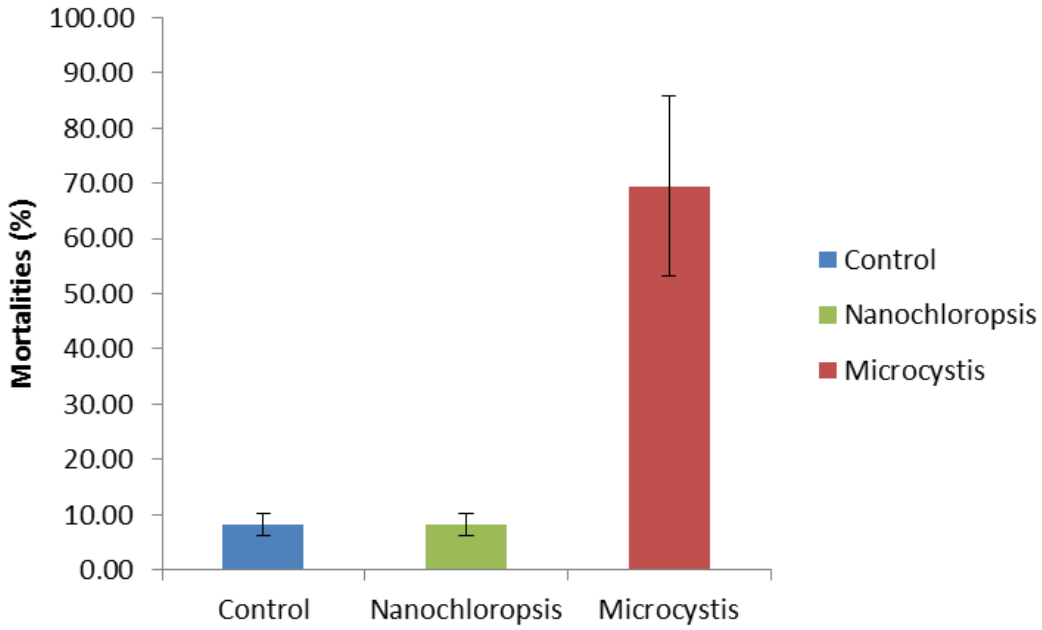


Figure 2.2.3 Medaka embryos exposed to no phytoplankton (Control), *Nanochloropsis* (Nano), and microcystin producing *Microcystis* (Micro) from the San Francisco Estuary. The average % mortalities are reported for each treatment.



Mortalities and swimming behavior are recorded and summarized in Table 2.2.2. Poor swimming behavior began on day 3 for Micro (6 very poor swimmers). Poor swimming behavior was characterized by slow to lethargic swimming compared to the active swimming of normal larvae. By the 96th hour the Micro treatment exhibited significantly greater incidences in poor swimming behavior. The increased prevalence of poor swimming behavior suggests that larvae are significantly affected by direct exposure to *Microcystis*.

Table 2.2.2 Medaka larvae exposed to no phytoplankton (Control), *Nanochloropsis* (Nano), and microcystin producing *Microcystis* (Micro) from the San Francisco Estuary. The number of medaka exhibiting poor swimming behavior and that had died were counted after 96 hrs of exposure.

Treatment	Replicate	n	# of Poor Swimmers	Mortalities
Control	1	30	0	0
Control	2	30	0	0
Control	3	30	1	0
Nano	1	30	0	0
Nano	2	30	0	0
Nano	3	30	1	0
Micro	1	30	2	0
Micro	2	30	3	0
Micro	3	30	1	0

Task 2.3. Dietary exposure of TFS to *Microcystis*

Materials and Methods

Groups of juvenile TFS from the Tracy Fish Collection Facility (Tracy, CA) were fed diets containing *Microcystis* harvested from the SFE during the bloom season in 2007. *Microcystis* biomass was examined for MC-LR content by LC/MS and then used to prepare different diets containing 0 (D0), 5 (D5) and 10 (D10) mg/kg of MC-LR. TFS were placed in a recirculating system equipped with sand, particle, and charcoal filters and a UV sterilizer. The fish were fed the different diets at 3% body weight twice/day. The water was analyzed for dissolved MC by ELISA to confirm the effectiveness of the biofilters to remove dissolved MC from the water. A 100 % water exchange was conducted daily to ensure that MC concentration was below the minimum detection limit of 0.5 µg/L. Each treatment group contained three replicates each with 10 fish. The experiment was terminated at 58 days post exposure to the MC-spiked diets. TFS were sacrificed with an overdose of MS 222 and immediately assessed for condition factor and presence of external signs. From each replicate, fish (N=5) were also fixed in 10% NBF for histopathology, immunohistochemistry, and in situ hybridization. The remaining fish were frozen for biochemistry. The 5 fish examined for histopathology were analyzed by one-way ANOVA to determine if gender was a significant factor in the MC-dietary effects.

Results

Gross Morphology and Growth

Gross examination of TFS at the end of 58 days exposure revealed that 10% of the fish in the D5 (5 mg/kg MC-LR) and 27% in the D10 (10 mg/kg MC-LR) diets were emaciated. Severe emaciation is associated with a condition known as cachexia, which in this study is characterized by the loss of body weight and muscle degeneration or atrophy (Fig. 2.3.1). The muscle atrophy is likely due to the failure of the liver to synthesize protein resulting in increased muscle protein catabolism. Exposure to MC can result to malnutrition due to liver damage, utilization of energy for detoxification, and reduced feeding. Under significant exposure to MC, malnutrition can be severe forcing the fish to use its muscle tissues as energy source resulting in severe emaciation or cachexia. A closer inspection of the internal anatomy revealed remarkably enlarged and dark colored gall bladder.

Growth as determined by body length (BL), body weight (BW) and condition factor (CF) was compared between treatments and between genders within each treatment. There was no significant difference ($P>0.05$) in BL of fish in tanks receiving the same diet concentrations and between tanks exposed to different MC-LR diet concentrations (Fig. 2.3.2). The BW decreased in the following order: 0>D5>D10 (Fig. 2.3.3), but the difference was not significant. While the CF was not significantly different between TFS fed with the *Microcystis*-spiked diets (D5 = 0.75; D10 = 0.71), only the high MC-LR diet concentration D10 versus the control (DO = 0.84; wild samples = 0.94) were significantly different as shown in Fig. 2.3.4. The lowest CF (0.33) was found in one fish in the highest concentration (D10). There was no significant effect of MC dietary treatment between males and females.

Histopathological examination of the liver revealed significant effects from MC exposure. Microcystin inhibits the activity of serine/threonine protein phosphatase 1 (PP1) and 2A (PP2A) and upon inactivation, the enzymes cause hyperphosphorylation of structural proteins. Cytoplasmic eosinophilic droplets or proteinaceous materials (EDP) accumulating in the cytoplasm of hepatocytes (liver cells) are likely due to the failure of the liver cells to process and export these denatured proteins. In addition to direct toxic stress from MC, the loss of appetite and increased energy needs may have resulted in significant glycogen depletion (GD) and single cell necrosis (SCN) in the liver. Protein kinases can be significantly unregulated in MC-exposed fish resulting in the loss of structural integrity within and between cells. Inhibition of PPI and PP2A can cause lack of cell to cell binding, single cell necrosis (SCN) and hemorrhaging in the liver, which will impair the circulatory system resulting in sinusoidal congestion (SC). Microscopic changes in the liver were observed among TFS exposed to increasing concentrations of MCs in the current study such as severe glycogen depletion (GD) and sinusoidal congestion (SC) (Fig. 2.3.5). Glycogen depletion suggests potential MC toxicity on energy reserves that may impair activity and susceptibility to disease, predation and starvation. Sinusoidal congestion, SCN and EDP indicate impaired liver function and were observed in greater prevalence among fish exposed to 10 mg/kg MC-LR (D10) compared to 5 mg/kg MC-LR (D5) as shown in Fig. 6. Although there was a significant difference in CF between genders among the histology samples, there was no significant interaction between sex and treatment on GD, SCN, SC and EDP. Gonadal development in males was not significantly affected but females showed a significant increase in the number of fish with severe ovarian atresia (OA)

(Figs. 2.3.6, 7). The extent and severity of OA may provide clues on gonadal development and reproductive viability. The incidence of OA among female TFS exposed to MC-laden diets may reflect gonadal development and reproduction rendering long-term effects to TFS populations in the SFE.

Figure 2.3.1. The top micrograph shows 4 abnormal and 1 normal threadfin shad (TFS) fed 10 mg/kg MC-LR diet (D10) at 58 days post exposure. The bottom micrographs of lateral and dorsal view of the abnormal TFS indicate severe emaciation characteristic of cachexia (severe ill health and malnutrition). Arrows point to degenerating dorsal muscle tissues observed during necropsy.

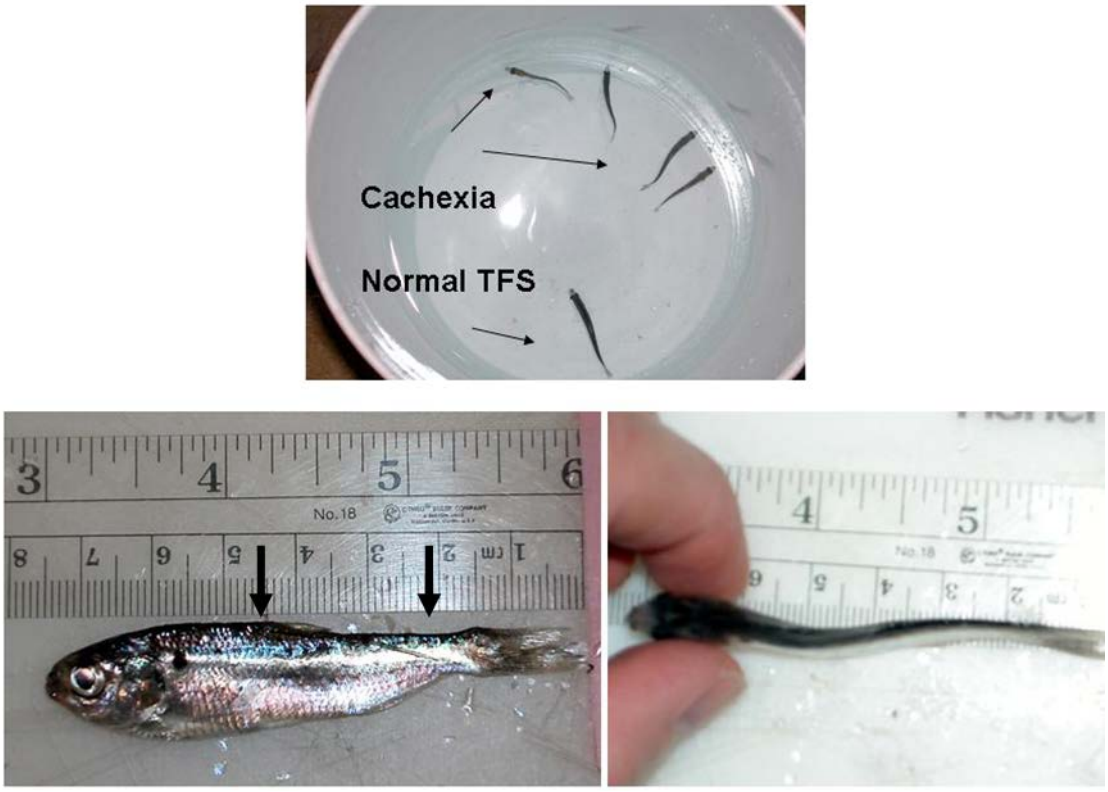


Figure 2.3.2. Body length of threadfin shad 58 days after feeding different concentrations of MC-LR-spiked diets. Similar letters between groups denote insignificant difference (P>0.05, Tukey pairwise comparison).

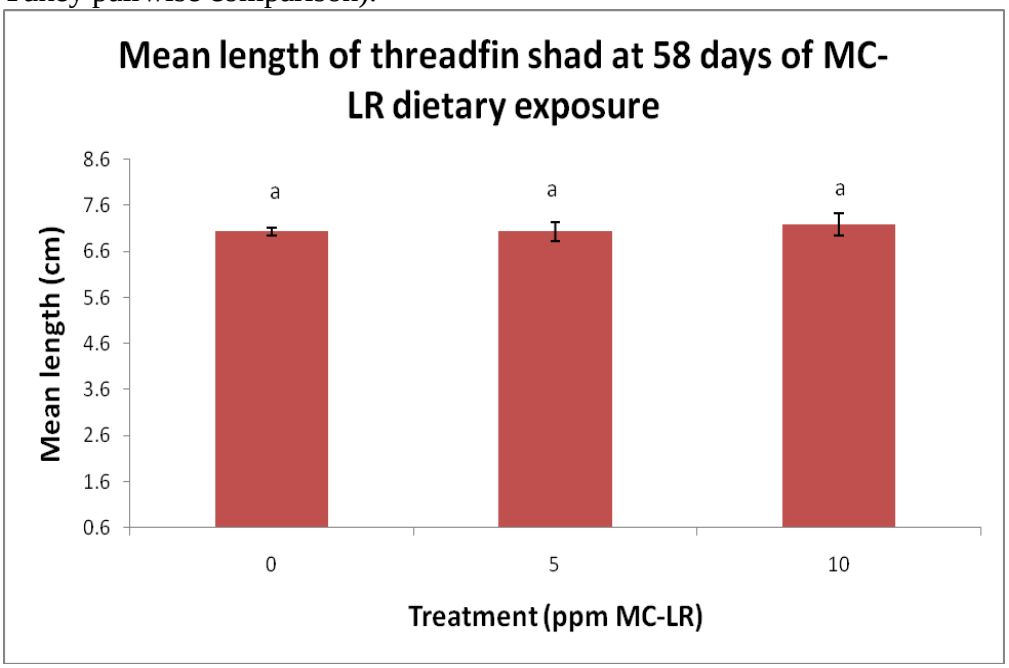


Figure 2.3.3. Body weight of threadfin shad 58 days after feeding different concentrations of MC-LR-spiked diets. Similar letters between groups denote insignificant difference (P>0.05, Tukey pairwise comparison).

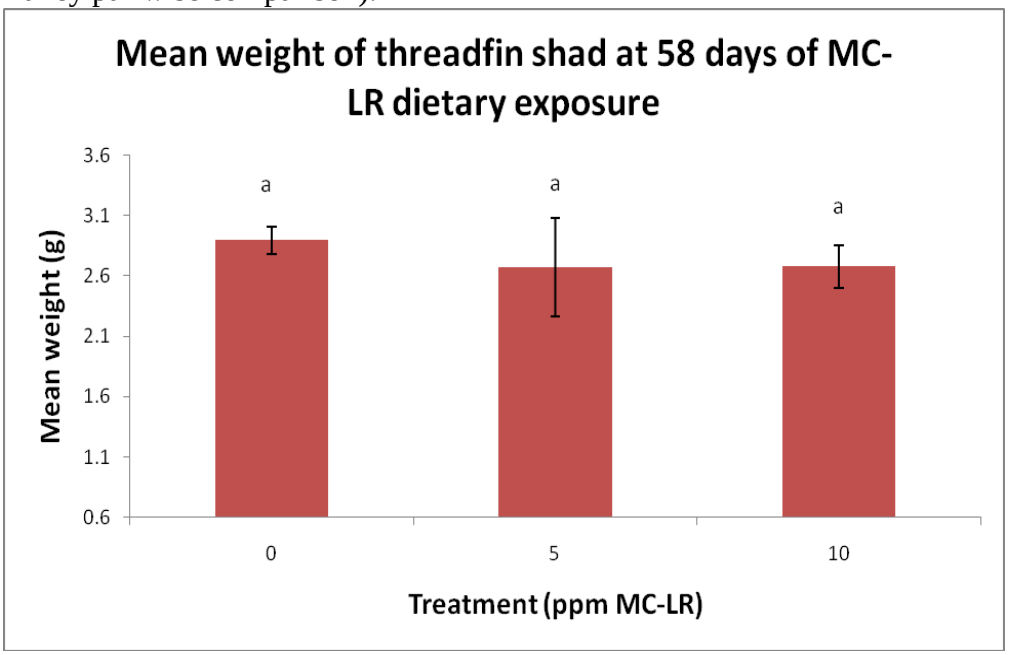


Figure 2.3.4. Condition factor in threadfin shad fed different concentrations of MC-LR-spiked diets after 58 days. Using Tukey pairwise comparison, different letters between groups denote significant difference.

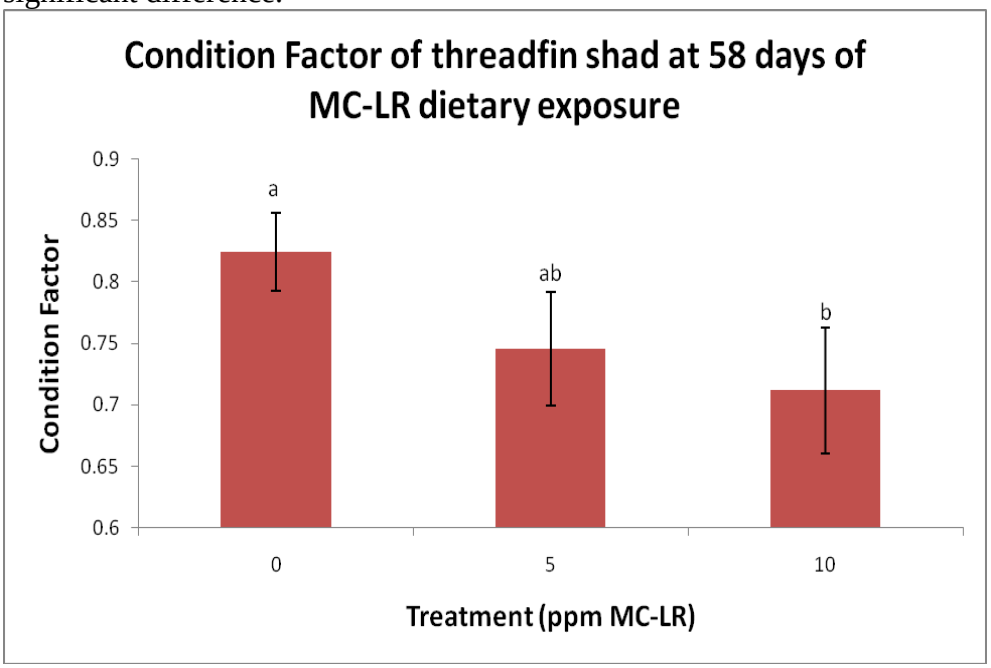


Figure 2.3.5. Threadfin shad liver sections stained with H & E. A) Normal glycogen-rich liver; B) higher magnification of A showing normal liver architecture. Note that the sinusoids (S) are usually one red blood cell thick; C) moderate sinusoidal congestion (arrows) and glycogen depletion in liver of TFS fed 10 mg/kg MC-LR diet for 58 days; D) higher magnification of C showing hepatocellular degeneration (arrows) and sinusoid congestion (SC) in a glycogen-depleted liver. Hepatocellular degeneration is characterized by cell injury which can be reversible or irreversible depending on the severity of the MC toxic stress. Irreversible cell injury usually leads to cell necrosis or apoptosis; E) severe eosinophilic protein droplets (arrows) and moderate singe cell necrosis (SCN) in liver of TFS exposed to 5 mg/kg MC-LR diet for 58 days.

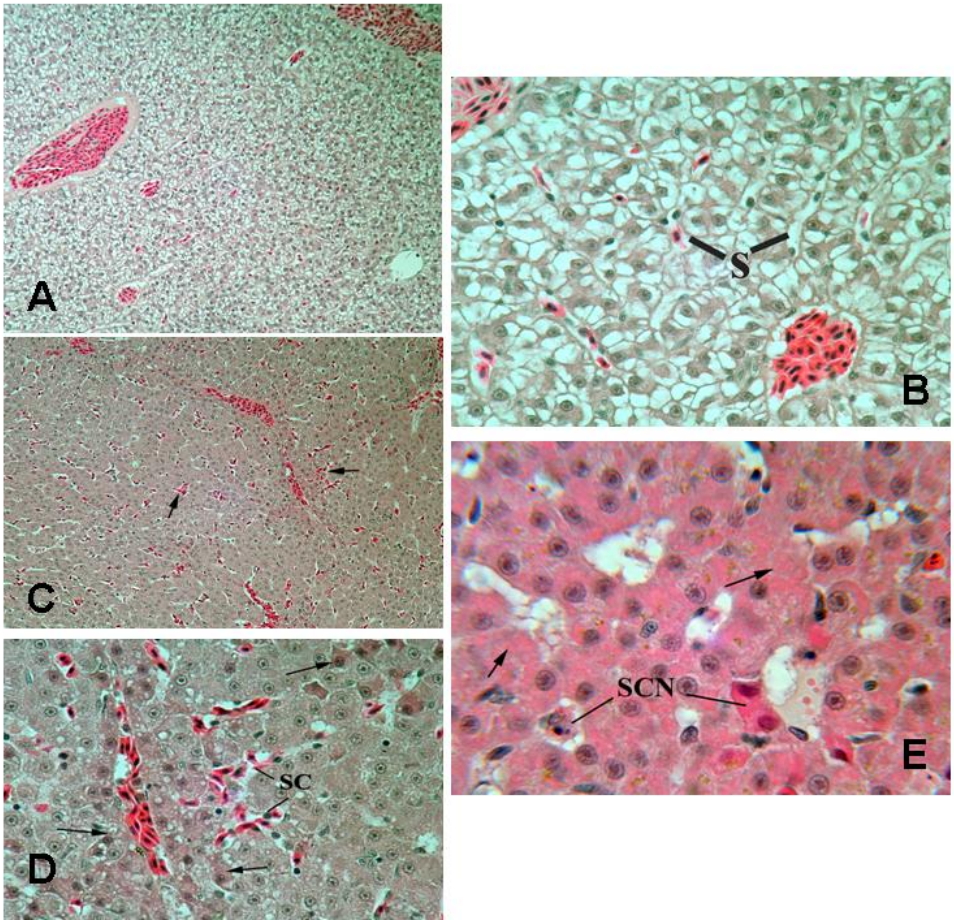


Figure 2.3.6. Mean lesion scores in the liver of threadfin shad exposed to *Microcystis* diets with 5 mg/kg MC-LR (D5) or 10 mg/kg (D10), and controls (no MC-LR or D0) at 58 days post exposure. Liver lesions were scored for: glycogen depletion (GD), single cell necrosis (SCN), sinusoidal congestion (SC), eosinophilic droplets or proteinaceous material (EDP) including ovarian atresia (OA) in female TFS. SCN, SC, EDP, and OA were not observed in control fish. There were no testicular lesions observed in the control and exposed fish.

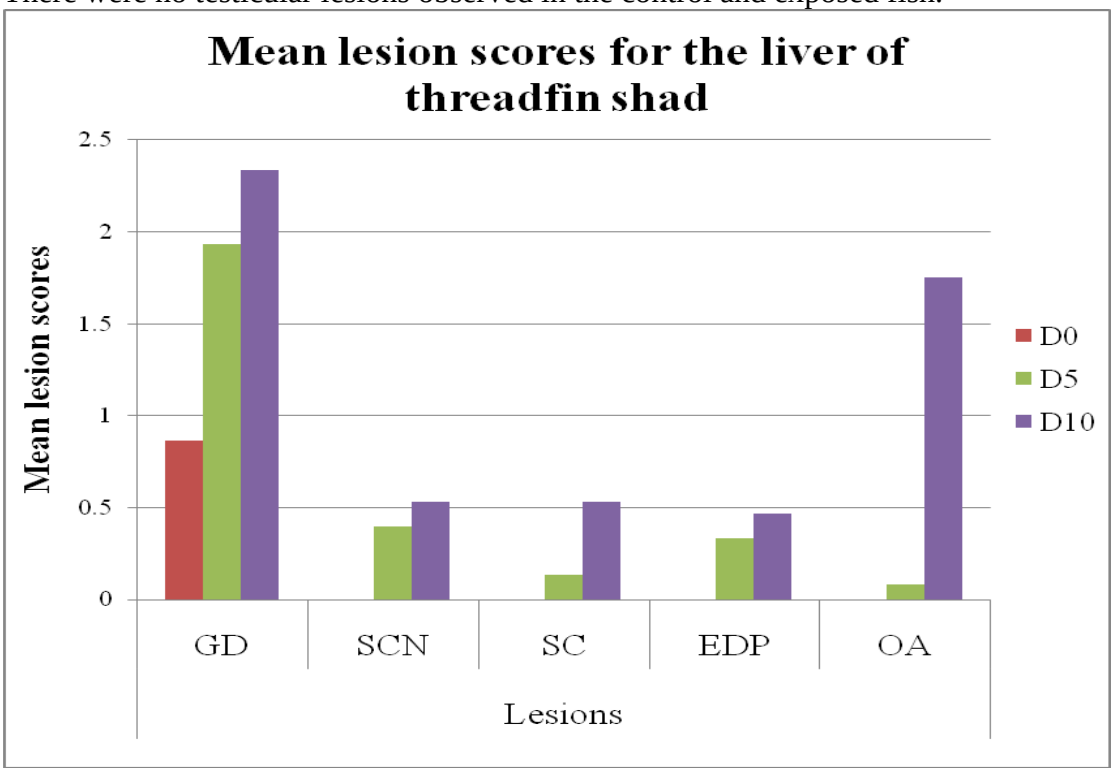
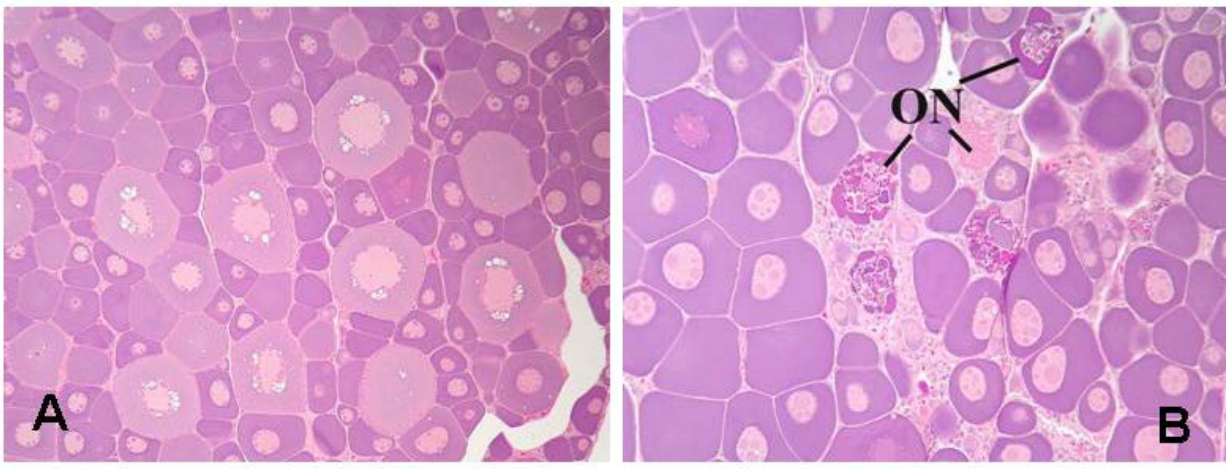


Figure 2.3.7. Threadfin shad ovarian sections stained with H & E. A) Normal ovary; B) severe stage II oocyte necrosis (ON) in TFS 58 days after exposure to 10 mg/kg MC-LR.



Immunohistochemistry

Using immunohistochemical analysis, threadfin shad samples were assessed for protein phosphatase 2A (PP2A) activity, the presence of microcystin-LR (MC-LR) and CYP1A activity. Nine samples were screened for PP2A and CYP1A activity and twelve samples were screened for the presence of MC-LR. Both control and exposed threadfin shad samples showed similar PP2A activity. As such, PP2A is an insensitive biomarker for field exposed fish. CYP1A activity was more sensitive with enhanced staining detected in the kidneys of exposed fish compared to controls (**Figure 2.3.8**). The use of the general biomarker CYP1A was used to confirm the potential for other toxic effects of *Microcystis* cells. The lipopolysaccharides of cyanobacterial cell walls, such as in *Microcystis*, can have significant toxic effects such as inflammatory response and oxidative stress. Using a commercial monoclonal antibody specific to microcystin-LR (MC10E7, Axxora Biochemicals, San Diego, CA) and secondary antibody in a standard immunohistochemical assay, MC-LR binding sites were detected in the intestine and kidney of TFS receiving high and low concentrations of microcystin-spiked diets (**Figure 2.3.9**). MC-LR positive sites were not observed in control fish. This result suggests that 1) MC-LR is absorbed through the intestines, 2) the toxin affected the target organ (liver), and 3) MC-LR is depurated in the kidneys indicating detoxification by glutathione. The presence of MC-LR and prevalent lesions in the liver confirm the toxic effects of MC-LR in the liver of threadfin shad upon ingesting the toxin. The presence of hepatic lesions indicates that the threadfin shad were unable to sufficiently detoxify MC-LR to prevent damage to the liver. MC-LR binding sites, as those observed among TFS receiving MC-spiked diets, were also prevalent from TFS collected in the estuary **(Figure 2.3.10)**. Severe cachexia among TFS fed with MC-laden diets suggests that dietary MC exposure is a factor affecting the growth and survival of TFS.

Figure 2.3.8. CYP1A (Cytochrome P450) immunohistochemistry in threadfin shad receiving microcystin-spiked diets

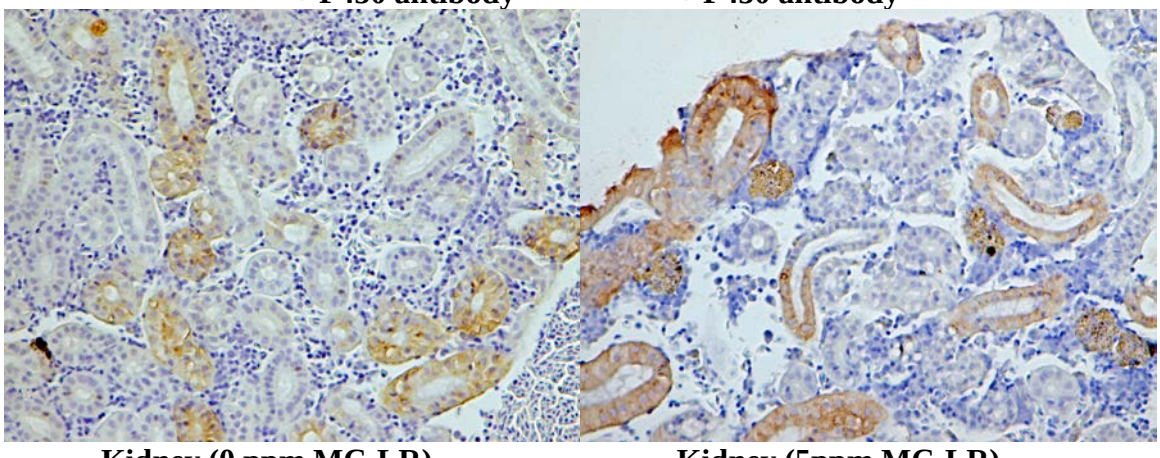
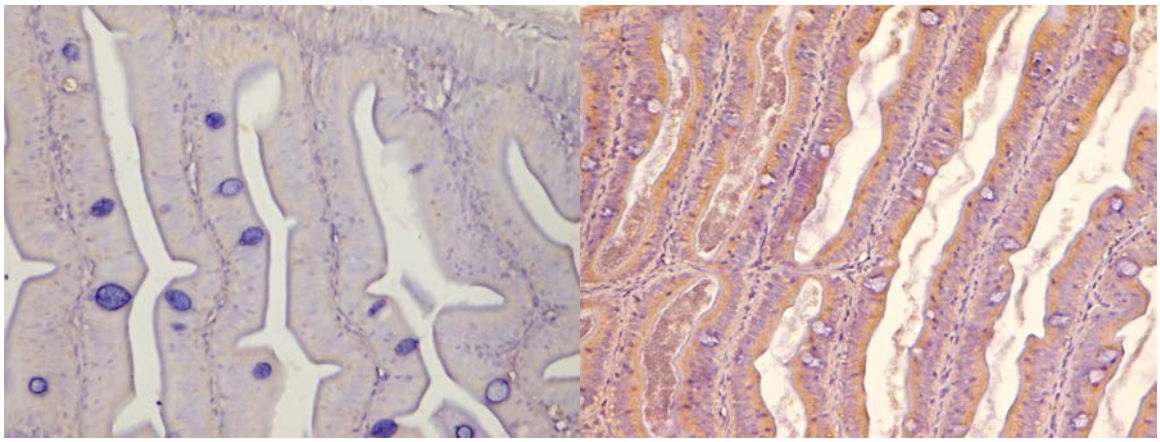


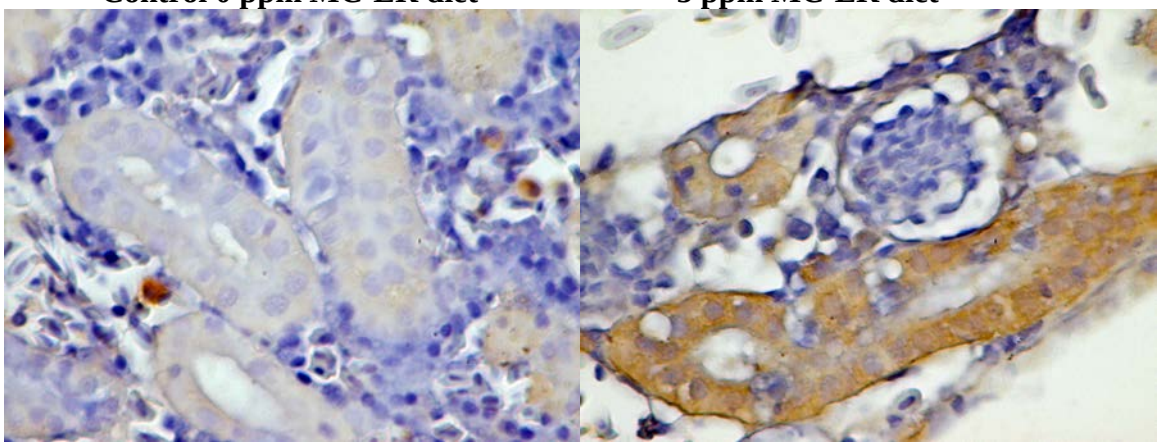
Figure 2.3.9. MC-LR immunohistochemistry of threadfin shad receiving microcystin-spiked diets

Control 0 ppm MC-LR diet **5 ppm MC-LR diet**



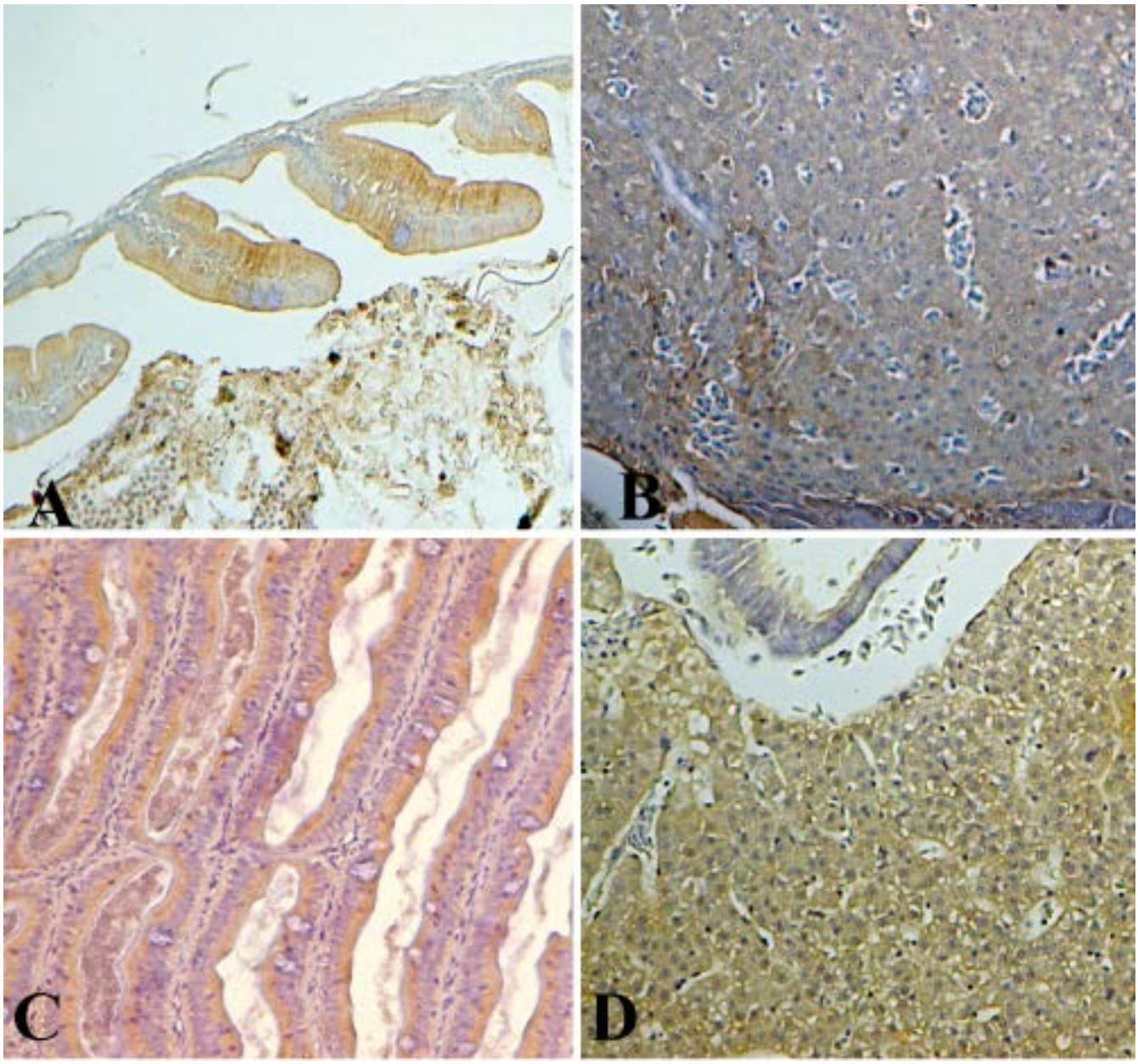
Intestine + MC-LR antibody

Control 0 ppm MC-LR diet **5 ppm MC-LR diet**



Kidney + MC-LR antibody

Figure 2.3.10. MC-LR localization by immunohistochemistry: A) intestine and B) liver of TFS from the SFE; and from intestine (C) and liver (D) of TFS in laboratory dietary exposures.



In situ hybridization (ISH)

One question that we want to answer was: Can we prove the ingestion of *Microcystis* in zooplankton and fish, particularly in threadfin shad, tissues? So we used a technique called in situ hybridization (ISH). Briefly this technique uses a labeled complementary DNA to localize a specific DNA sequence in target tissues. In other words, this technique can provide a precise anatomic localization of *Microcystis* DNA in affected organisms. Using the 16S rDNA sequences of *Microcystis* found in the SFE (Baxa et al. 2010), we designed DNA probes specific to *Microcystis* to reveal target organs of *Microcystis* exposure or ingestion in TFS.

Using ISH, we looked at *Microcystis* ingestion in TFS following exposure to diets spiked with *Microcystis* from the 2007 blooms. The purple precipitates in the intestines and gut contents **(Figure 2.3.11)** indicate the *Microcystis* DNA from the diets. Corresponding sections stained with unlabeled probes did not show these signals. We also examined TFS collected from the estuary during the peak of the bloom season in 2007. Aggregates of *Microcystis* cells were shown in the stomach contents and adjacent intestinal lining of threadfin shads collected from Mildred Island **(Figure 2.3.12)** demonstrating direct ingestion of *Microcystis* in the estuary.

Figure 2.3.11. In situ hybridization of threadfin shad showing ingestion of *Microcystis*-spiked diets in the laboratory. Purple precipitates indicate the *Microcystis* DNA.

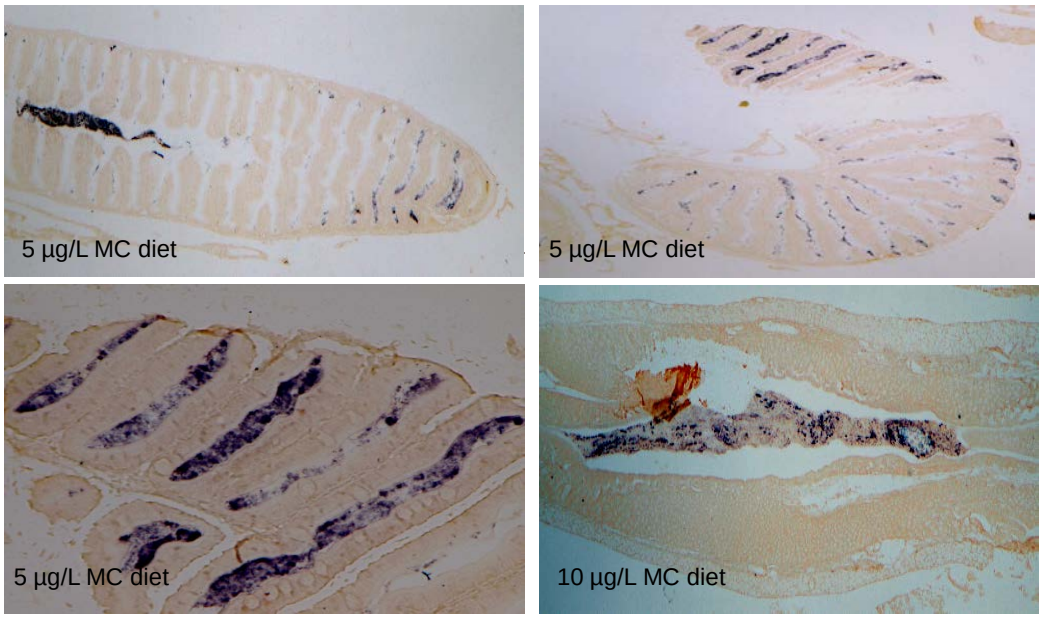
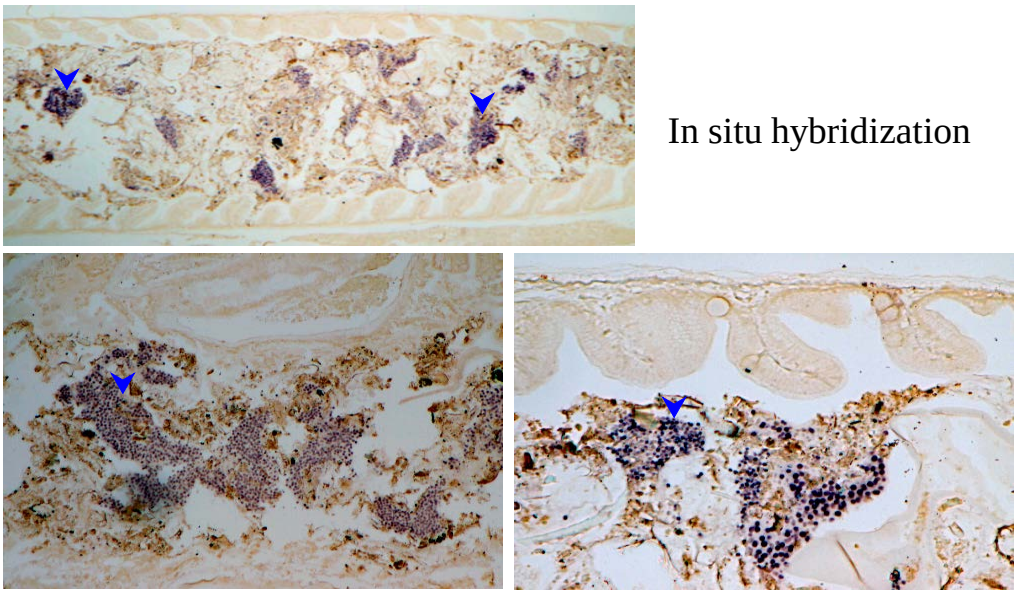


Figure 2.3.12. In situ hybridization of threadfin shad from Mildred Island in 2007 showing the ingestion of *Microcystis* cells (arrows) in the gut contents. The lower panels show a closer view of the localized *Microcystis* (DNA) cells.

Threadfin shad (Mildred Island 2007): *Microcystis* ingestion



Task 3: Field surveys to determine bioaccumulation and fate of microcystins in TFS

Field studies involving collection of *Microcystis*, TFS, and analyses of MC concentrations in *Microcystis* and fish tissues were coordinated with Dr. Peggy Lehman (DWR) during a monitoring program in 2007 funded by CALFED Bay-Delta Program. TFS and *Microcystis* were collected intermittently from *Microcystis*-impacted locations at the SFE during the bloom season in 2007. Sampling locations were chosen as previously designated by DWR for *Microcystis* collection and monitoring.

Field samples of TFS were analyzed for nutritional status, gonadal development, and histopathological changes as endpoints for assessing reproductive fitness. Presence of pathogens or diseases was also assessed. During the course of the field studies, a real-time quantitative polymerase chain reaction (qPCR) test was inevitably developed to estimate the spatial and temporal variations in the distribution of toxic versus nontoxic *Microcystis* across locations in the SFE (see Baxa et al. 2010). Approach and results of several studies in Task 3 are described below.

Task 3.1. Nutritional status of TFS

In collaboration with DWR, groups of TFS were caught by beach seining from 4 locations in the SFE between August 29 and September 12, 2007 **(Table 3.1.1).** Our goal was to determine if there are geographic differences in the nutritional fitness of TFS found at different sites in the estuary. To address this objective, we evaluated the nutritional status of TFS samples based on growth, somatic indicators including condition factor (CF) and hepatosomatic index (HSI), DNA/RNA ratio, and nutritional composition (protein, lipid, moisture and energy levels). Data are presented as mean ± SE by one way ANOVA and Duncan test to determine if differences among sampling locations were significant. Different letters denote significant differences ($P<0.05$) between or among fish from the different sampling locations.

Table 3.1.1. Number and size of threadfin shad collected from the San Francisco Estuary for evaluation of nutritional status

Location	Total fish no.	No. fish examined	Mean wt/length (g/mm)
Sherman Island (SI)	133	55	6.9/89
Brannon Island (BI)	62	30	8.5/94
Stockton (STK)	65	30	6.5/87
Mildred Island (MI)	36	13	1.4/58

Results

The body weight (BW) and body length (BL) of threadfin shad were similar from Sherman Island (SI), Brannon Island (BI) and Stockton (STK) ($P>0.05$) although fish from Mildred Island (MI) was smaller ($P<0.05$) than fish from the above three locations **(Fig. 3.1.1)**. Due to the small number and size of fish from MI, nutritional composition from this location was not compared with the other three locations. The condition factor (CF) was significantly lower in fish from BI than those from SI and STK **(Fig. 3.1.2).** The hepatosomatic index (HSI) decreased in the following order: SI>BI>STK, but the difference was not significant **(Fig. 3.1.2**). The RNA/DNA ratio in the muscle, as an indicator of protein synthesis reflecting effects of nutritional feeding or potential stressors, was significantly higher in fish from SI than fish from BI and STK **(Fig. 3.1.3)**. The nutritional composition based on protein, lipid, moisture and energy levels of whole fish was different among the three locations **(Figs. 3.1.4)**. Fish from SI had generally higher lipid, protein, energy and low moisture contents compared to fish from BI. Fish from STK showed the lowest protein, lipid and energy but high moisture content. Based on these preliminary findings, threadfin shad from Sherman Island indicate more favorable nutrition indices compared to fish from Stockton.

Figure 3.1.1. Body weight (BW) and length (BL) of threadfin shad collected from different sites in the San Francisco Estuary in 2007 for nutrition analyses. SI=Sherman Island, BI=Brannan Island, STK= Stockton, MI=Mildred Island

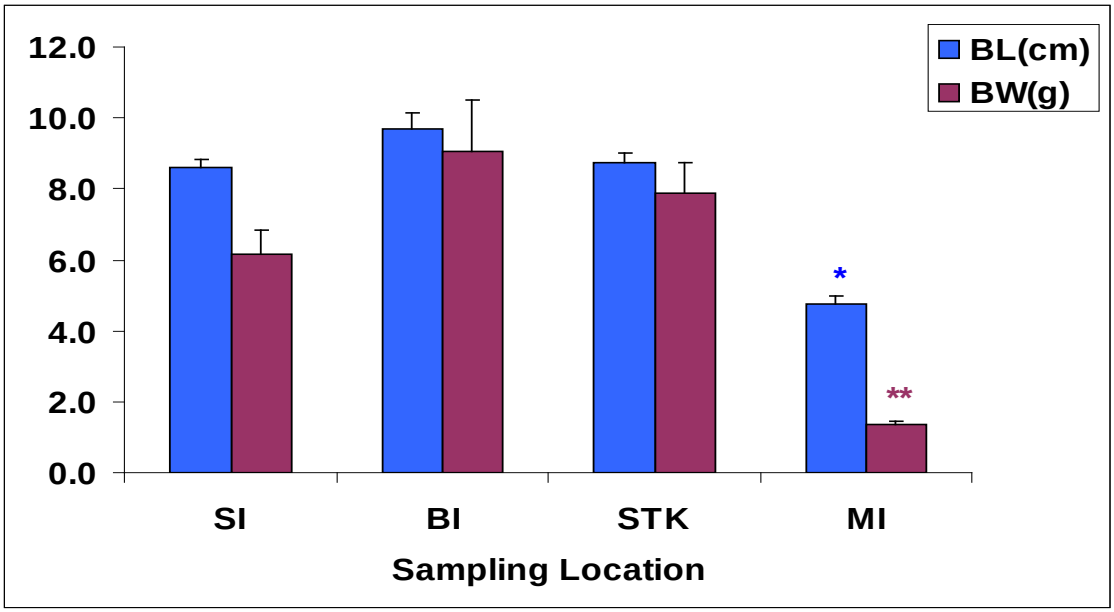


Figure 3.1.2. Hepatosomatic index (HSI) and condition factor (CF) of threadfin shad collected from different sites in the San Francisco Estuary in 2007 for nutrition analyses. SI=Sherman Island, BI=Brannan Island, STK= Stockton

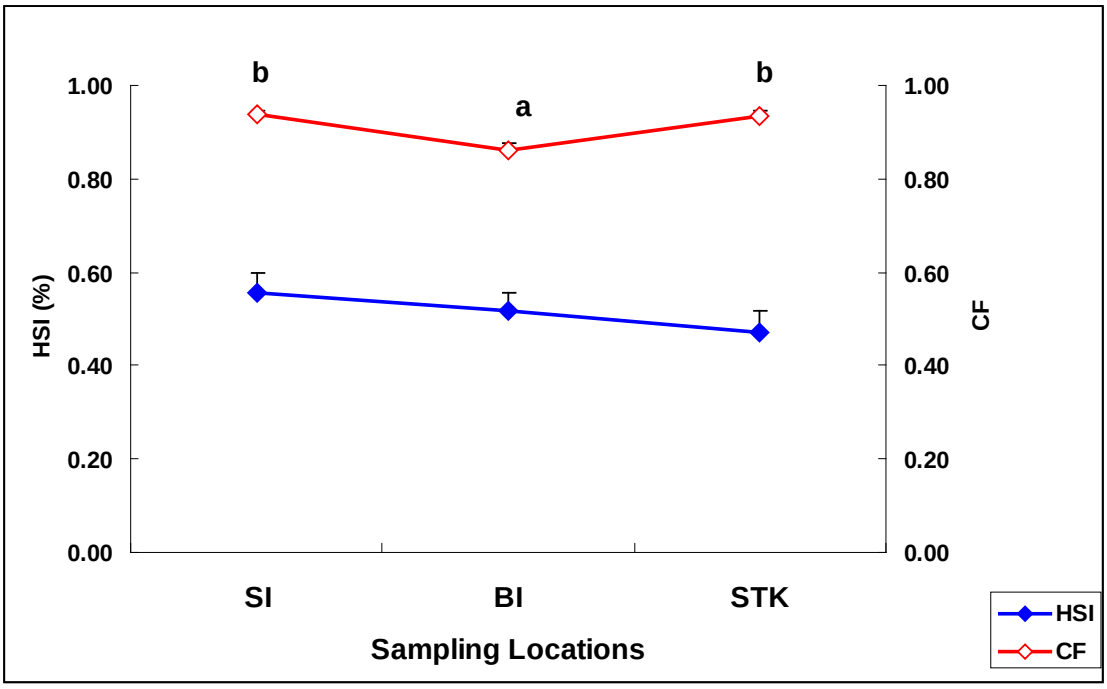


Figure 3.1.3. RNA/DNA ratio of threadfin shad collected from different sites in the San Francisco Estuary in 2007. SI=Sherman Island, BI=Brannan Island, STK= Stockton

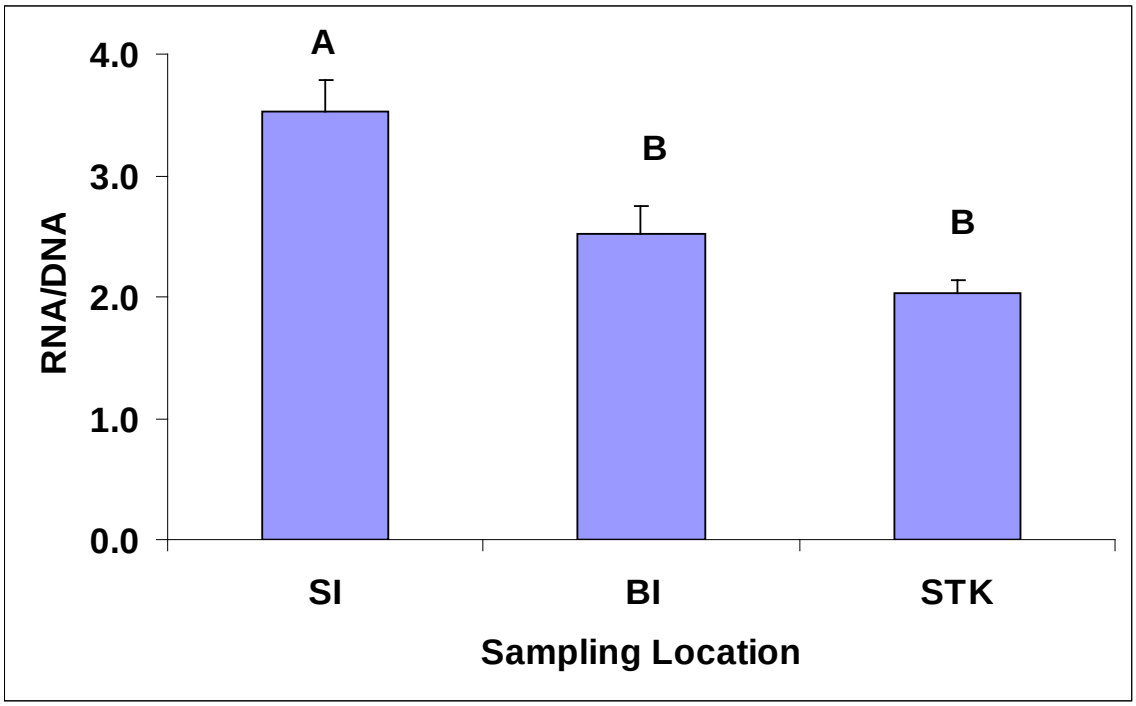


Figure 3.1.4. Lipid and moisture contents in threadfin shad collected from different sites in the San Francisco Estuary in 2007. SI=Sherman Island, BI=Brannan Island, STK= Stockton

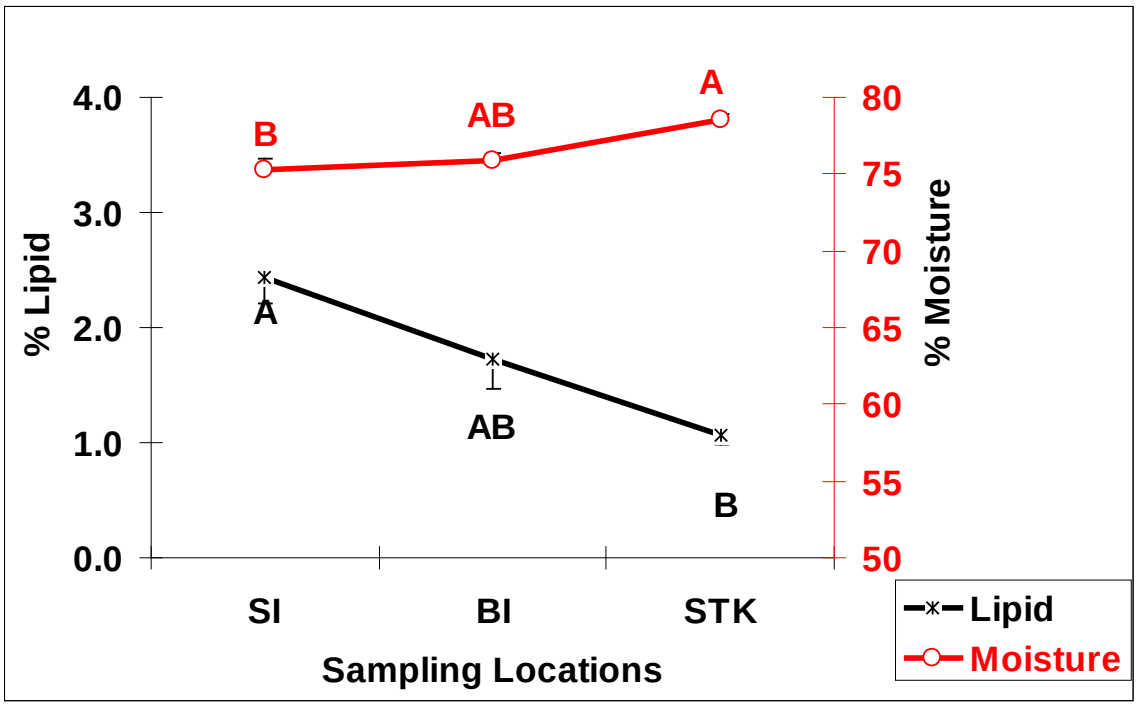
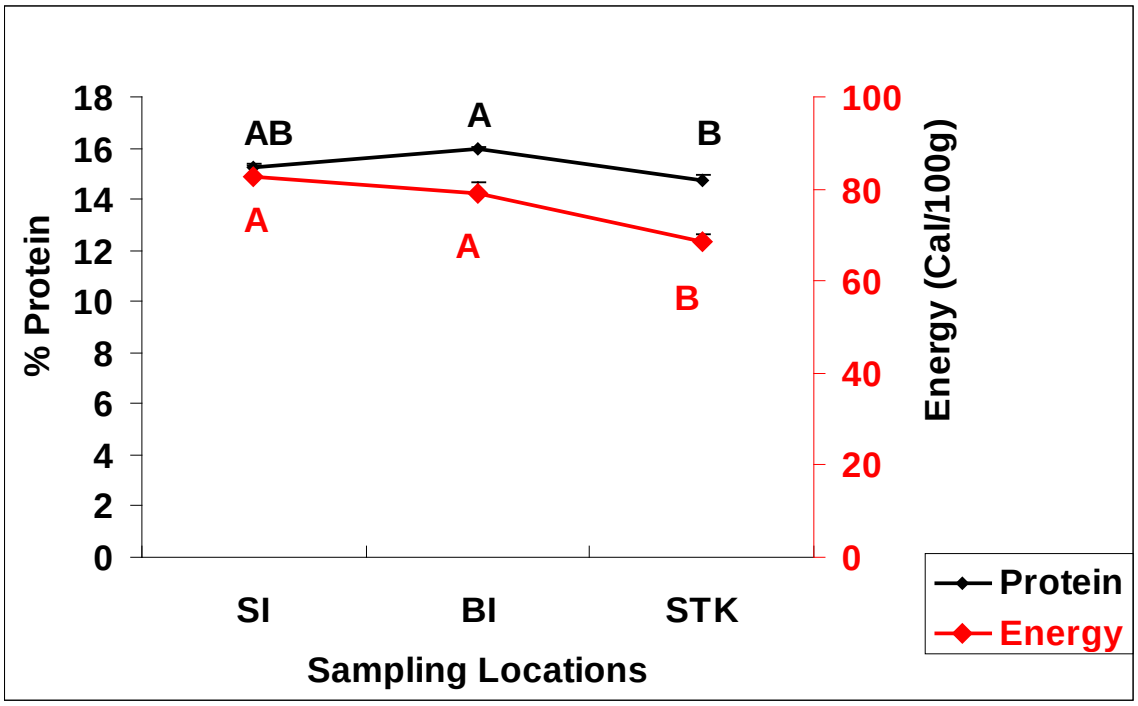


Figure 3.1.5. Protein and energy contents of threadfin shad collected from different sites in the San Francisco Estuary in 2007. SI=Sherman Island, BI=Brannan Island, STK= Stockton



Task 3.2. Development of a real-time quantitative PCR (qPCR) for *Microcystis*

Cyanobacterial samples were collected from different sites in the San Francisco Estuary during bloom development from July to September 2007 using tow nets and van Dorn to include both colonial and single algal cells. Standard polymerase chain reaction (PCR) employing primers designed for the conserved *Microcystis*-specific 16S ribosomal DNA (rDNA) was initially used to establish the presence of microcystin-producing (MC+) or toxic *Microcystis* in cyanobacterial and water samples **(Fig. 3.2.1).** The amplification of the MC toxin synthetase genes *mcy*B and *mcy*D established *Microcystis* toxicity. We developed a real-time quantitative PCR (qPCR) based on the 16S rDNA and *mcy*D gene sequences of *Microcystis* found in the SFE to quantify the proportion of toxic *Microcystis* with *mcy*D genes among total *Microcystis* or cyanobacterial population **(Table 3.2.1)**.

Cyanobacterial samples collected by diagonal net tows of the water column showed that the ratio of gene copies was dominant for *Microcystis* among cyanobacteria (28 – 96%), and *Microcystis* carrying *mcy*D genes formed 0.4 – 20% of the total *Microcystis* spp. (calculated from Table 2). Total *Microcystis* was also abundant (7.7×10^4– 9.9×10^7 cells L^{-1}) in ambient surface waters, and the range of *Microcystis* cell equivalents with *mcy*D genes (4.1×10^2–2.2×10^7 cells L^{-1}) indicated a large variation in the ratio of toxic *Microcystis* among total *Microcystis* (0.01 – 27%) (refer to Table 3). Differences in the proportion of toxic and nontoxic *Microcystis* were observed

across the sampling locations and seasons where concentrations of total MCs (0.007 – 10.81 μg/L) also varied **(Table 3.2.2).**

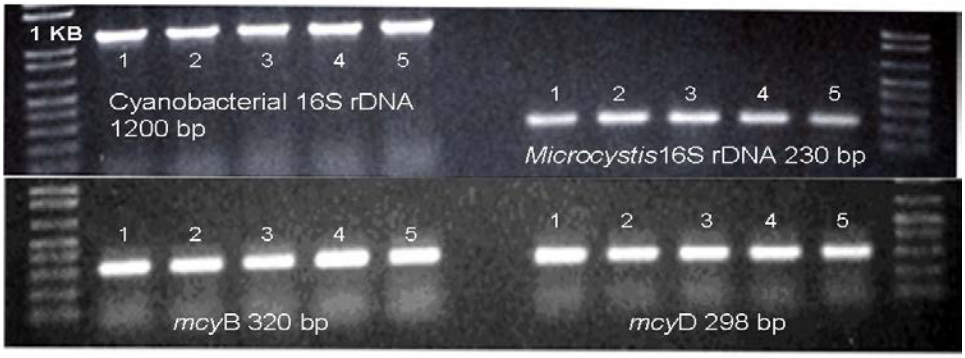
Microcystis was dominant in all of the sites examined but relatively more abundant in Brannon Island, San Joaquin, and Antioch. Although minimal, toxic Microcystis was present in all locations examined which formed up to 20% of the total *Microcystis* spp. particularly in Antioch at the beginning and towards the end of the bloom season **(Fig. 3.2.2).**

As in the algal tissue samples, the abundance of total and toxic *Microcystis* in surface waters fluctuated across sites and sampling dates but generally more abundant in Antioch, Brannon Island, and Mildred Island during the bloom season in 2007 **(Fig. 3.2.3).**

Quantifying the relationship between total *Microcystis* and MC+ *Microcystis* using qPCR in combination with chemical methods such as PPIA can offer greater accuracy for understanding the source and variability of different MC producers and bloom toxicity. Past methods of monitoring the toxicity of *Microcystis* blooms in the SFE have relied on assessment of cell density and chemical detection of MC concentrations in algal samples using PPIA. As measurements such as cell density do not correlate well with toxicity generally as well as in the SFE, the qPCR assay can circumvent the limitation of this technique. The cost and the length of time needed for analysis of chemical assays such as PPIA may limit large-scale application and rapid management decisions needed to protect humans and wildlife. By revealing trends in the proportion of MC producing cells, qPCR can identify the sources and magnitude of MC producers among mixed populations of *Microcystis* or cyanobacterial species present in the bloom. In this context, qPCR can contribute to rapid risk assessment and prediction of strategies designed to manage the adverse effects of blooms in this important ecosystem. See Baxa et al. (2010) for details of the qPCR development.

Figure 3.2.1. Standard PCR for initial screening of *Microcystis* samples indicating the amplification of the target genes: 16S rDNA for cyanobacteria and *Microcystis* and toxin synthetase genes mcyB and mcyD.

PCR amplification of target genes from *Microcystis* in the SFE



1:Antioch, 2:Chipps Island, 3:Mildred Island, 4:Venice Cut and 5:San Joaquin

Table 3.2.1. Quantitative real-time PCR: Gene targets based on 16S rDNA sequences of *Microcystis* found in the SFE

Target	**Probe**	**Sequence**
Microcystis 16S rRNA	MIC16S P	TTC CCC ACT GCT GCC
*Microcystis mcy*D	*mcy*D P*	ATG CTC TAA TGC AGC AAC GGC CAA A
Cyanobacteria 16S rRNA	CYA604 P	CTG ACA CTC AGG GAC G

Primers and probes were designed from 16S rDNA sequences of *Microcystis* spp. from the SFE; *mcyD probe is based from Rinta-Kanto et al. 2005

Table 3.2.2. Mean copy numbers of toxic *Microcystis* (*mcy*D), total *Microcystis* (16S rDNA) and cyanobacterial (16S rDNA) genes from cyanobacterial tissues collected by diagonal net tows in the San Francisco Estuary. Mean total microcystin concentrations (as determined by PPIA) of cyanobacterial samples are shown from each site.

Site Date	Total microcystin (µg/L)	Toxic *Microcystis* *mcy*D (Gene copies/µg DNA)	Total *Microcystis* 16S rDNA (Gene copies/µg DNA)	Cyanobacteria 16S rDNA (Gene copies/µg DNA)
AT-8/07/07	1.65 ± 0.08	$1.4 \pm 1.24 \times 10^5$	$4.1 \pm 0.28 \times 10^6$	$7.5 \pm 0.32 \times 10^6$
AT-8/21/07	0.28 ± 0.02	$1.2 \pm 0.11 \times 10^6$	$3.5 \pm 0.21 \times 10^7$	$6.1 \pm 0.44 \times 10^7$
AT-9/05/07	0.02 ± 0.001	$6.4 \pm 0.55 \times 10^4$	$5.1 \pm 0.04 \times 10^6$	$1.4 \pm 0.03 \times 10^7$
AT-9/18/07	0.16 ± 0.000	$2.4 \pm 1.78 \times 10^7$	$3.6 \pm 0.31 \times 10^7$	$5.0 \pm 0.16 \times 10^7$
BI-8/07/07	3.06 ± 0.052	$3.8 \pm 1.81 \times 10^4$	$3.3 \pm 0.81 \times 10^5$	$6.3 \pm 0.61 \times 10^5$
BI-8/21/07	0.176 ± 0.03	$1.1 \pm 0.08 \times 10^6$	$4.1 \pm 0.26 \times 10^7$	$5.2 \pm 0.23 \times 10^7$
BI-9/05/07	0.007 ± 0.000	$3.6 \pm 0.07 \times 10^6$	$5.5 \pm 0.58 \times 10^7$	$5.7 \pm 0.38 \times 10^7$
CI-7/25/07	0.046 ± 0.002	$5.0 \pm 2.57 \times 10^5$	$3.9 \pm 0.05 \times 10^6$	$5.5 \pm 0.19 \times 10^6$
MI-7/25/07	0.021 ± 0.001	$1.1 \pm 0.07 \times 10^6$	$2.9 \pm 0.14 \times 10^7$	$5.3 \pm 0.82 \times 10^7$
MI-8/08/07	0.736 ± 0.036	$8.0 \pm 0.42 \times 10^4$	$1.6 \pm 0.05 \times 10^6$	$2.6 \pm 2.77 \times 10^6$
MI-8/22/07	0.091 ± 0.005	$6.2 \pm 0.18 \times 10^5$	$1.8 \pm 0.38 \times 10^7$	$1.8 \pm 0.12 \times 10^7$
MI-9/04/07	0.032 ± 0.000	$1.8 \pm 0.11 \times 10^5$	$9.9 \pm 0.76 \times 10^6$	$2.6 \pm 0.78 \times 10^7$
MI-9/18/07	0.016 ± 0.005	$2.2 \pm 0.08 \times 10^6$	$1.1 \pm 0.01 \times 10^7$	$1.8 \pm 0.04 \times 10^7$
OR-8/08/07	0.696 ± 0.000	$2.4 \pm 0.40 \times 10^5$	$1.0 \pm 0.01 \times 10^7$	$1.8 \pm 0.16 \times 10^7$
OR-8/22/07	Not done	$3.8 \pm 0.74 \times 10^5$	$5.9 \pm 0.41 \times 10^6$	$2.0 \pm 0.11 \times 10^7$
OR-9/04/07	0.032 ± 0.000	$5.6 \pm 0.66 \times 10^5$	$1.5 \pm 0.19 \times 10^7$	$2.4 \pm 0.06 \times 10^7$
SJ-7/25/07	Not done	$2.1 \pm 0.26 \times 10^5$	$3.7 \pm 0.11 \times 10^7$	$3.8 \pm 0.09 \times 10^7$
VC-7/25/07	10.807± 0.227	$1.4 \pm 0.12 \times 10^5$	$3.4 \pm 0.29 \times 10^7$	$8.6 \pm 0.07 \times 10^7$

Sampling sites: AT=Antioch, BI=Brannan Island, CI=Chipps Island, MI=Mildred Island, OR=Old River, SJ=San Joaquin, VC=Venice Cut

Figure 3.2.2. Ratio of gene copies from algal tissues from the 2007 blooms in the SFE. Sampling sites: AT=Antioch, BI=Brannan Island, CI=Chipps Island, MI=Mildred Island, OR=Old River, SJ=San Joaquin, VC=Venice Cut

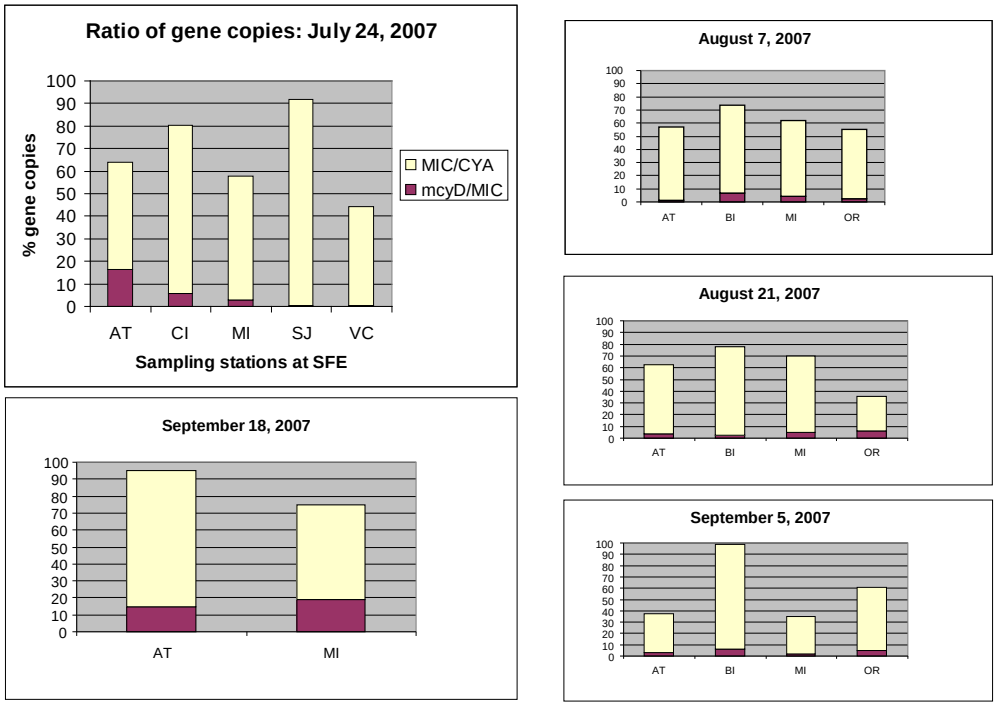


Figure 3.2.3. Abundance of toxic and nontoxic *Microcystis* in surface water samples at different sites in the SFE during the 2007 blooms.
Sampling sites: AT=Antioch, BI=Brannan Island, MI=Mildred Island, OR=Old River, FT=Frank's tract

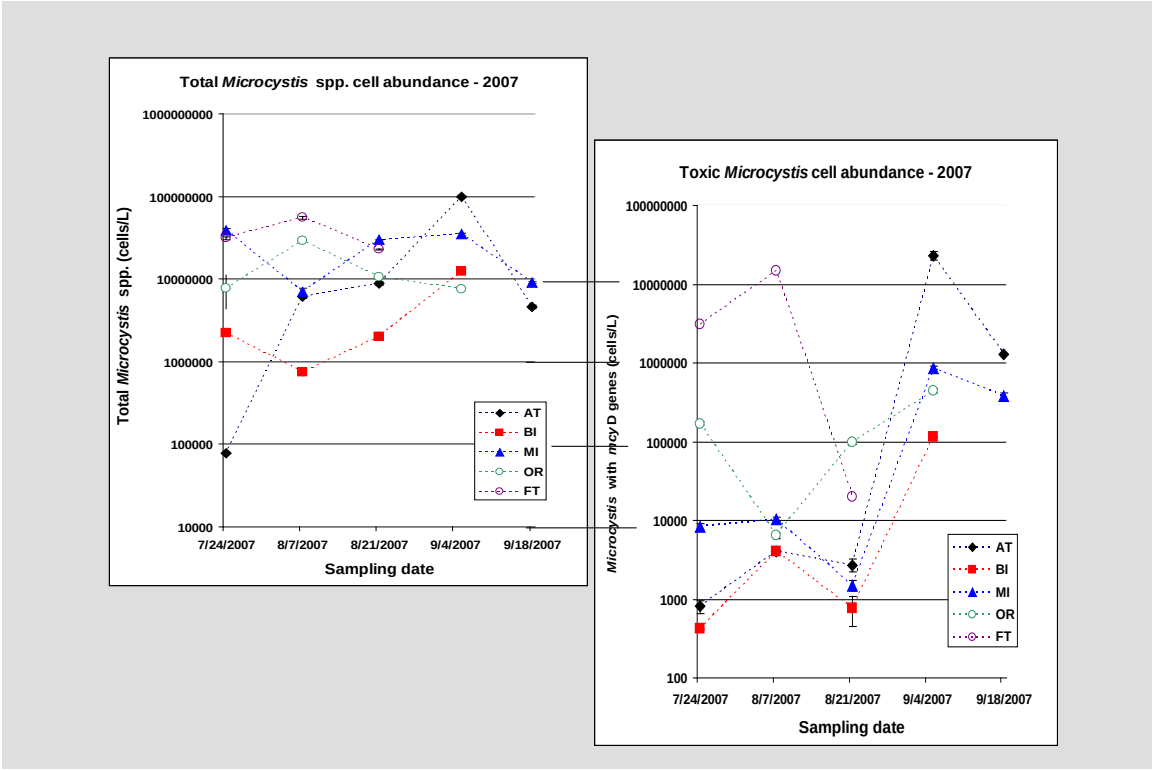


Table 3.2.3. Cell equivalents of toxic *Microcystis* (*mcy*D) and total *Microcystis* from ambient surface water samples in the San Francisco Estuary.

Site Date	Toxic *Microcystis* (Cell equivalents L^{-1}) *	Total *Microcystis* (Cell equivalents L^{-1})	Proportion (%) Toxic *Microcystis*
AT-7/24/07	$8.1 \pm 2.55 \times 10^2$	$7.7 \pm 0.04 \times 10^4$	1.03 ± 0.32
AT-8/07/07	$4.1 \pm 0.15 \times 10^3$	$6.1 \pm 0.42 \times 10^6$	0.06 ± 0.005
AT-8/21/07	$2.7 \pm 0.88 \times 10^3$	$8.8 \pm 0.90 \times 10^6$	0.03 ± 0.007
AT-9/05/07	$2.2 \pm 0.46 \times 10^7$	$9.9 \pm 0.28 \times 10^7$	23.08 ± 4.84
AT-9/18/07	$1.2 \pm 0.06 \times 10^6$	$4.6 \pm 0.36 \times 10^6$	27.67 ± 2.20
BI-7/24/07	$4.1 \pm 0.16 \times 10^2$	$8.9 \pm 1.68 \times 10^6$	0.01 ± 0.009
BI-8/07/07	$4.0 \pm 0.02 \times 10^3$	$7.3 \pm 1.20 \times 10^5$	0.55 ± 0.10
BI-8/21/07	$7.7 \pm 5.60 \times 10^2$	$1.9 \pm 0.06 \times 10^6$	0.03 ± 0.02
BI-9/04/07	$1.1 \pm 0.01 \times 10^5$	$8.9 \pm 6.73 \times 10^6$	3.89 ± 5.16
CI-7/24/07	$2.6 \pm 0.55 \times 10^3$	$1.9 \pm 0.10 \times 10^6$	0.13 ± 0.02
CI-8/07/07	$1.4 \pm 0.35 \times 10^4$	$5.9 \pm 0.06 \times 10^5$	2.43 ± 0.62
FT-8/01/07	$7.7 \pm 0.16 \times 10^5$	$3.1 \pm 0.20 \times 10^7$	2.48 ± 0.11
FT-8/08/07	$1.4 \pm 0.008 \times 10^7$	$5.5 \pm 0.35 \times 10^7$	26.46 ± 1.85
FT-8/21/07	$1.9 \pm 0.49 \times 10^4$	$2.2 \pm 0.07 \times 10^7$	0.08 ± 0.02
MI-7/25/07	$8.3 \pm 1.46 \times 10^3$	$3.8 \pm 0.37 \times 10^7$	0.02 ± 0.002
MI-8/08/07	$1.0 \pm 0.11 \times 10^4$	$6.9 \pm 1.30 \times 10^6$	0.15 ± 0.02
MI-8/22/07	$1.4 \pm 0.39 \times 10^3$	$2.9 \pm 0.01 \times 10^7$	0.005 ± 0.001
MI-9/04/07	$8.6 \pm 0.77 \times 10^5$	$3.4 \pm 0.25 \times 10^7$	2.48 ± 0.22
MI-9/18/07	$3.7 \pm 0.78 \times 10^5$	$9.0 \pm 0.34 \times 10^6$	4.14 ± 0.74
OR-8/08/07	$1.6 \pm 0.12 \times 10^5$	$1.0 \pm 0.04 \times 10^7$	1.55 ± 0.16
OR-8/22/07	$6.4 \pm 0.98 \times 10^3$	$2.8 \pm 0.37 \times 10^7$	0.02 ± 0.005
OR-9/04/07	$9.8 \pm 1.07 \times 10^4$	$1.0 \pm 0.07 \times 10^7$	0.94 ± 0.13
OR-9/18/07	$4.4 \pm 0.46 \times 10^5$	$7.5 \pm 0.48 \times 10^6$	5.84 ± 0.59
SJ-7/25/07	$6.3 \pm 1.27 \times 10^4$	$1.7 \pm 1.33 \times 10^7$	0.98 ± 1.21
SJ-8/22/07	$4.3 \pm 0.11 \times 10^3$	$3.2 \pm 0.08 \times 10^7$	0.01 ± 0.003
VC-7/25/07	$1.2 \pm 0.08 \times 10^6$	$1.0 \pm 0.06 \times 10^7$	12.03 ± 0.87
VC-8/22/07	$3.9 \pm 0.71 \times 10^5$	$3.3 \pm 0.21 \times 10^6$	11.91 ± 2.45

Sampling sites: AT=Antioch, BI=Brannan Island, CI=Chipps Island, FT=Franks Tract, OR=Old River, SJ=San Joaquin, VC=Venice Cut

*Cell equivalents of total and toxic *Microcystis* was calculated from the copy number of *Microcystis* 16S rDNA (two copies per genome, Kaneko et al., 2007) or *mcy*D gene (one copy per genome, Kaebernick et al., 2002), respectively.

Task 3.3. Application of PCR to determine the ingestion of *Microcystis* among zooplankton in the SFE

Using the PCR assay specific to *Microcystis*, zooplankton populations from different sites in the SFE were examined in 2007 and 2008 to determine if they ingest *Microcystis* in the field. Results showed the amplification of *Microcystis* genes **(Table 3.3.1)** suggesting the ingestion of *Microcystis*. A negative PCR result indicated that the zooplankton were eating other foods but not Microcystis or were not eating anything at all at the time of collection **(Table 3.3.1).**

The next question that we want to answer was: how much MIC is being ingested in each copepod in the estuary? To address this question, we conducted a qPCR assay on representative zooplankton samples collected from the estuary. Results showed that in Antioch for example, a single copepod can ingest as much as 10^6 *Microcystis* cells with a considerable proportion of toxic cells **(Table 3.3.2).** Some zooplanktons will only ingest nontoxin producing Microcystis such as in Mildred Island on 8/17/08. The proportion of toxic *Microcystis* ingested was relatively higher in July compared to the other sampling dates **(Table 3.3.2).**

To verify the ingestion of fish with *Microcystis*-laden zooplankton, we examined archived tissues of delta smelt collected in Antioch in 2008 using ISH. The *Microcystis* PCR primers were labeled with a molecule called DIG and used as probe. This DIG-labeled probe bound to *Microcystis* DNA in the tissues as shown in the gut of the delta smelt ingesting zooplankton-laden *Microcystis* **(Fig. 3.3.1).** Localized *Microcystis* DNA were also demonstrated in TFS tissues exposed to *Microcystis* in the field and in the laboratory **(Figs. 2.3.**and **2.3.).** The implication of these findings underscores the importance of PCR-based molecular tools for the rapid and accurate diagnosis of *Microcystis* abundance, toxicity and species-specific food web interactions with zooplankton that may affect pelagic fishes and other aquatic organisms in the SFE.

Table 3.3.1. Presence of *Microcystis* in zooplankton collected from the SFE in 2007 and 2008 as determined by standard PCR.

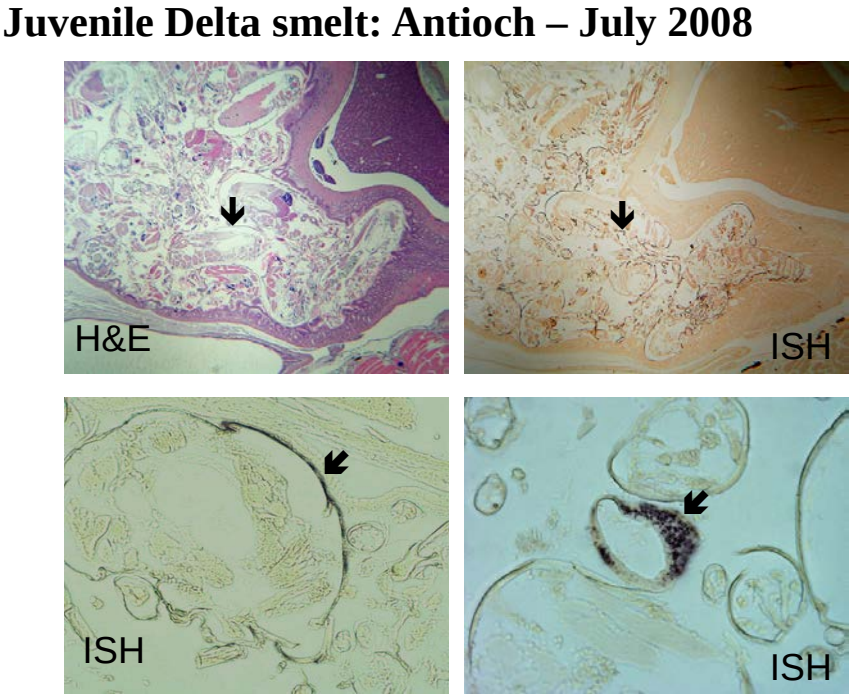
Location	Date	Total *Microcystis*	Toxic *Microcystis* mcyD
San Joaquin–1	7/24/07	-	-
San Joaquin–2	7/24/07	+	+
San Joaquin	8/21/07	-	-
Mildred Island	8/21/07	+	+
Collinsville	8/21/07	+	+
Mildred Island	9/4/07	-	-
Venice Cut	9/4/07	+	+
Antioch	6/23/08	+	+
Antioch	7/07/08	+	+
Antioch	8/04/08	+	+
Antioch	8/17/08	+	+
Antioch	9/29/08	+	+
Mildred Island	6/23/08	+	+
Mildred Island	7/07/08	+	+
Mildred Island	8/04/08	+	+
Mildred Island	8/17/08	-	-
Mildred Island	9/29/08	+	+

+ = positive DNA amplification; − denotes absence of specific gene target; no amplified DNA
Zooplankton (N=40) were pooled from each site, rinsed in sterile distilled water to ensure amplified DNA are from the guts and not from the body surface of zooplanktons.

Table 3.3.2. Estimated quantity of *Microcystis* ingested by zooplankton from representative sites in the San Francisco Estuary. qPCR was conducted using probes specific to 16S rDNA sequences of *Microcystis* found in the estuary.

Location	Date	*Microcystis*/ copepod	Toxic *Microcystis* per copepod	%Toxic *Microcystis*
Antioch	8/04/08	1.3×10^6	3.1×10^5	23
Mildred Island	7/07/08	6.2×10^3	2.1×10^3	33
Mildred Island	8/17/08	8.2×10^4	0	0
Mildred Island	9/29/08	1.5×10^4	3.9×10^3	25
San Joaquin	7/24/07	1.9×10^3	6.8×10^2	36

Figure 3.3.1. Juvenile delta smelt collected in Antioch in July 2008 showing sections of the gut stained by H&E and in situ hybridization, ISH using DIG-labeled *Microcystis*-specific probes. Arrows in in the lower panel indicate *Microcystis* DNA in the zooplankton ingested in the gut of the delta smelt. Purple precipitate indicates *Microcystis* DNA following specific binding of *Microcystis* DIG-labeled probes with *Microcystis* DNA in target tissues.



Task 3.4. Screening for pathogens and diseases in TFS and development of specific cell lines for viral isolation

On three sampling occasions, the presence of pathogens or diseases was examined in juvenile and adult TFS from different locations: Sherman Island (N=9), Mildred Island (N=4), and Stockton (N=20). Using standard necropsy procedures including observation of external and internal signs and conventional microbiological isolation techniques, all of the fish examined did not reveal the presence of significant pathogens or diseases or overt clinical signs. The dominant bacteria isolated were from the genera *Aeromonas*, which are Gram negative rods and are normally considered ubiquitous in the environment or in gut contents of fish.

We have developed a cell line from TFS to enhance the isolation of viruses that may be present from this fish species from the SFE. Other cell lines have also been developed including for delta smelt and striped bass.

Investigating other goals associated with the potential impact of blooms in the SFE

1. Impacts of *Microcystis* to key zooplankton species in the SFE

Based on preliminary histological analysis of juvenile TFS stomach contents in our lab, TFS can ingest a wide range of particles including detrital and other non-nutritious food including *Microcystis* spp. Several questions that need to be addressed were: Can TFS larvae survive during a *Microcystis* bloom by actively avoiding *Microcystis* cells? Is this response determined by the ratio of *Microcystis* in the diet? If ingested, how toxic is *Microcystis* to TFS larvae? Will TFS larvae feed on copepods in the presence of *Microcystis*? To address these questions, two studies were conducted in our laboratory to demonstrate the impacts of *Microcystis* to two key zooplankton species in the SFE, *Pseudodiaptomus forbesi* and *Eurytemora affinis*. Please refer to Ger et al. (2009a, 2009b) for detailed findings of these studies.

2. Development of local *Microcystis* cultures

In an effort to establish local *Microcystis* cultures in our laboratory, we initiated the process of isolating and testing cyanobacterial cells from the 2007 and 2009 blooms to determine whether they are toxin producing or nontoxin producing *Microcystis*. We observed that other cyanobacteria such as *Aphanizomenon* and other filamentous bacteria are present in our cultures. These cyanobacteria were collected in the delta along with *Microcystis* and are being propagated in our lab cultures together with *Microcystis*. These cyanobacteria are potentially toxin producing in addition to *Microcystis*.

3. Pilot study to determine the effect of water temperature and salinity on *Microcystis* toxicity

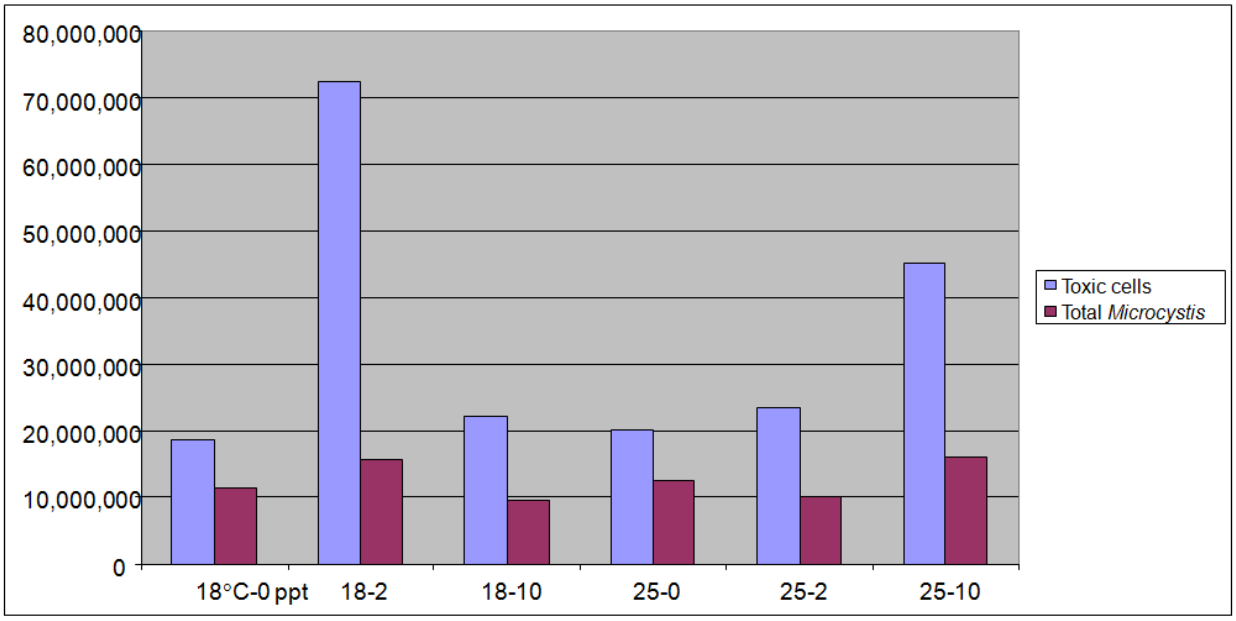
Using mixed *Microcystis* populations that we have been propagating in the laboratory, we devised a pilot study to determine how key environmental factors such as salinity and temperature can make local *Microcystis* more or less toxic in the presence of other cyanobacteria. In this initial study, we used mixed *Microcystis* populations from the 2009 blooms and exposed them to different salinities (0, 2, and 10 ppt) and temperatures (18°C, 25°C) that are relevant in the estuary. qPCR analysis was conducted at 14 days post exposure to determine the abundance of toxin producing cells compared to nontoxin producing cells.

In the presence of other toxin-producing cyanobacteria, the growth of toxic cells is greatly enhanced at 18°C/2 ppt and at 25°C/10 ppt treatments **(Fig. 3.4.1)**. The mcyD qPCR assay that we used most likely quantified other toxin producing cyanobacteria in the Delta such as the filamentous cyanobacteria and *Aphanizomenon*, which can also produce microcystins and other neurotoxins. As such, toxic cells were more abundant than the total number of *Microcystis* as shown in **Fig. 3.4.1.**

This is a pilot study and that careful interpretation of these results is strongly cautioned until further analysis can be conducted on the identity and toxin-producing potential of other cyanobacteria that are present in the estuary. Importantly, as these cultures have been well adapted to laboratory conditions, *Microcystis* populations that are freshly collected from the SFE will be used to verify their usefulness for the study of responses to threshold environmental

parameters (e.g., 10 ppt and 25°C). Under culture conditions, biotic interactions are markedly altered relative to the environment from which they were isolated driving the evolution and/or stability of certain algal traits (Lakeman et al. 2009).

Figure 3.4.1. Effect of salinity and temperature on the mean abundance of toxin-producing cells and total *Microcystis* using mixed cyanobacterial cultures propagated in the laboratory.



One relevant finding is that *Microcystis* morphology was altered at the different temperature and salinity treatments **(Table 3.4.1)**. At 0 and 2 ppt, single cells were observed at 18°C compared to colonial forms at 25°C. Although with minimal growth, the opposite was observed at 10 ppt: single cells at 25°C and colonial forms at 18°C. These results have important implications in the ability of zooplankton to ingest *Microcystis* cells in the field.

Table 3.4.1. *Microcystis* morphology at different temperature and salinity treatments

Salinity (ppt)	Temperature (°C)	Morphology
0	18	cells
	25	colonies
2	18	cells
	25	colonies
10	18	few colonies
	25	few cells

4. DNA barcoding of algal bloom composition in the SFE and Clear Lake (Kurobe et al. 2013 SpringerPlus in press)

- This work showed an apparent shift (during the course of the study) to *Aphanizomenon flos-aquae* from *Microcystis aeruginosa* that have dominated the recurring blooms in the delta in the past decade
- Species-specific identification of dominant species in the blooms including *Microcystis aeruginosa*, *Aphanizomenon flos-aquae*, *Dolichospermum* (formerly *Anabaena*) *lemmermannii*, *Dolichospermum* spp., *Limnoraphis* (formerly *Lyngbya*) *robusta*, *Limnoraphis* spp., and *Synechococcus* spp.
- DNA barcoding documented the presence of *Lm. robusta* in Clear Lake, Northern California. To date, this harmful cyanobacterium has only been reported from Lake Atitlan in Guatemala where the climate is different from that in California. It is important to understand the factors affecting the emergence of *Lm. robusta* in California and the potential link promoting the growth of the cyanobacterium between the two geographically distant water bodies.
- The identification of prokaryote assemblages by DNA barcoding will enhance the current cyanobacterial monitoring efforts by allowing us to develop specific quantitative PCR (qPCR) assays using the sequences obtained in this study. We are currently validating the reliability and reproducibility of the qPCR tests for estimating the abundance of key cyanobacterial species with potential toxin production.
- Assessment of cyanobacterial assemblages using an interdisciplinary approach (i.e. DNA barcoding and qPCR supported by morphological identification) will aid in formulating effective mitigation measures by addressing the specific identity of cyanobacteria, their corresponding physiological features, and determining the effects of fundamental environmental factors on species-specific toxicity.

Summary and Conclusions

1. Medaka was utilized for embryo and larval exposures due to the lack of TFS embryos and larvae from various sources such as the field, laboratory spawning, and fish culturists. Medaka was used as a surrogate since it is an established testing species with a reliable supply of embryos and larvae.

Microcystis and microcystin-LR were examined for two life stages of medaka: embryo and larvae. The embryo studies examined the effects of MC-LR at increasing concentrations and *Microcystis* exposure until all the embryos either hatched or died. For the MC-LR exposure no effect was detected on the embryos for heart rate, hatch date, or % mortalities. The results were consistent with previous studies that showed the chorion being a sufficient barrier to the cyanotoxin, MC-LR.

The *Microcystis* study showed a significant effect on heart rate and mortalities of larval stages of medaka. Waterborne exposure of larvae to graded concentrations to the pure form of the toxin and to strains of *Microcystis* cultured from the San Francisco Estuary was toxic. Increasing concentrations of MC-LR showed a dose response for poor swimming behavior and mortalities. This suggests that larval fish from the SFE may experience toxicity from MC-LR that can impair

their ability to prey on zooplankton or avoid predators. Mortalities were also significant suggesting that MC-LR may be a significant threat to the health of larval fish in the SFE during *Microcystis* blooms. For the *Microcystis* exposure, larvae exposed to toxic algae exhibited significant swimming impairment compared to the other treatments. In addition, *Microcystis* appeared to smother the medaka embryos resulting in slowed hatching time and increased mortalities. *Microcystis* blooms occur throughout the SFE (Lehman et al 2008), which potentially exposes larval fish from the SFE to toxic effects from the cyanobacteria. *Microcystis* from the SFE may not be a threat to embryos but it may be a significant threat to larval stages of various fish species.

2. Initial field studies involving the collection of juvenile and adult TFS during the cyanobacterial blooms in 2007 revealed variable responses of TFS from the different sites in terms of growth and nutritional status. The bioaccumulation and fate of MCs in these samples were proposed to be determined (Task 3). TFS tissue samples for MC concentration analysis were sent to Dr. Greg Boyer at State University of New York, College of Environmental, Science and Forestry in 2007. Unfortunately, the analysis was not conducted and results were not returned despite multiple and rigorous requests. As a consequence, we used other alternative methods to determine the biological fate and accumulation of MCs in the fish.

The use of immunohistochemistry has confirmed the presence of MC (LR) in the intestines, liver and kidneys. The presence of MC-LR in these organs prove that MCs are absorbed in the intestines and accumulate in the liver where the toxin is either detoxified by glutathione transferase and then transports to the kidney before depuration or resulted in liver damages which can impair liver function. Integrating histopathology and immunohistochemistry of the liver sections from field samples of threadfin shad demonstrates the direct effects of bloom toxicity.

3. An unexpected but positive outcome of the field studies conducted in 2007 was the opportunity to estimate the spatial and temporal variations of toxic and nontoxic *Microcytis* in the SFE by developing a real-time quantitative PCR (qPCR) (Baxa et al. 2010).

- The development of standard and qPCR techniques also enabled us to initially determine the ability of zooplankton to ingest *Microcystis* in the field suggesting MC bioaccumulation via food web organisms of the SFE.
- The qPCR provided an initial estimate on the abundance of *Microcystis* that zooplankton can ingest in the SFE
- The PCR/qPCR will be useful as a monitoring and research tool with broad applications in studies designed to analyze the relationship between environmental factors, and MC synthesis and impacts.
- Localization of *Microcystis* (DNA) was also demonstrated in gut contents of field samples of threadfin using an in situ hybridization technique that utilized specific 16S rDNA sequences of local *Microcystis* strains

4. Exposures to low (5 mg/kg) and high (10 mg/kg) concentrations of MC-LR spiked diets have demonstrated definitive effects in juvenile TFS on key indicators of health and biomarkers as outlined below:

- Growth – abnormal growth such as cachexia, loss of body weight, and decreased condition factor
- Reproduction – impaired gonad development affecting reproductive viability in female TFS
- Histopathology – presence of liver lesions associated with MC toxicity such as cytoplasmic eosinophilic droplets or proteinaceous materials (EDP), glycogen depletion (GD), single cell necrosis (SCN) and sinusoidal congestion (SC). These pathologies are consistent with our laboratory studies in the laboratory fish model (medaka) and Sacramento splittail (Deng et al. 2010; Acuna et al. in preparation).
- Immunohistochemistry – confirmed the presence of MC-LR in the intestines, liver and kidney of TFS receiving MC-LR-spiked diets.
- Nutrition – impaired nutritional status as indicated by histopathological changes and severe cachexia

5. Using *in situ* hybridization that utilized DNA probes specific to *Microcystis* provided evidence that zooplankton and TFS can ingest *Microcystis* following exposures to blooms in the SFE or to MC-LR spiked diets in the laboratory.

6. In addition to TFS, we have also recently described the adverse effects of *Microcystis*/MCs in other species such as the Sacramento splittail (Acuña et al. 2008), the quart medaka (Deng et al. 2010), key copepods of the SFE (see Ger et al. 2009a, 2009b) including impacts of *Microcystis* blooms to inland silverside and striped bass in the SFE (Lehman et al. 2010). Taken together, findings from these studies conducted in our laboratory demonstrate the potential threats of *Microcystis* to important organisms in the SFE food web.

7. Our studies showed that the TFS is an ideal indicator species in view of the timing of their larval development during peaks of cyanobacterial blooms in the SFE. In this context, investigating the effects of *Microcystis* in TFS particularly on early life stages is critical including range of effects in this species that can be determined under field and laboratory conditions.

8. An extension to continue with the project was requested beyond October 2010 to conduct the *Microcystis*/microcystin (MC) exposures of embryonic and larval stages of threadfin shad due to the difficulties encountered with culturing and finding other sources of early life stages. For this reason, medaka (*Oryzias latipes*) was used as a surrogate species for the exposure trials. This fish shares common features with threadfin shad and was deemed appropriate as a model for understanding the effects of *Microcystis* and MC on embryonic and larval stages of threadfin shad and other fish species.

Manuscripts generated from the research project:

1. Baxa DV, Kurobe T, Ger KA, Lehman PW, Teh SJ. 2010. Estimating the abundance of toxic *Microcystis* in the San Francisco Estuary using quantitative real-time PCR. Harmful Algae, 9(3): 342-9. http://www.sciencedirect.com/science/article/pii/S1568988310000028

2. Deng DF, Zhang K, Teh FC, Lehman PW, Teh SJ. 2010. Toxic threshold of dietary microcystin (-LR) for quart medaka. Toxicon 4:787-794

3. Acuña S, Baxa D, Teh S. 2012. Sublethal dietary effects of microcystin producing *Microcystis* on threadfin shad, *Dorosoma petenense*. Toxicon, 60(6): 1191-202. http://www.sciencedirect.com/science/article/pii/S0041010112007179

4. Kurobe T, Baxa DV, Mioni CE, Kudela RM, Smythe TR, Waller S, Chapman AD, Teh SJ. 2013. Identification of harmful cyanobacteria in the Sacramento-San Joaquin Delta and Clear Lake, California by DNA barcoding. SpringerPlus, in press. http://www.springerplus.com/content/pdf/2193-1801-2-491.pdf

5. Acuña S, Deng D-F, Lehman P, Teh S. 2012. Sublethal dietary effects of *Microcystis* on Sacramento splittail, *Pogonichthys macrolepidotus*. Aquatic Toxicology, 110-111: 1-8. http://www.sciencedirect.com/science/article/pii/S0166445X11003389

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