FINAL REPORT

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

To
Dr. Ted Sommer
Aquatic Ecology Section
Department of Water Resources
Contract No. 4600008137

By
Swee J. Teh, Dolores V. Baxa, Shawn Acuña
Aquatic Toxicology Program,
Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine,
University of California, Davis
November 10, 2010

Table of Contents **Page**

Executive Summary…….. **4**

Background …….. **5**

Research Methods, Results, and Discussion by Task

Task 1 – Laboratory Culture of Threadfin Shad …….. **6**
Fish collection, transport, and maintenance …….. **6**
Broodstock development …… ……..**7**
Culture system …… ……..**8**
Induced spawning …… **8**
Natural spawning …… ……..**9**

Task 2 – Lethal and Sublethal Effects of *Microcystis* on TFS …… **10**
Task 2.1. Exposure of TFS embryo and larvae to MC-LR …….. **10**
Task 2.2. Exposure of TFS embryo and larvae to local *Microcystis* … **10**
Task 2.3. Exposure of TFS to *Microcystis*/MC-LR diets ……. **10**

Task 3 – Field Survey of *Microcystis* and Effects on Wild TFS ……. **22**
Task 3.1. Assessment of health and nutritional status of TFS using protein, lipid, and energy levels …….. **23**
Task 3.2. Screening for pathogens and diseases (development of specific TFS cell lines for future viral isolation) ……. **28**
Task 3.3. Development of PCR and qPCR for *Microcystis* …… **28**
Task 3.4. Verification of *Microcystis* ingestion in zooplankton using PCR and qPCR ……. **34**

Investigating other Research Associated Goals …….. **36**
Impacts of *Microcystis* to key zooplankton species in the SFE …… **36**
Initial establishment of local *Microcystis* cultures …… **37**
Pilot study: effects of temperature and salinity on abundance of toxin producing *Microcystis* ……**37**

General Summary …… **40**

Justification for request of funding extension …… **42**

Proposed Work to Address Future Challenges …… **42**

Study 1. Comprehensive characterization of key cyanobacterial toxin producers and green algae in the SFE using DNA microarray and specific qPCR …….. **42**

Study 2. Estimating the presence and abundance of *Eurytemora affinis* and *Pseudodiaptomus forbesi* in ambient surface waters and in gut contents of larval species in the SFE…….…… **43**

Study 3. Field studies to evaluate the potential impacts of exposure to contaminants (metals, pesticides) and *Microcystis* on fish health ……**43**

Study 4. Laboratory studies to evaluate the potential relationship between exposure to environmental contaminants (metals, pesticides) including *Microcystis* and health of key pelagic and other economically important fish species …..…… **44**

Literature Cited …… **45**

List of publications generated from the research project …… **47**

Appendix– PDF of products/publications…… **48-87**

Executive Summary

Threadfin shad, TFS (*Dorosoma petenense*), a member of the family Clupeidae, is one of two clupeid species found in California's freshwater. Previously one of the four most abundant pelagic fishes in the upper San Francisco Estuary (SFE), TFS abundance has been in decline since 2000. Recurring harmful algal blooms, due mainly to *Microcystis aeruginosa*, were first observed in the SFE in 1999 and have since increased in distribution and magnitude across habitats where pelagic fish have reached historically low levels of abundance (i.e. Pelagic Organism Decline, POD). Because of the overlapping occurrence of blooms and POD, potential links between these two factors have been strongly hypothesized but actual mechanisms of interaction remain to be determined. This study was therefore conducted to address the potential effects of *M. aeruginosa* on TFS.

Laboratory investigation on effects of exposures to diets spiked with *M. aeruginosa* collected from the SFE demonstrated detrimental effects on health of TFS including: 1) Decreased growth and condition factor, 2) Cachexia or malnutrition resulting in significant loss of body weight and muscle degeneration or atrophy, 3) Severe liver lesions such as cytoplasmic eosinophilic droplets or proteinaceous materials, glycogen depletion, single cell necrosis, and sinusoidal congestion, and 4) Increased ovarian atresia which may impair reproduction. In lieu of costly direct toxin chemical analysis of TFS tissues, alternative methods such as immunohistochemistry (IHC) and in situ hybridization (ISH) were developed to validate the fate and accumulation of dietary microcystin (MC)/*Microcystis* in TFS. IHC confirmed the presence of MC (LR) in the intestines, liver and kidneys while ISH demonstrated the localization of ingested *Microcystis* in the stomach and intestines of TFS exposed to *Microcystis*/MC-spiked diets.

Field investigation of TFS (N = 296) from Brannon Island (BI), Sherman Island (SI), Mildred Island (MI), and Stockton (STK) employed the evaluation of condition factor, hepatosomatic index, nutritional status, and histopathology to establish health status. Based on these criteria, TFS in SI were found to be relatively healthier compared to TFS from STK and BI. Although TFS from STK and BI were generally of poor health, histopathologic analysis revealed significant lesions in TFS from STK that are more likely related to effects of contaminant stressors than to *Microcystis* toxicity. Among TFS in BI, severe intestinal epithelial cell necrosis and the localization of MCs in liver (IHC) and *Microcystis* in stomach and intestine (ISH) strongly indicated effects of *Microcystis* intoxication. The TFS collected from the field were identified as immature sub-adults as determined by length and gonadal development.

In conclusion, our findings establish that TFS consumed *Microcystis*, absorbed and localized MCs in target tissues, and toxic effects were linked to MC exposure. This project incorporates other research inputs including 1) Development of molecular tools (PCR/qPCR) to identify and quantify *Microcystis* in algal tissues and ambient waters, 2) Detection, quantification, and localization of *Microcystis* as ingested by zooplankton in the field, and 3) Establishment of local *Microcystis* cultures to initially characterize toxicity due to environmental factors (e.g. temperature and salinity) that trigger the growth of toxin producers. Future challenges and proposed studies to address the fundamental health of fish due to recurring *Microcystis* blooms in the SFE are presented. A total of 5 peer-reviewed publications (plus one in progress) were generated from this project.

Background

Threadfin shad, TFS (*Dorosoma petenense*), a member of the family Clupeidae, is one of 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). TFS feed on small zooplankton, phytoplankton, and detritus particles via filter feeding but will also feed on larger zooplanktons when available. Spawning occurs between May to August and peaks in June (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 that may affect 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.

Recurring blooms in the SFE are composed primarily of colonial forms and single cells of *Microcystis* 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, heptapeptides liver toxin) that are released into water during or upon bloom senescence. 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). MC-LR is one of the most toxic variants of the more than 80 congeners of the protein (Zurawell et al. 2005). Although MC concentrations have been detected 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 the gills during breathing. 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 are at risk of exposure to the toxins.

Fish are hypothetically exposed to MCs through: 1) direct consumption of *Microcystis* and other MC-producing cyanobacteria, 2) interaction of organisms in the food web by feeding on grazers consuming *Microcystis* and other toxin-producing cyanobacteria, and 3) exposure to dissolved MCs during or at the senescence of *Microcystis* and other toxin-producing cyanobacteria. Induced MC toxicity has been evaluated in other fish species such as 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. 2009).

Because of the widespread impact of blooms, the Interagency Ecological Program Management Team listed *Microcystis* 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 *Microcystis*

and the general health of TFS is necessary to demonstrate the threat of the cyanobacterium. The impacts of *Microcystis*/MC exposure in TFS was evaluated using a combination of histopathology, immunohistochemistry, toxin analysis, and molecular techniques. This research approach can be used to examine other pelagic organisms that are potentially at risk from the adverse effects of exposure to MCs.

While bloom toxicity 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. 2007, Feyrer et al. 2009). To date, studies have not conclusively established the direct effects of MCs/*Microcystis* 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, 2010), it is important to evaluate the biological fate and potential risk of the toxins to fish and other food web organisms. The purpose of this study is to determine the potential threat of *Microcystis* to threadfin shad, *Dorosoma petenense*, by utilizing this pelagic fish as an indicator of the risks from exposure to *Microcystis* blooms in the SFE.

Task 1 − Laboratory Culture of Threadfin Shad

Fish collection, transport, and maintenance

2008

Groups of TFS were collected for broodstock development from two locations in the 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 at water temperatures of 20ºC and 22ºC, respectively. 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 was 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

SFE: Wild broodstock was collected on several occasions in the SFE with the help of Dr. Ted Sommer, Kevin Reece and other DWR staff. On May 5, Sherman and Brannon Islands and Stockton were sampled. No fish were found in Sherman or Brannon Island. A total of 40 fish was collected at Stockton that were transported to CABA for broodstock development as described below. On May 15 at Stockton, only 32 fish were collected to determine if there were any ripe females and males. The males spermiated but none of the females were mature. Out of 32 fish, 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 Game and in other labs at UC Davis (Drs. Sih, Moyle and Cech) but did not receive any new information on TFS distribution.

Reservoir: Additional sites for obtaining broodstock for larvae were recommended by Teejay Orear from Dr. Peter Moyle's laboratory. With Orear'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 in these locations.

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 well water from CABA and Tracy and added with 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

SFE: TFS were collected in the field by electrofishing with the aid of Dr. Andy Sih's laboratory at UC Davis and equipment from the Department of Fish and Game. On June 17, 2010, TFS (N=3) were collected in Sugar Slough and from Grant Line Slough (N=3). The TFS from Sugar Slough included one gravid female that 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 TFS from Sugar Slough were males but only one responded to stripping. The sperm was collected from the responsive male and added to the eggs from the female using dry fertilization. River water was added to the sperm. The resulting embryos became adhesive and sticked to the sides of the nalgene container and to each other. The embryos were transported in a Coleman cooler to the Aquatic Toxicology Program laboratory 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/week 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 immature. 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.

Culture system

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.

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°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 30 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, 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 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 1 at 8:30 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 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. 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°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, 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 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 TFS matured but did not exhibit synchronous 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 9 and 15 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 TFS relative to untreated TFS and males could be stripped but the females would never progress to the final stage of oocyte maturation. The TFS 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 culture 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 the literature for culturing TFS and developing salmonid broodstock, may not have been suitable for broodstock development in TFS. Spawn Tek was added to the water to promote spawning behavior but the TFS tended to avoid the material. Acclimating the broodstock to the Spawn Tek or utilizing another form of spawning aid may be necessary.

Natural spawning

Natural spawning of TFS was attempted in May 2009. Adult TFS (N=26) collected in the SFE 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. Induced spawning was initiated one week following unsuccessful natural spawning.

Captive TFS broodstock (N= 90) originally acquired from the Tracy Fish Collection Facility in 2009 were transported to Chris Miller at the Contra Costa Mosquito and Vector Control (CCMVC) in August 2010. Mr. Miller's experience with spawning techniques provided some insights on difficulties encountered with TFS. Significant mortalities (ca. 85%) occurred

during transport and upon arrival at CCMVC. The remaining broodstock (N=14) was placed into flow through tanks supplied with green water at 20°C. From this broodstock, the presence of juvenile TFS (N<100) in September 2010 was verified by Chris Miller and Shawn Acuña (UC Davis) to have been spawned from the broodstock shortly after transfer to CCMVC. The juveniles are currently maintained at the CCMVC for use as potential broodstock. All of the remaining broodstock from UC Davis, CABA were transported to CCMVC in September 2010 to duplicate the natural spawning but was unsuccessful. Specific conditions and techniques used at CCMVC that promoted successful spawning are unknown but natural spawning of captive TFS broodstock maintained in this facility will reconvene in the spring of 2011.

Task 2 − Lethal and Sublethal Effects of *Microcystis* on TFS

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

We were unable to conduct the studies outlined in Tasks 2.1 due to the unavailability of embryo and larvae from laboratory and wild spawns of broodstock as would have been generated from Task 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 17, 2010 from the SFE. The embryos were placed in 18°C water baths. After 6 days, over 1,100 embryos hatched. Because there were few larvae and the potential for significant mortality, the embryos were reserved for Task 2.2.

Task 2.2. Water exposure of embryo and larval TFS to *Microcystis* from the SFE

This task was initially conducted using larvae obtained from fertilized embryos from the SFE. Prior to feeding with *Microcystis*, the larvae were maintained in beakers with green water (green algae *Nannochloris*) and fed daily with rotifers. Larvae were microscopically examined at 6 days but did not reveal any rotifers or other foods in guts. No larvae survived after 7 days. Loss of the larvae was most likely due to starvation and poor water quality. The green water and rotifers in the beakers decreased the water quality by producing ammonia and reducing dissolved oxygen. Future attempts to culture the larvae will utilize a flow-through system and improved food quality.

All of our options to recover broodstock and to generate embryos and larval stages of TFS from the SFE and from private fish farms have been exhausted in 2010. The spawning techniques and larval rearing methods will be further improved upon availability of broodstock to complete the objectives outlined in Task 2.2.

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 and verified independently at SUNY–Dr. G. Boyer for MC-LR content by LC/MS and then used to prepare different diets containing 0 (D0), 4.4 (D5) and 10 (D10) ppm of MC-LR. Dr. Greg Boyer and Dr. Birgit Pushner–UC Davis verified the diet

concentrations. TFS were placed in a recirculating system equipped with sand, particles, 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% neutral buffered formalin for histopathology, immunohistochemistry, and in situ hybridization. The remaining fish were frozen for biochemistry. The results were analyzed by one-way ANOVA to determine if there was a significant effect of MC dietary exposure.

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 (4.4 ppm MC-LR) and 27% in the D10 (10 ppm 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. 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 from malnutrition due to liver damage, utilization of energy for detoxification, and reduced feeding. Significant exposure to MC can lead to malnutrition forcing the fish to use its muscle tissues as an energy source resulting in severe emaciation or cachexia. A closer inspection of the internal anatomy revealed remarkably enlarged and dark colored gall bladders. Severe cachexia among TFS fed with MC-laden diets suggests that dietary MC exposure is a factor affecting the growth and survival of TFS.

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)**. The BW decreased in the following order: 0>D5>D10 **(Fig. 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. 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.

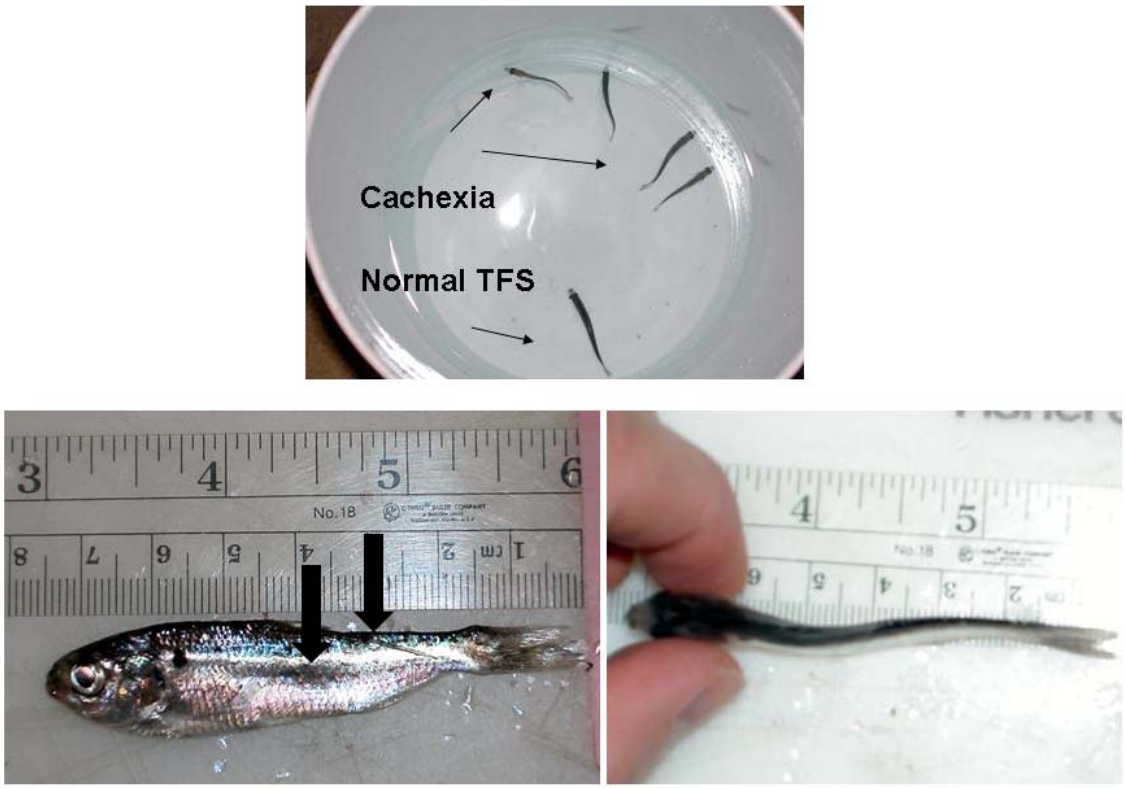

Figure 1. The top micrograph shows 4 abnormal and 1 normal threadfin shad (TFS) fed 10 ppm MC-LR diet (D10) at 58 days post exposure. The bottom micrographs of lateral and dorsal view of the abnormal TFS indicate severe emaciation characteristic of cachexia (severe ill health and malnutrition). Arrows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

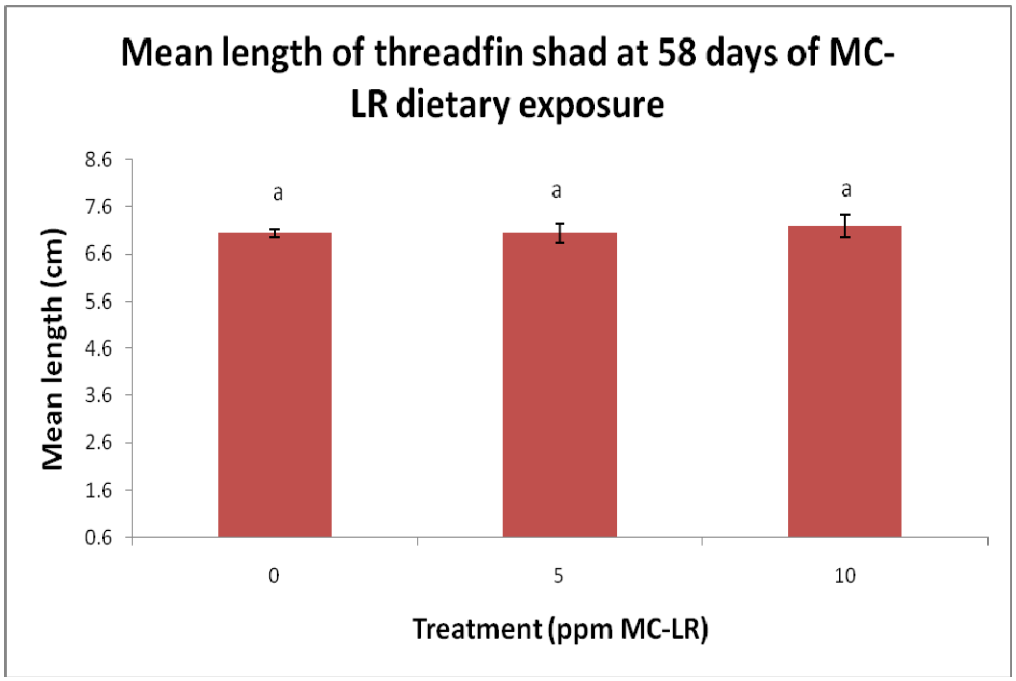

Figure 2. Body length of threadfin shad 58 days after feeding different concentrations of MC-LR-spiked diets. Similar letters between groups denote insignificant difference (P>0.05, Tukey pairwise comparison).

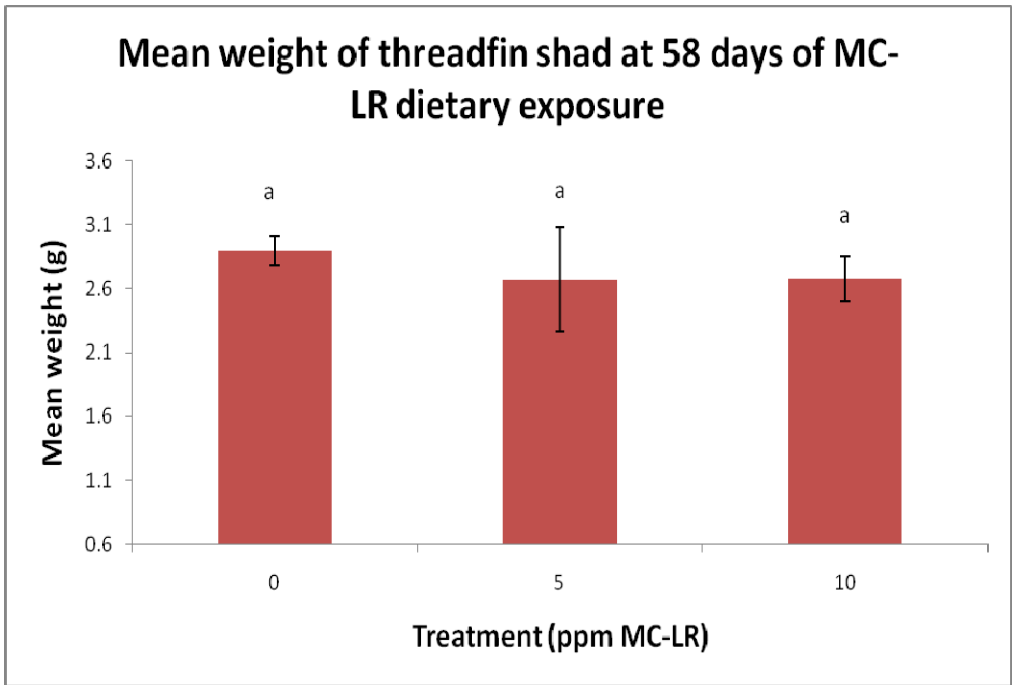

Figure 3. Body weight of threadfin shad 58 days after feeding different concentrations of MC-LR-spiked diets. Similar letters between groups denote insignificant difference (P>0.05, Tukey pairwise comparison).

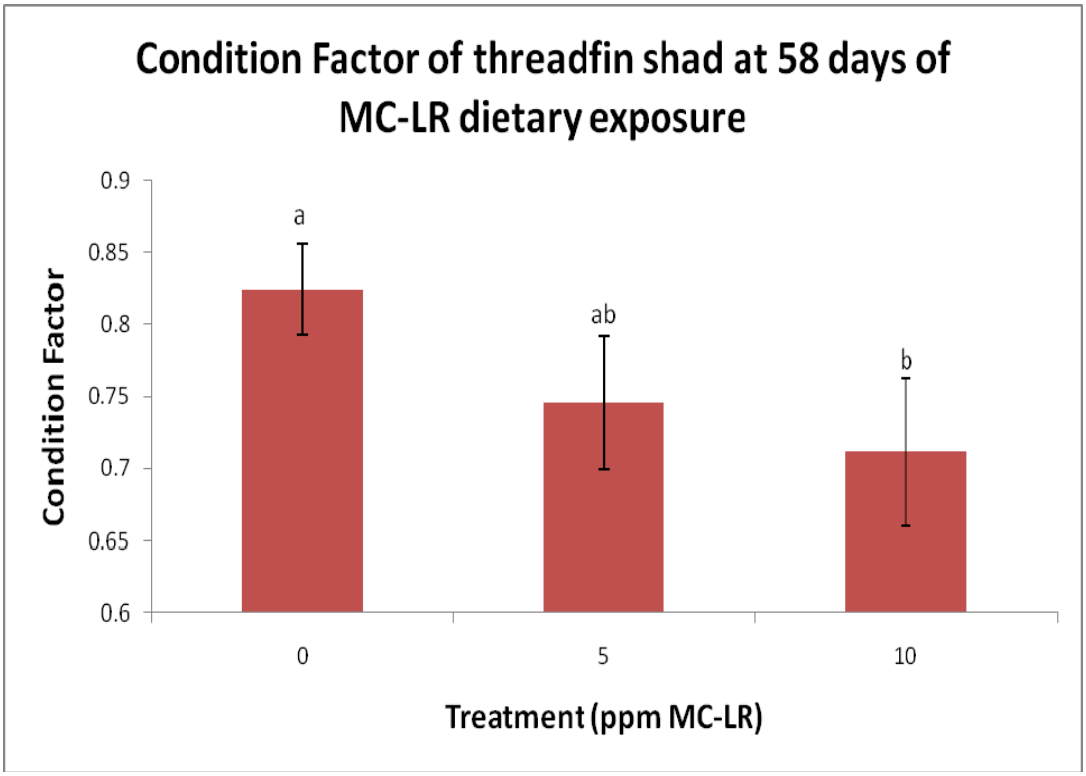

Figure 4. Condition factor in threadfin shad fed different concentrations of MC-LR-spiked diets after 58 days. Different letters between groups denote significant difference using Tukey pairwise comparison.

Histopathology

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 result in reduced cell to cell binding, increased single cell necrosis (SCN) and hemorrhaging in the liver. Hemorrhaging from an impaired circulatory system may result in sinusoidal congestion (SC).

TFS exposed to increasing concentrations of MCs showed microscopic changes in the liver such as severe glycogen depletion (GD) and sinusoidal congestion (SC) **(Fig. 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 ppm MC-LR (D10) compared to 4.4 ppm MC-LR (D5) as shown in **Fig. 6.** Although there was a

significant difference in CF between genders in 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 there were more females showing a significant increase in severe ovarian atresia (OA) **(Figs. 6, 7).** The extent and severity of OA may provide clues on gonadal development and reproductive viability. The incidence of OA in female TFS exposed to MC-laden diets may reflect impaired gonadal development and reproduction that may suggest long-term effects to TFS populations in the SFE.

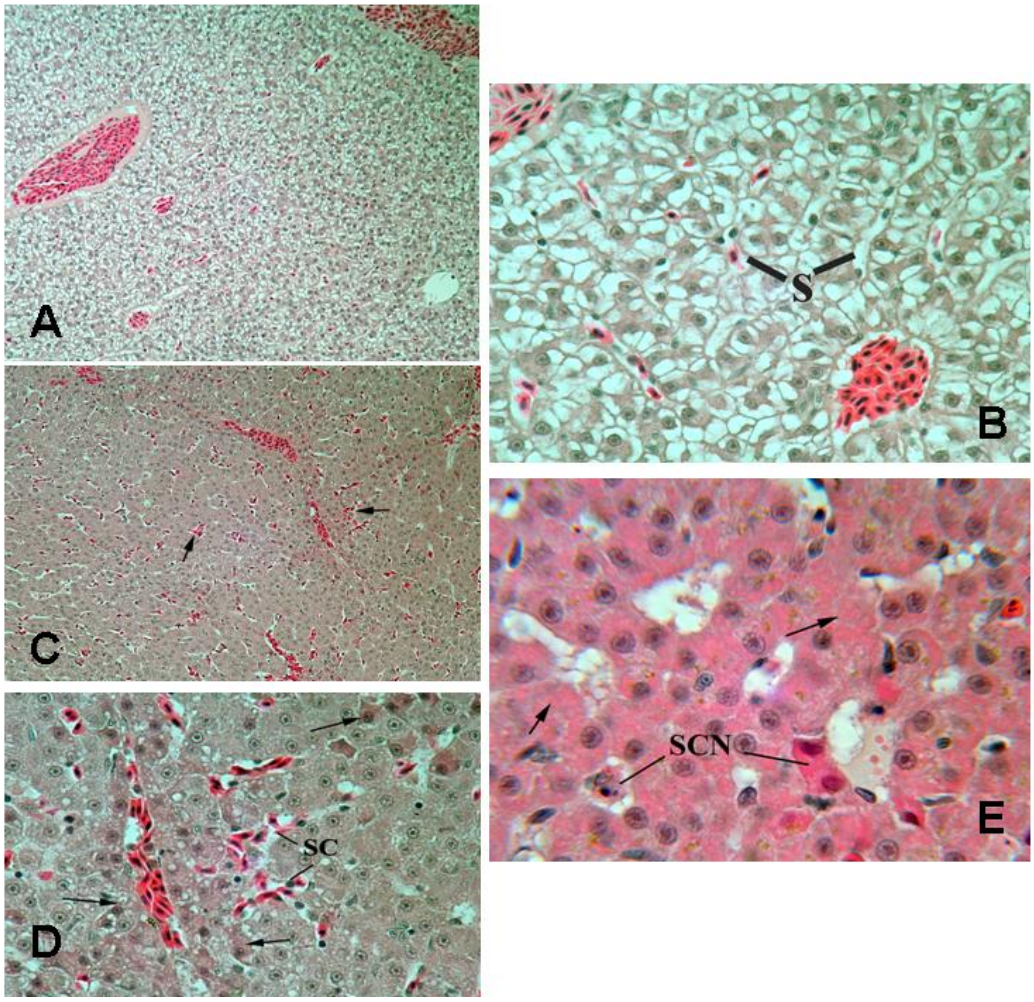

Figure 5. Threadfin shad liver sections stained with H & E. A) Normal glycogen-rich liver; B) higher magnification of normal liver architecture. Note that the sinusoids (S) are usually one red blood cell thick; C) moderate sinusoidal congestion (arrows) and glycogen depletion in liver of TFS fed 10 ppm 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 (single cell necrosis) SCN in liver of TFS exposed to 4.4 ppm MC-LR diet for 58 days.

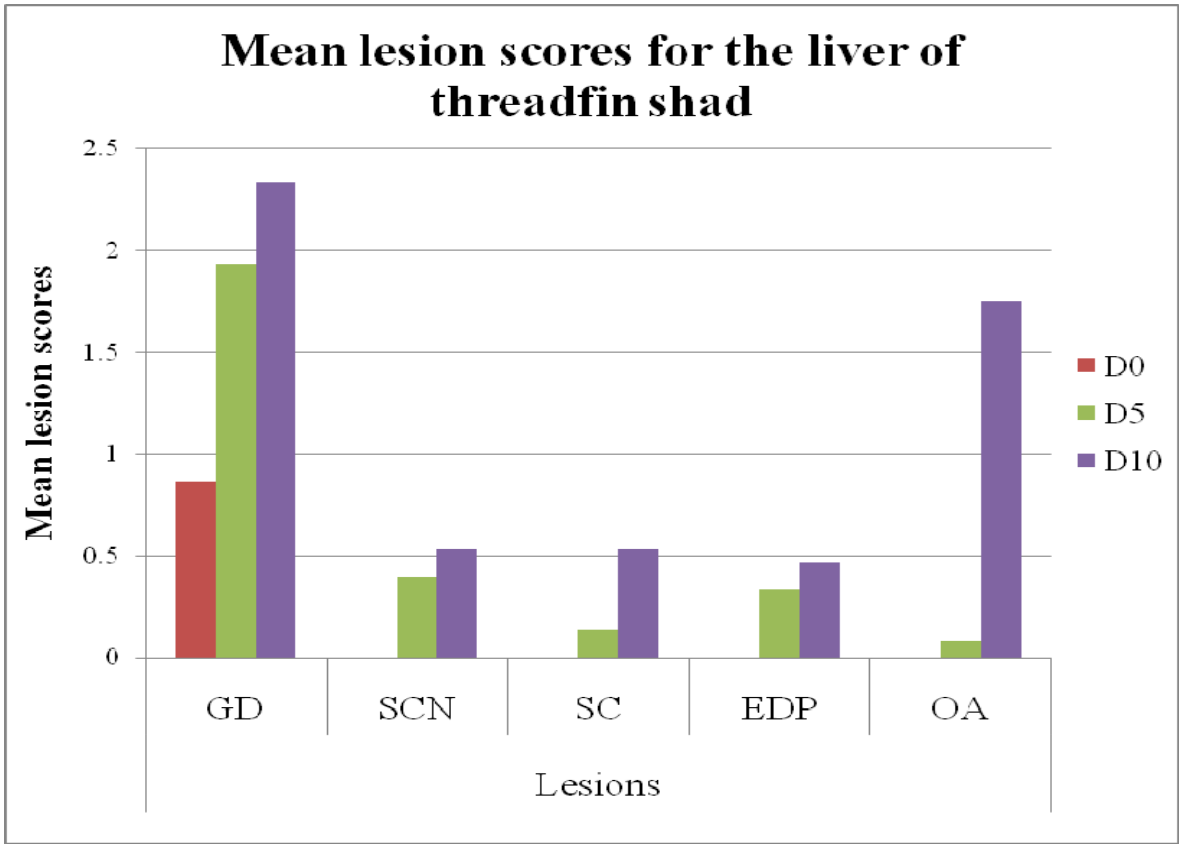

Figure 6. Mean lesion scores in the liver of threadfin shad exposed to *Microcystis* diets with 4.4 ppm MC-LR (D5) or 10 ppm (D10), and controls (no MC-LR or D0) at 58 days post exposure. Liver lesions were scored for glycogen depletion (GD), single cell necrosis (SCN), sinusoidal congestion (SC), eosinophilic 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.

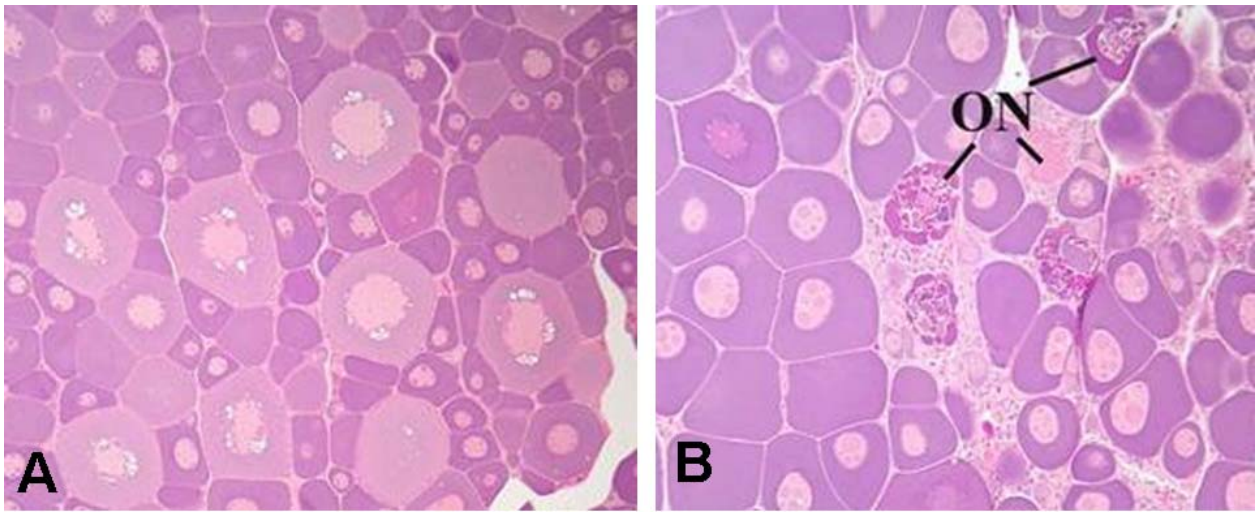

Figure 7. Threadfin shad ovarian sections stained with H & E. A) Normal ovary; B) severe stage II oocyte necrosis (ON) in TFS 58 days after exposure to 10 ppm MC-LR.

Immunohistochemistry (IHC)

Using IHC, TFS samples were assessed for protein phosphatase 2A (PP2A) activity (N=9), the presence of microcystin-LR (MC-LR) (N=12), and CYP1A activity (N=9). Both control and exposed TFS showed similar PP2A activity. As such, PP2A IHC was determined to be an insensitive biomarker for assessment of MC exposure in TFS. CYP1A activity was more sensitive with enhanced staining detected in the kidneys of exposed fish compared to controls (**Fig. 8**). The use of the general biomarker CYP1A was used to confirm other toxic effects of *Microcystis*. The lipopolysaccharides of cyanobacterial cell walls 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 IHC assay, MC-LR binding sites were detected in the intestine, liver and kidney of TFS receiving high and low concentrations of MC-spiked diets (**Fig. 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 TFS upon ingesting the toxin. The presence of hepatic lesions indicates that the TFS 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 **(Fig. 9)**, were also prevalent from TFS collected in the estuary **(Fig. 10)**.

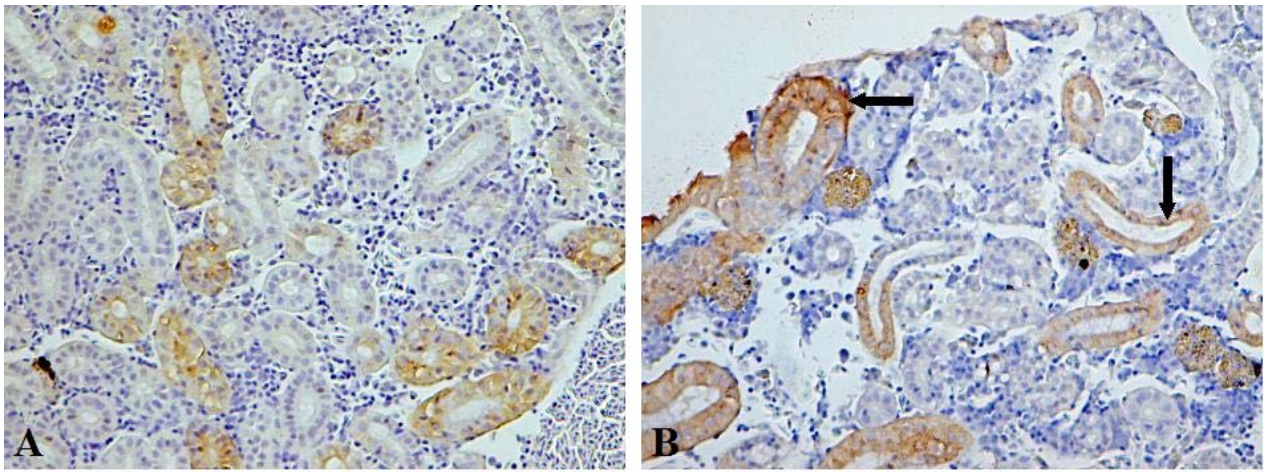

Figure 8. CYP1A (Cytochrome P450) immunohistochemistry in kidney of threadfin shad fed with A) 0 ppm, and B) 4.4 ppm MC-LR spiked diets. Note that CYP1A activity is more enhanced (arrows) in exposed compared to control fish. CYP1A is an active generalized biomarker that was used to confirm other toxic effects of *Microcystis*. For example, the lipopolysaccharides of cyanobacterial cell walls can have significant toxic effects such as inflammatory response and oxidative stress and as such, CYP1A activity is enhanced in fish receiving diets with MC-LR.

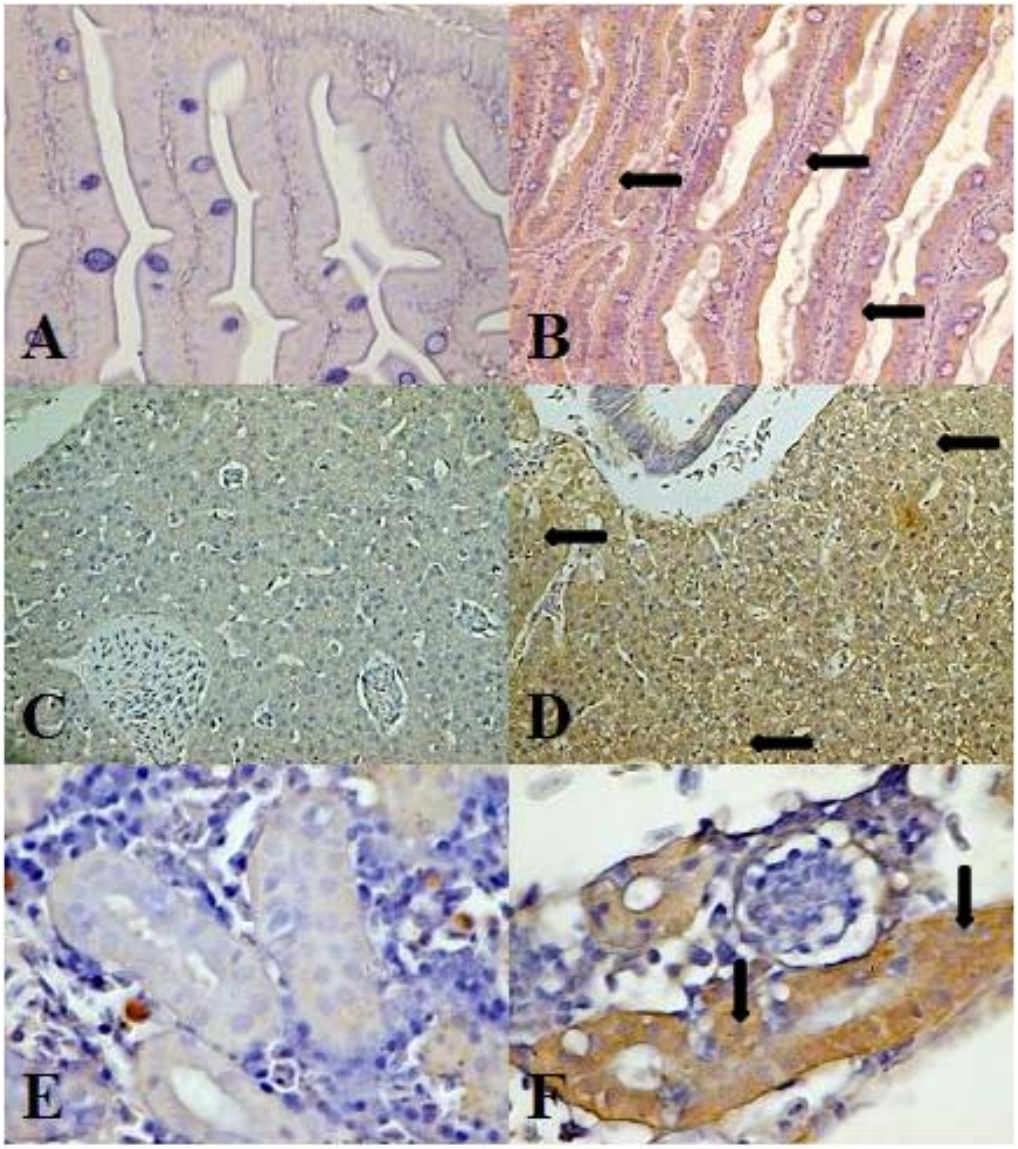

Figure 9. Immunohistochemistry: MC-LR localization in A) intestine C) liver and E) kidney of threadfin shad receiving no MC-LR; and B) intestine D) liver and F) kidney of threadfin shad receiving 4.4 ppm MC-LR spiked diet. Arrows indicate positive brownish stains of MC-LR.

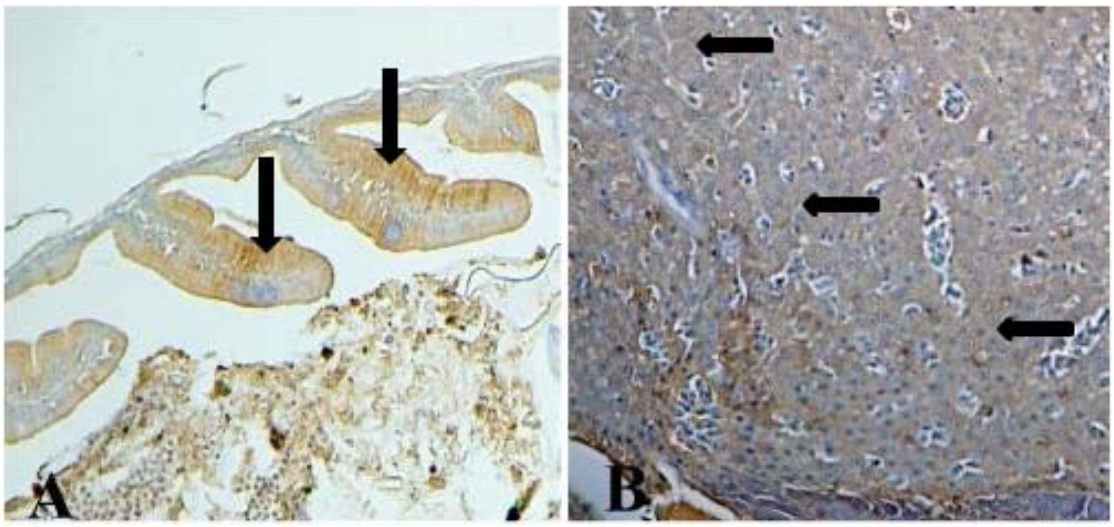

Figure 10. Immunohistochemistry: MC-LR localization in A) intestine and B) liver of threadfin shad from the SFE. Arrows indicate positive brownish stains of MC-LR.

***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 TFS? So we used a technique called ISH that utilizes 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, 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 **(Fig. 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 TFS collected from Mildred Island **(Fig. 12)** demonstrating direct ingestion of *Microcystis* in the SFE.

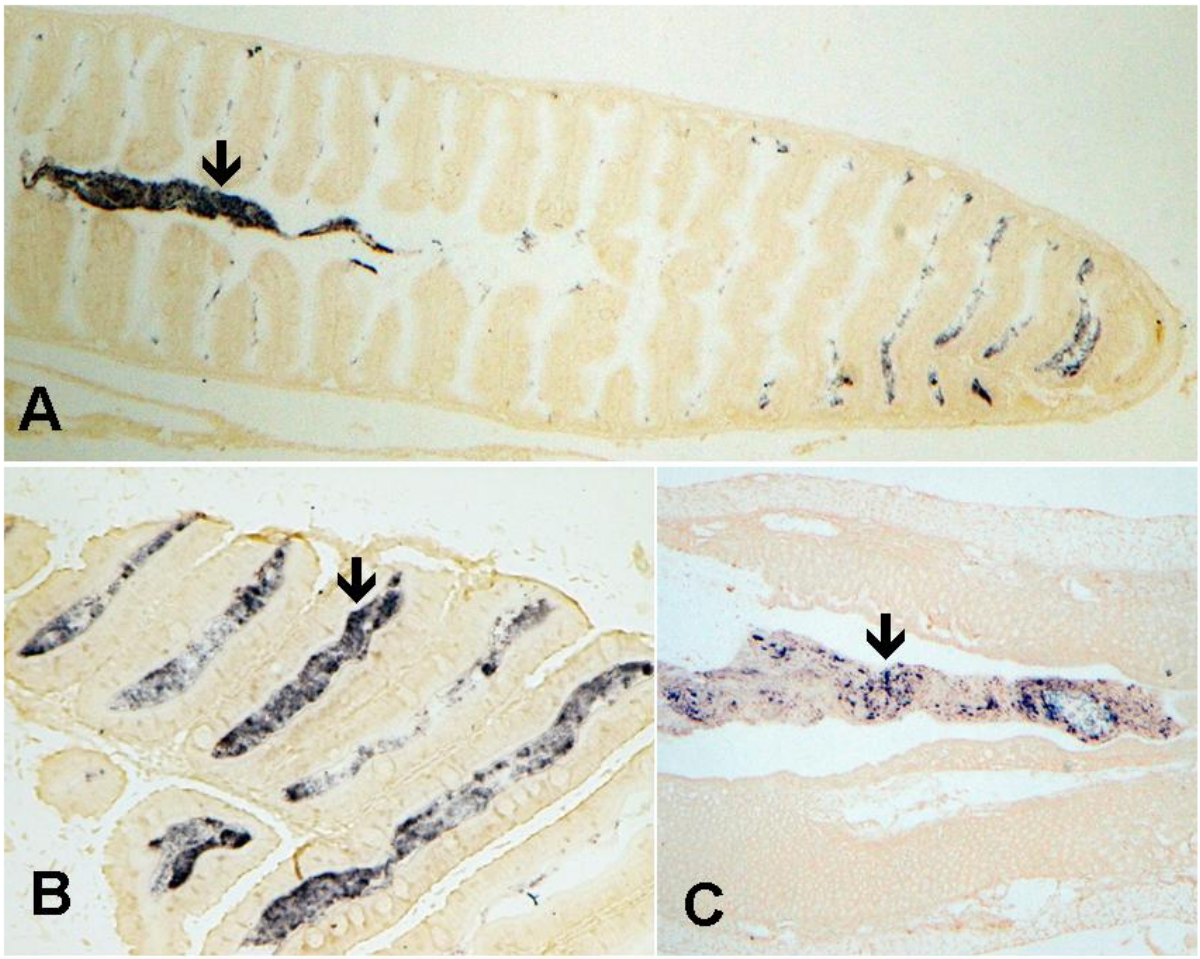

Figure 11. In situ hybridization of threadfin shad showing ingestion of *Microcystis*-spiked diets (arrows) in intestines (A, B) of fish fed with 4.4 ppm MC-LR diet and gut contents (C) of fish fed with 10 ppm MC-LR.

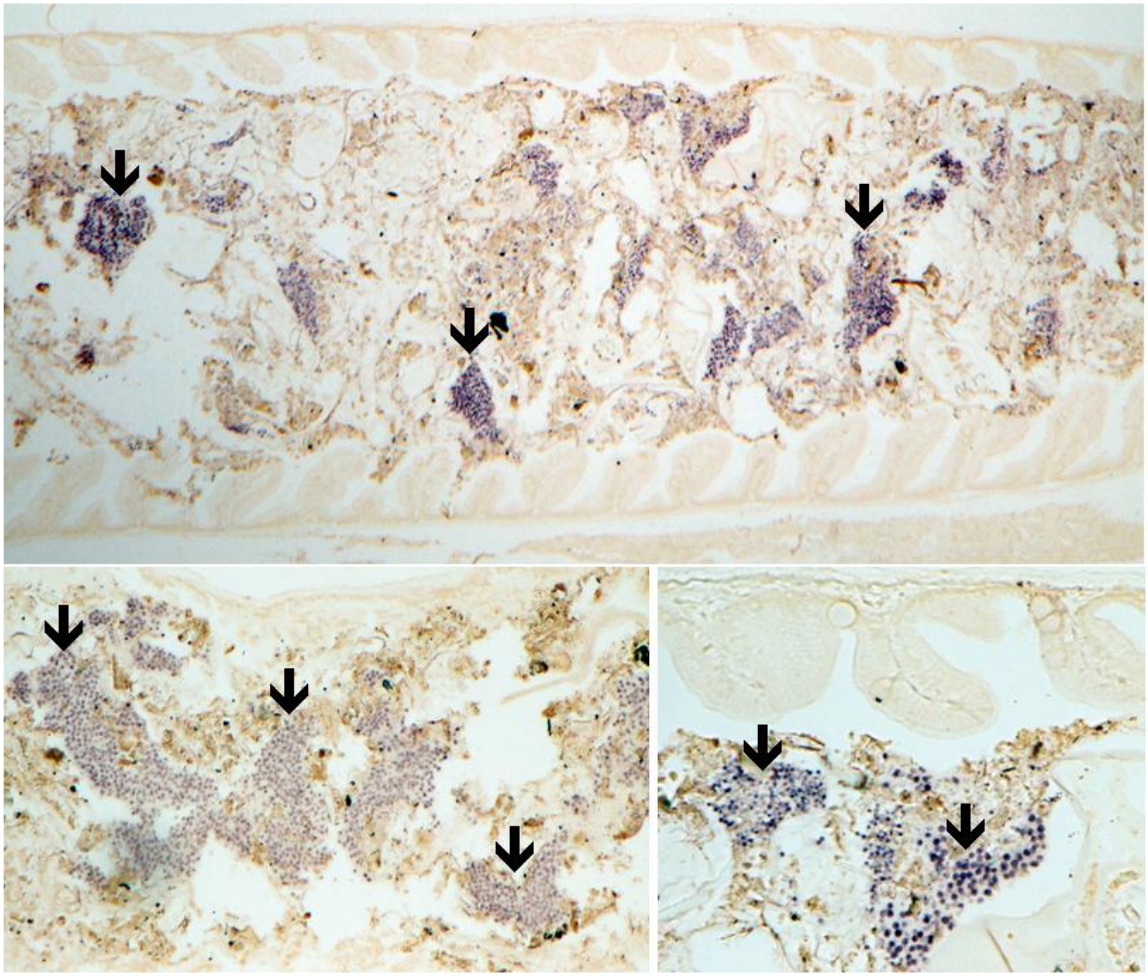

Figure 12. In situ hybridization of threadfin shad from Mildred Island showing ingestion of *Microcystis* cells (arrows) in gut contents.

Task 3 − Field Survey of *Microcystis* and Effects on Wild TFS

Collection of *Microcystis* and 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. Assessment of health and nutritional status of wild 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 for assessment of health and nutritional status **(Table 1).** Our goal was to determine if there are geographic differences in the health and 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). In addition, 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 1. Threadfin shad collected from the San Francisco Estuary for evaluation of health and nutritional status. The total number of fish used for each analysis is indicated.

Location	No. fish collected	Nutrition	Histopathology	Disease	Histochemistry MC-LR	Mean wt/length (g/mm)
Sherman Island (SI)	133	55	15	9	4	6.9/89
Brannon Island (BI)	62	30	15	9	4	8.5/94
Stockton (STK)	65	30	15	20	4	6.5/87
Mildred Island (MI)	36	13	15	4	4	1.4/58

Results and Discussion

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. 13)**. 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. 14).** The hepatosomatic index (HSI) decreased in the following order: SI>BI>STK, but the difference was not significant **(Fig. 14**). 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. 15)**. The nutritional composition based on protein, lipid, moisture and energy levels of whole fish was different among the three locations **(Figs. 16 and 17)**. 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.

Table 2 illustrates the sex ratio and prevalence of liver and intestinal lesions in TFS. The female (F)/male (M) sex ratios were: 33F/67M (SI); 40F/60M (BI, STK, and MI). TFS collected from BI had the most significant lesions where: 1) 7 of 15 fish had moderate to severe glycogen depletion (LGD), and 2) 4 of 15 fish had moderate to severe liver cell necrosis (LCN) and fatty vacuolar degeneration (LLIP). In addition, 5 of 15 fish collected from BI had severe intestinal epithelial cell necrosis. TFS collected from STK had significant glycogen depletion (14 of 15 fish) and liver cytoplasmic inclusion bodies (LCIB; 10 of 15 fish). All TFS (15 of 15 fish collected from MI had significant glycogen depletion. TFS collected from SI were in good health where only 1 of 15 fish showed LGD, LLIP, and LCIB.

In summary, results from the histopathologic and nutritional findings indicate that TFS collected from Sherman Island are relatively healthier compared to TFS from STK, MI, and BI. Although TFS collected from STK and BI were nutritionally impaired, histopathologic analysis revealed significant lesions in TFS from STK that are likely related to the effect of other contaminant stressors than to *Microcystis* toxicity. Severe intestinal epithelial cell necrosis **(Fig. 18)** and further supported by the localization of *Microcystis* in gut contents of TFS collected from BI strongly suggest *Microcystis* intoxication. Lastly, histological analysis indicated that the TFS collected from the SFE were immature sub-adults as determined by gonadal development and length.

Table 2. Histopathological analysis of TFS in the SFE

Location	N	% Prevalence of significant lesions					F/M ratio	% Maturity
		LGD	LCN	LLIP	LCIB	IN		
Sherman Island	15	6.7	0	6.7	6.7	0	5/10	6.7
Brannon Island	15	46.7	26.7	26.7	0	33.3	6/9	13.3
Stockton	15	93.3	0	0	66.7	0	6/9	0
Mildred Island	15	100	0	0	0	0	6/9	0

LGD= liver glycogen depletion; LCN = liver cell necrosis; LLIP = lipidosis or fatty vacuolar degeneration in liver; LCIB = cytoplasmic inclusion bodies; IN= intestinal epithelial cell necrosis; F/M ratio = female to male ratio.

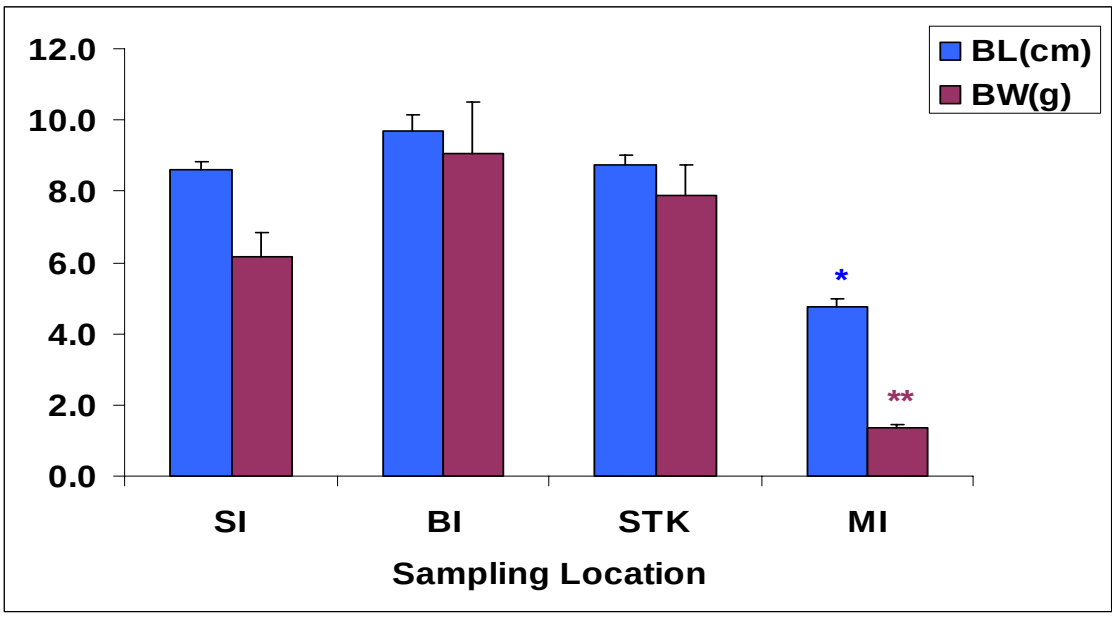

Figure 13. Body weight (BW) and length (BL) of threadfin shad collected from different sites in the San Francisco Estuary in 2007 for nutrition analyses. SI=Sherman Island, BI=Brannon Island, STK= Stockton, MI=Mildred Island

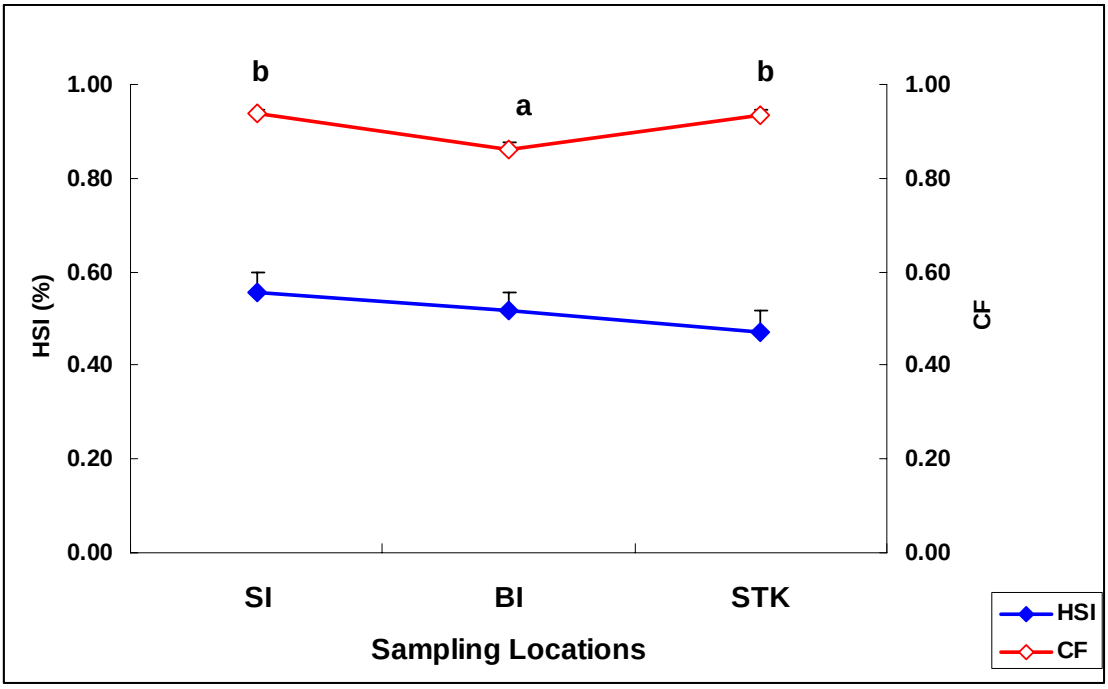

Figure 14. Hepatosomatic index (HSI) and condition factor (CF) of threadfin shad collected from different sites in the San Francisco Estuary in 2007 for nutrition analyses. SI=Sherman Island, BI=Brannon Island, STK= Stockton

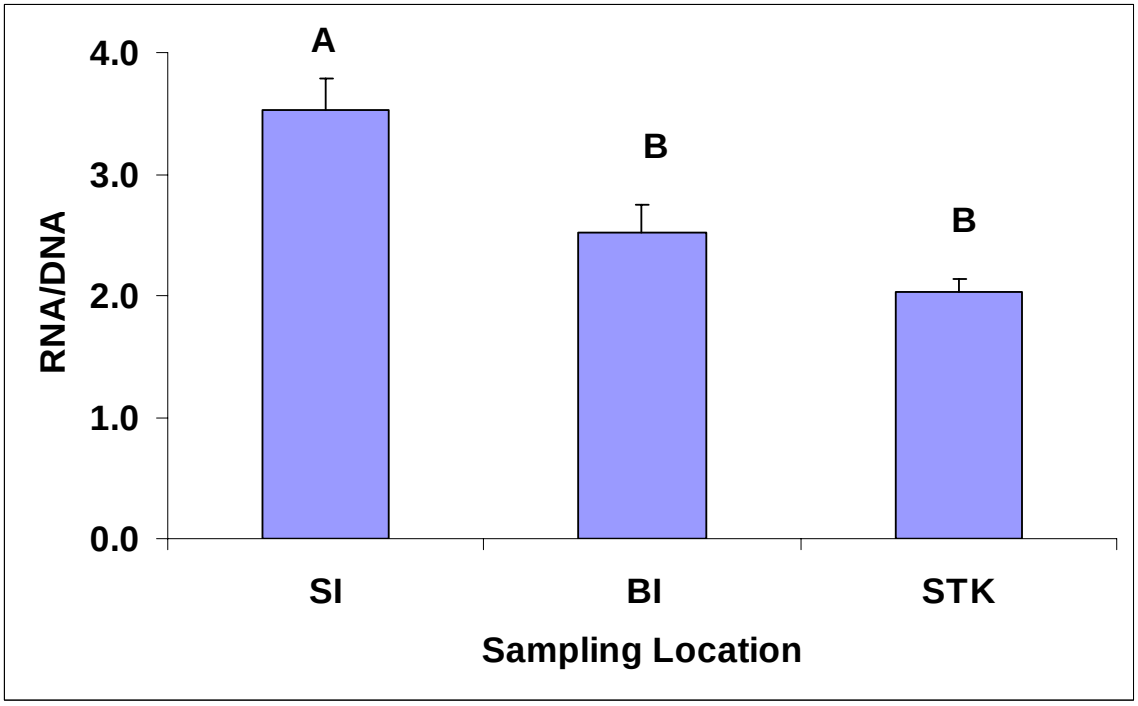

Figure 15. RNA/DNA ratio of threadfin shad collected from different sites in the San Francisco Estuary in 2007. SI=Sherman Island, BI=Brannon Island, STK= Stockton

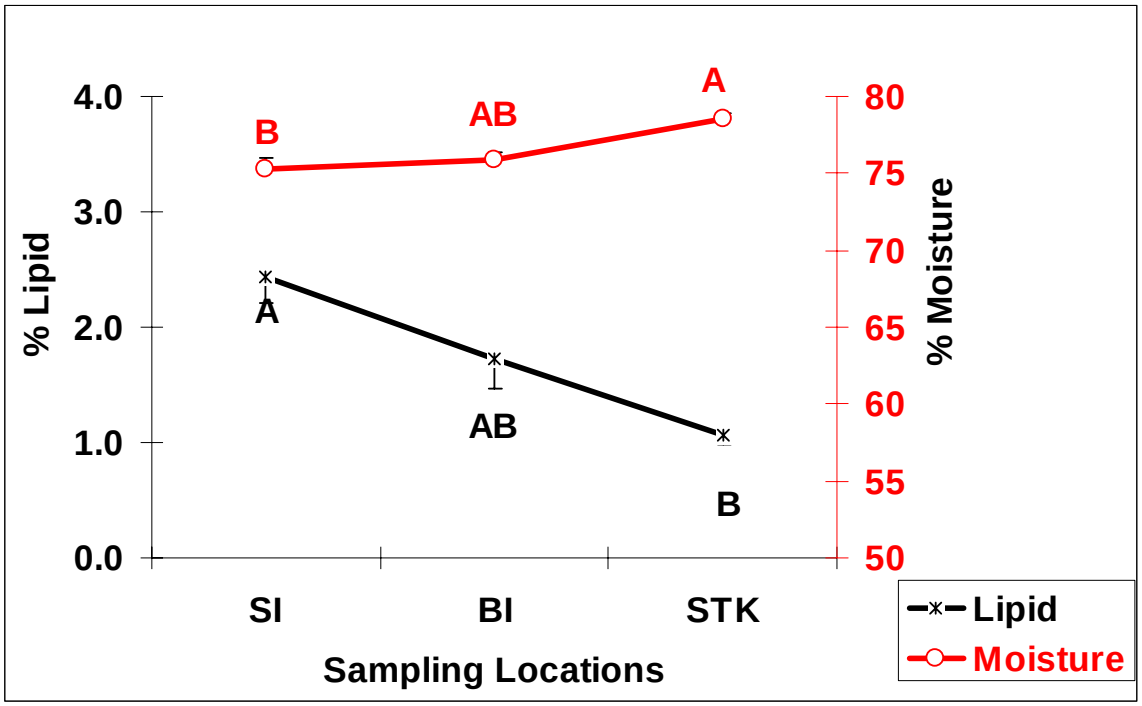

Figure 16. Lipid and moisture contents in threadfin shad collected from different sites in the San Francisco Estuary in 2007. SI=Sherman Island, BI=Brannon Island, STK= Stockton

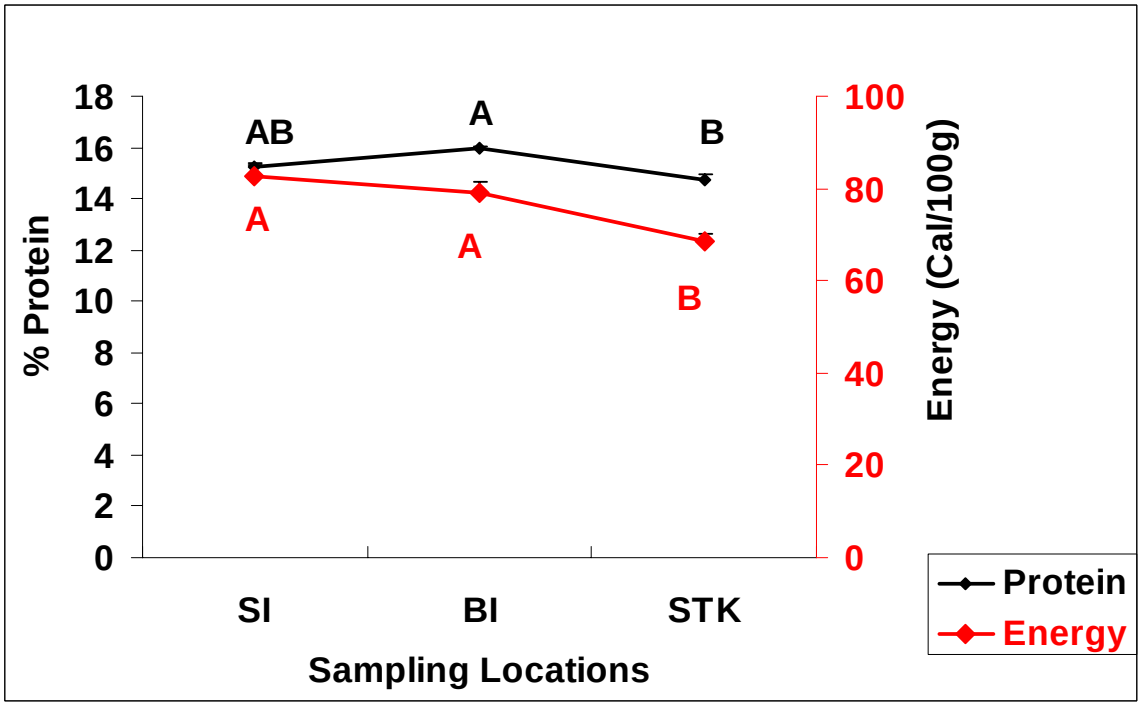

Figure 17. Protein and energy contents of threadfin shad collected from different sites in the San Francisco Estuary in 2007. SI=Sherman Island, BI=Brannon Island, STK= Stockton

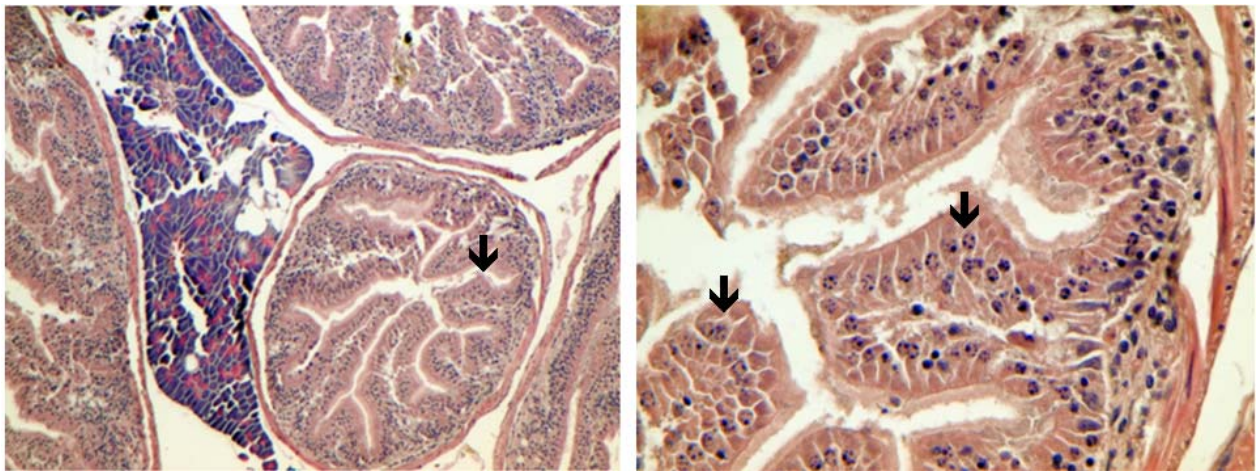

Figure 18. Histopathology: Severe epithelial cell necrosis in threadfin shad collected from Brannon Island suggestive of *Microcystis*/microcystin exposure. Arrows indicate sections of the intestine containing necrotic cells.

Task 3.2. Screening for pathogens and diseases in TFS and development of specific cell lines for viral isolation

The presence of pathogens or diseases was examined in juvenile and adult TFS from different locations: Sherman Island (N=9), Brannon Island (N=9), Mildred Island (N=4), and Stockton (N=20) **(Table 1)**. 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 are currently developing viral cell lines to enhance the specific isolation of viruses that may be potentially present from pelagic fish species in the SFE including the TFS, delta smelt, striped bass and the native Sacramento splittail.

Task 3.3. Development of PCR and real-time quantitative PCR (qPCR) for *Microcystis*

Cyanobacterial samples were collected from different sites in the SFE 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) **(Fig. 19)** employing published primers (see Baxa et al. 2010) for conserved *Microcystis*-specific 16S ribosomal DNA (rDNA) was initially used to establish the presence of *Microcystis* in cyanobacterial tissues and water samples**.** The amplification of the MC toxin synthetase genes *mcy*B and *mcy*D established the presence of toxin-producing *Microcystis*. 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* populations. The gene targets and probes for qPCR are shown in **Table 3**.

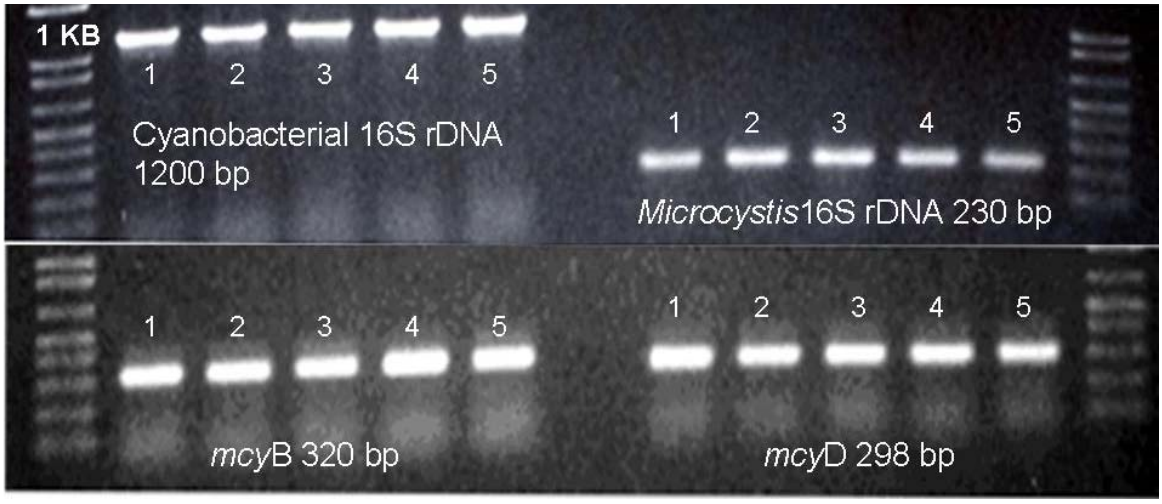

Figure 19. Standard PCR for initial screening of *Microcystis* showing the amplification of target genes: 16S rDNA for cyanobacteria and *Microcystis*, and toxin synthetase genes *mcy*B and *mcy*D. *Microcystis* were collected from different sites in the SFE: 1) Antioch, 2) Chipps Island, 3) Mildred Island, 4) Venice Cut, and 5) San Joaquin.

Table 3. Quantitative real-time PCR gene targets and probes based on 16S rDNA sequences of *Microcystis* found in the SFE.

Target	Probe	Sequence
Microcystis 16S RDNA	MIC 16S P	TTC CCC ACT GCT GCC
*Microcystis mcy*D	*mcy*D P	ATG CTC TAA TGC AGC AAC GGC CAA A
Cyanobacteria 16S rDNA	CYA 604 P	CTG ACA CTC AGC GAC G

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

Cyanobacterial samples collected by diagonal net tows of the water column showed that *Microcystis* was dominant among cyanobacteria (28 – 96%), and toxic *Microcystis* (*mcy*D gene carriers) formed 0.4 – 20% of the total *Microcystis* spp. **(Fig. 20, Tables 4, 5)**. Total *Microcystis* was also abundant (7.7×10^4– 9.9×10^7 cells L^{-1}) in ambient surface waters **(Fig. 21),** 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%) (**Table 5**). Differences in the proportion of toxic and nontoxic *Microcystis* were observed across the sampling locations and seasons for *Microcystis* tissues **(Fig. 20, Tables 4, 5)** and water samples **(Fig. 21, Table 5)** where concentrations of total MCs (0.007 – 10.81 µg/L) also varied **(Table 4).**

Microcystis (from tow nets) 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 **(Fig. 20).**

As in the *Microcystis* 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 **(Fig. 21).**

Quantifying the relationship between nontoxin and toxin-producing *Microcystis* using qPCR in combination with direct toxin analysis using chemical methods such as PPIA can offer greater accuracy for understanding the source and variability of different MC producers and bloom toxicity. Traditional methods of monitoring the toxicity of *Microcystis* blooms in the SFE have relied on microscopic assessment of cell density and chemical detection of MC concentrations in water and 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 distribution 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.

Table4. Mean copy numbers of toxic (*mcy*D) and total *Microcystis* (16S rDNA) and cyanobacterial genes from cyanobacterial tissues. 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=Brannon Island, CI=Chipps Island, MI=Mildred Island, OR=Old River, SJ=San Joaquin, VC=Venice Cut

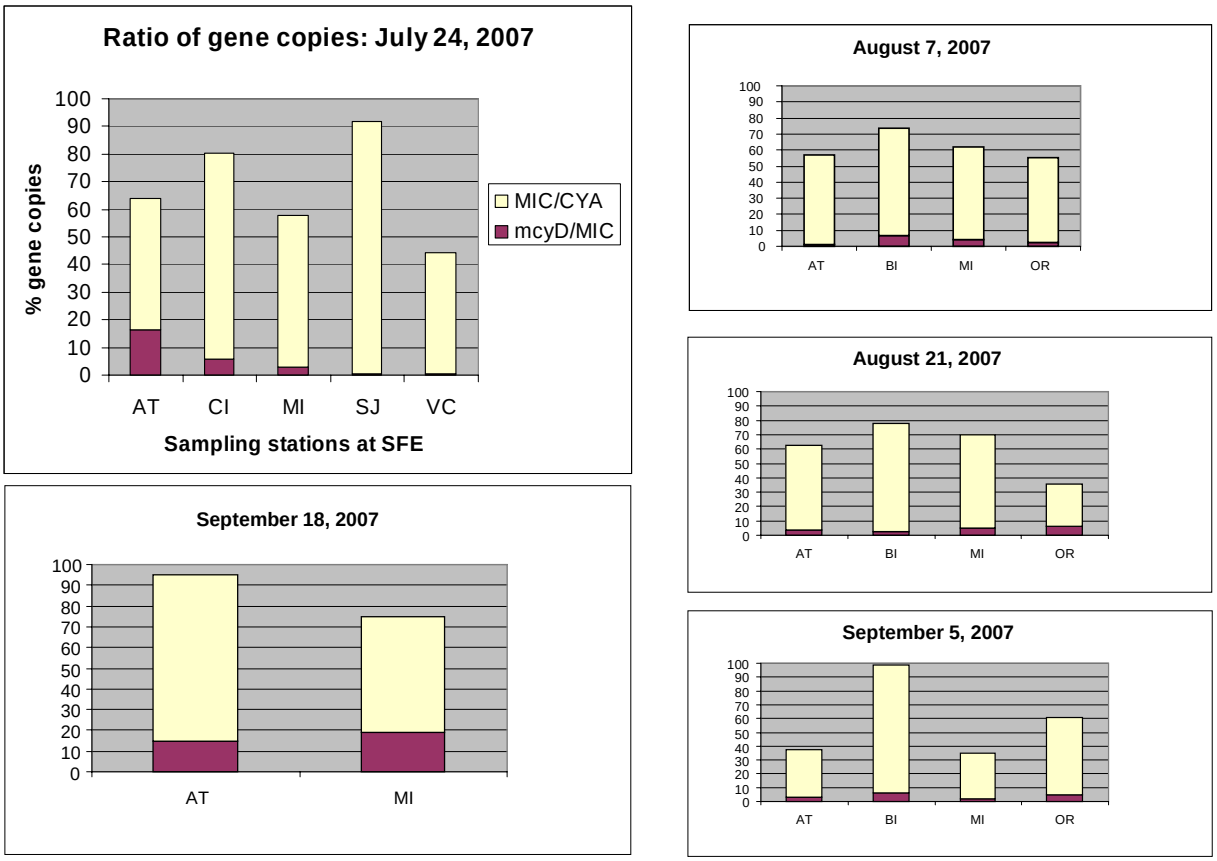

Figure 20. Ratio of gene copies from cyanobacterial tissues from the 2007 blooms in the SFE. (■) toxic *mcy*D carriers, (■) nontoxic *Microcystis*. Sampling sites: AT=Antioch, BI=Brannon Island, CI=Chipps Island, MI=Mildred Island, OR=Old River, SJ=San Joaquin, VC=Venice Cut

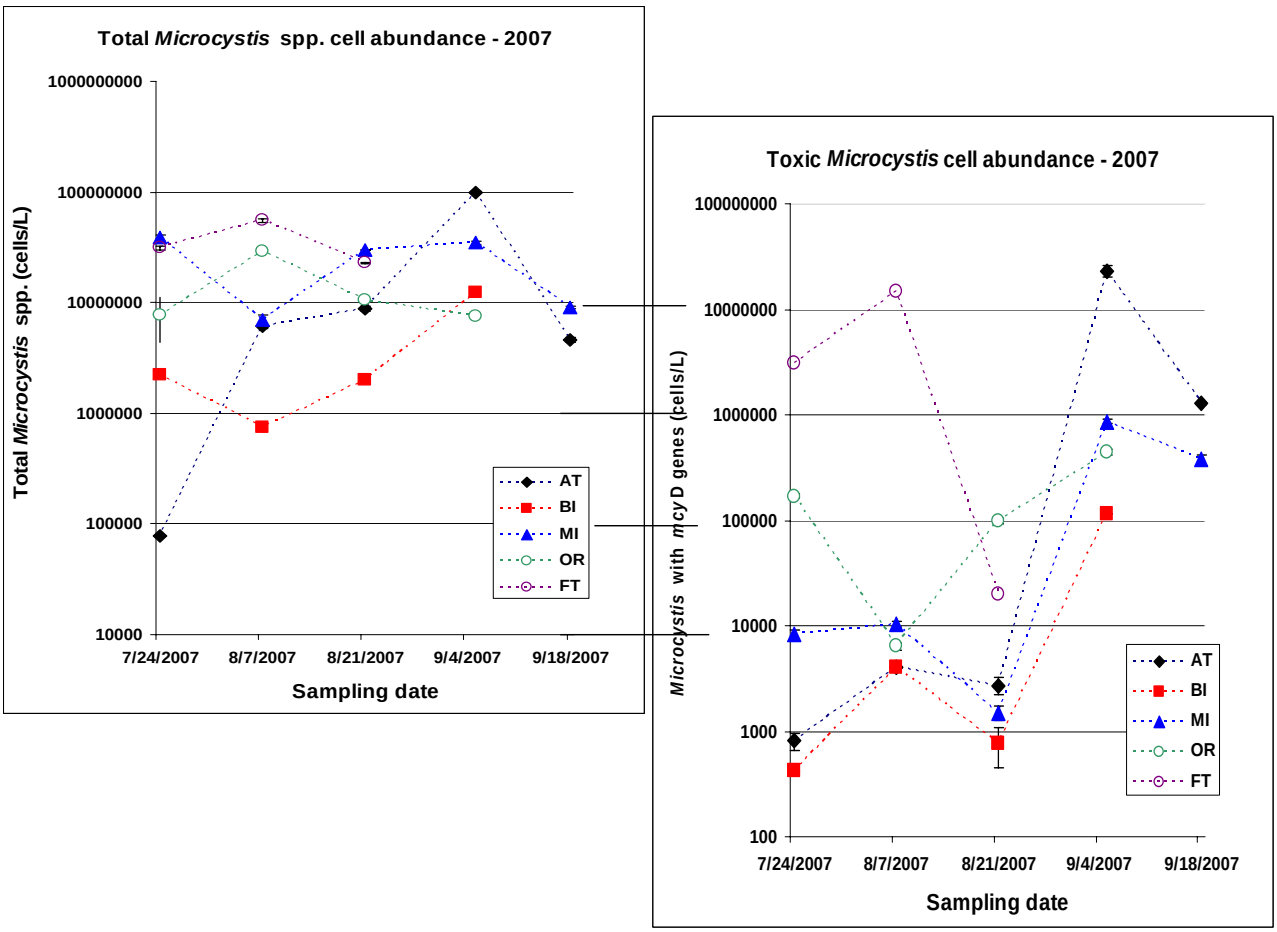

Figure 21. Abundance of total and toxic *Microcystis* in surface waters during the 2007 blooms in the SFE. Sampling sites: AT=Antioch, BI=Brannon Island, MI=Mildred Island, OR=Old River, FT=Franks Tract

Table 5. Cell equivalents of toxic (*mcy*D carriers) 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 3.4. Verification of *Microcystis* ingestion among zooplankton in the SFE using PCR and qPCR

Using standard PCR assays specific to *Microcystis* (Baxa et al. 2010), zooplankton populations from the SFE were examined in 2007 and 2008 to verify if they ingest *Microcystis* in the field. Results showed the amplification of *Microcystis* genes **(Table 6)** 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 6).**

The next question that we want to answer was: how much *Microcystis* are 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 1.3×10^6 *Microcystis* cells with a considerable proportion of toxic cells **(Table 7).** Some zooplankton 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 at least in Mildred Island (7/07/08) and San Joaquin (7/24/07) **(Table 7).**

To verify the ingestion of *Microcystis*-laden zooplankton in fish, we examined archived fish tissues showing suspicious gut contents such as Delta smelt. The Delta smelt was collected in Antioch in 2008 and examined by ISH using DIG-labeled *Microcystis* probe. Aggregates of *Microcystis* cells were localized in the gut of the delta smelt ingesting zooplankton-laden *Microcystis* **(Fig. 22).** Clusters of *Microcystis* cells were also demonstrated in TFS tissues exposed to *Microcystis* in the field and in the laboratory **(Figs. 11 and 12).** 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 6. Presence of *Microcystis* in zooplankton collected from the SFE in 2007 and 2008 as determined by standard PCR.

Location	**Date**	**Total *Microcystis***	**Toxic *Microcystis mcy*D**
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 or negative DNA amplification of total or toxic *Microcystis*

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 7. 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 SFE.

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

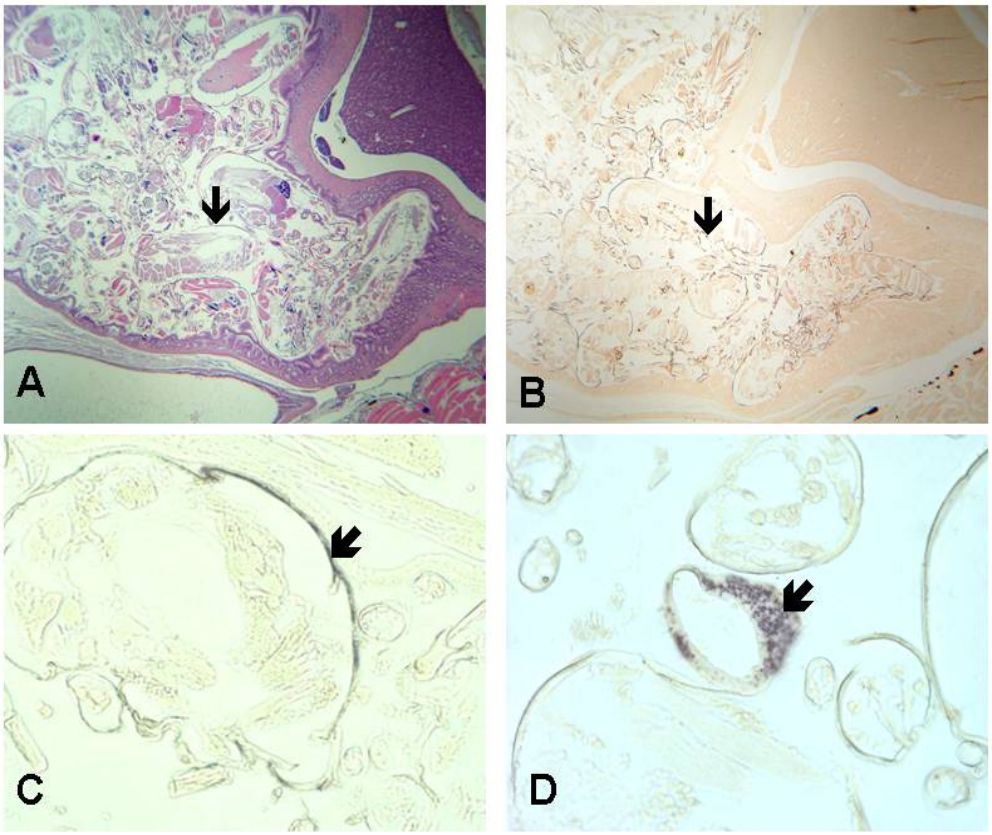

Figure 22. Juvenile delta smelt collected in Antioch in July 2008 showing sections of the gut stained by H&E (A) and in situ hybridization, ISH (B) using DIG-labeled *Microcystis*-specific probes. Arrows in C & D indicate *Microcystis* in the zooplankton ingested in the gut of the delta smelt.

Investigating other Research Associated Goals

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

Histological analysis of juvenile TFS stomach contents suggested that TFS can ingest a wide range of particles including detrital and other non-nutritious food including *Microcystis* spp. Several questions that needed 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, we conducted two separate studies demonstrating 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 specific findings of these studies.

2. Establishing local *Microcystis* cultures

Central to characterizing *Microcystis* toxicity is establishing local *Microcystis* cultures and determining which key environmental factors (i.e. temperature, salinity, pH, nutrients, and light) control their growth and toxicity. Manipulating these variables in the laboratory and measuring the abundance of the *mcy*D gene responsible for the production of the toxin by qPCR will enable us to predict conditions that enhance or decrease MC synthesis. Using controlled laboratory conditions, the interaction between key environmental factors and MC production will provide the knowledge to predict conditions that may alter *Microcystis* abundance and toxicity in the SFE.

Cultures were initially established by isolating and testing cyanobacterial cells from the 2007 and 2009 blooms by PCR/qPCR to determine whether they are toxin-producing or nontoxin-producing *Microcystis*. Other cyanobacteria such as *Aphanizomenon* and other filamentous bacteria were observed in the cultures **(Fig. 23)**. 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*, and their ability to produce toxins due to environmental factors can be evaluated under controlled laboratory conditions.

3. Pilot study: effect of temperature and salinity on abundance of toxin producing cells

Using the mixed *Microcystis* populations propagated in the laboratory, a pilot study was conducted 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 and 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. 24).** The *mcy*D qPCR assay that we used most likely quantified other toxin producing cyanobacteria in the Delta such as other 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*.
These results also suggest the broad range of salinity tolerance (e.g. *Microcystis* growth enhanced at 10 ppt) indicating that the *Microcystis* strain in the SFE ecosystem may be more halotolerant than other strains. Further investigation of these initial findings is warranted.

Investigating the identity and toxin-producing potential of other cyanobacteria present in the estuary is critically needed. Because these cultures have been well adapted to laboratory conditions, biotic interactions can be markedly altered relative to the environment from which they were isolated. As such, the evolution and/or stability of certain algal traits can occur (Lakeman et al. 2009). *Microcystis* populations that are freshly collected from the SFE will be used to verify their usefulness for the study of responses to genuinely stressful conditions (e.g., 10 ppt and 25°C). One relevant finding is that *Microcystis* morphology was distinctly altered at the different temperature and salinity treatments. At 0 and 2 ppt, single cells were formed at 18°C compared to colonial forms at 25°C **(Table 8, Fig. 25).** 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 certain morphological forms (single cells vs. colonies) of *Microcystis* that predominate in habitats at different salinity gradients.

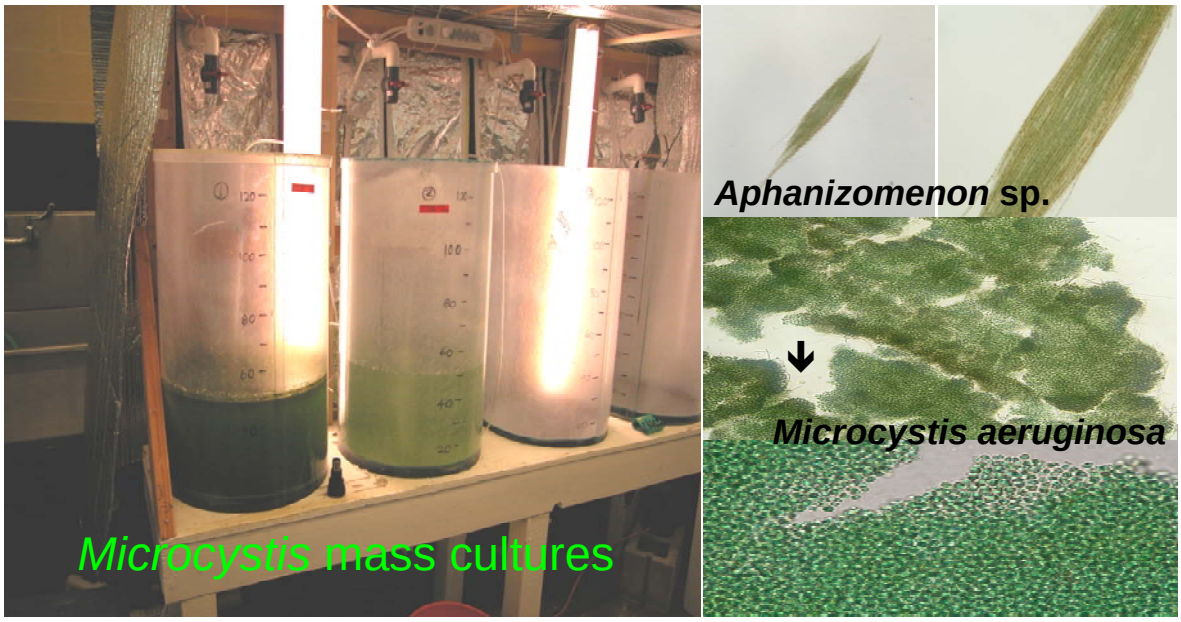

Figure 23. *Microcystis* cultures at UC Davis Aquatic Toxicology Program. Other cyanobacteria such as *Aphanizomenon* and filamentous bacteria (arrow) were collected along with *Microcystis* from the San Francisco Estuary in 2009.

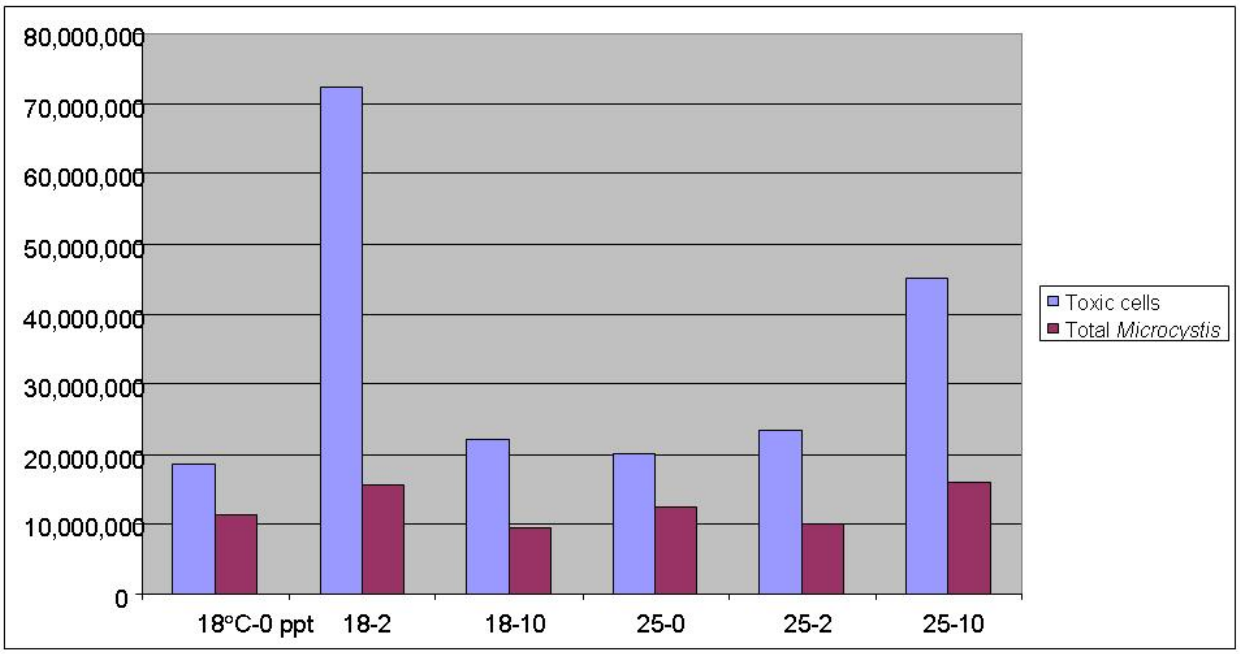

Figure 24. Effect of salinity and temperature on the mean abundance of toxin-producing cells and total *Microcystis* using local *Microcystis* cultures propagated in the laboratory.

Table 8. *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

Figure 25. *Microcystis* morphology showing the predominance of single cells at 0 and 2 ppt at 18°C compared to colonial forms at 25°C. The opposite was observed at 10 ppt: single cells at 25°C and colonial forms at 18°C.

General Summary

1. Determining the effects of *Microcystis* and MCs on early life stages (embryos and larvae, Tasks 2.1 and 2.2) could not be conducted because adult TFS were lacking during several collection dates from known distribution sites in the SFE in 2008, 2009, and 2010 and from facilities that raise TFS. In addition, we were unable to locate field spawned embryos even with the help of personnel from the Department of Fish and Game, Department of Water Resources, and the Bureau of Reclamation. Only one batch of embryos was collected in June 2010 after many attempts of collecting TFS in the SFE unfortunately, early progenies did not survive longer than one week after hatching in the laboratory. Larvae did not thrive due to poor feeding or stress from poor water quality. Several trials to induce spawning of captive broodstock did not result in the production of viable larvae.

2. Exposures to low (4.4 ppm) and high (10 ppm) concentrations of MC-LR spiked diets demonstrated adverse effects in juvenile TFS as outlined below:

- Growth – severe loss of body weight and muscle degeneration (cachexia) and decreased condition factor
- Reproduction – impaired gonad development in female TFS
- Histopathology – presence of liver lesions associated with MC toxicity such as cytoplasmic eosinophilic droplets or proteinaceous materials, glycogen depletion, single

cell necrosis and sinusoidal congestion. These pathologies are consistent with laboratory exposures of medaka (Deng et al. 2009) and Sacramento splittail (Acuña et al. in preparation) to MCs.

- Immunohistochemistry – presence of MC-LR was confirmed in the intestines, liver and kidney of TFS receiving MC-LR-spiked diets including wild TFS collected from the SFE.
- Nutrition – impaired nutritional status as indicated by histopathological changes and severe cachexia.
- *In situ* hybridization demonstrated the localization of *Microcystis* in stomach and intestines of TFS fed with MC/*Microcystis*-spiked diets including the localization of *Microcystis* cells in the gut contents of TFS collected from several sites in the SFE

3. Field investigations involved the collection of immature sub-adult TFS that were evaluated for general health using condition factor, nutritional status, and histopathology. Based on these criteria, TFS in Sherman Island (SI) were found to be relatively healthier compared to TFS from Stockton (STK) and Brannon Island (BI). While TFS from STK and BI were generally of poor health, significant lesions were observed in TFS from STK that are highly indicative of exposures to contaminant stressors than to *Microcystis* toxicity. In contrast, TFS in BI showed severe intestinal epithelial cell necrosis and the localization of MCs in liver (IHC) and *Microcystis* in stomach and intestine (ISH) strongly indicating effects of *Microcystis* intoxication. The TFS examined from these sites were immature sub-adult based on length and gonadal development. These results suggest that the general health status of TFS from the SFE may be due to the additive effects of *Microcystis* and contaminants.

Immunohistochemistry confirmed the presence of MC (LR) in the intestines, liver and kidneys of TFS collected from the SFE. The presence of MC-LR in these organs proves that MCs are absorbed in the intestines and accumulate in the liver and kidney. These results were further confirmed by the localization of ingested *Microcystis* in the gut of wild TFS (as demonstrated by in situ hybridization) collected during the 2007 blooms in the SFE.

4. In the field studies, *Microcystis* were collected from the different sites, in addition to TFS, that allowed us to develop a real-time quantitative PCR (qPCR) to estimate the spatial and temporal variations of toxic and nontoxic *Microcytis* and to demonstrate the ingestion of *Microcystis* by zooplankton.

- The development of qPCR based on local *Microcystis* gene sequences enabled us to confirm the ability of zooplankton to ingest *Microcystis* in the field. Further, the molecular tools can be used to predict and quantify the abundance of *Microcystis* in food web organisms of the SFE and then verified by standard immunohistochemistry (using commercial MC-LR antibodies) to localize MC-LR in target tissues.
- The qPCR provided a specific detection and quantification of the relative dominance of toxic and non toxic *Microcystis* that zooplankton can ingest in the SFE.

5. The adverse effects of MCs as observed in TFS have been observed in other species such as the Sacramento splittail (Acuña et al. in preparation), the quart medaka (Deng et al. 2010), key copepods (Ger et al. 2009a, b), and inland silverside and striped bass (Lehman et al. 2010). Taken together, these findings demonstrate the potential threats of *Microcystis* to important organisms in the SFE food web.

Justification for request of funding extension

We would like to request a one-year cost extension to this project. The absence of TFS in the SFE and from culture facilities in 2008, 2009, and 2010 hampered our ability to complete Tasks 2.1 and 2.2 (effect of *Microcystis*/MCs to TFS embryo and larvae). Adult spawners were not found in the SFE despite enormous assistance from Mr. Kevin Reece and Dr. Ted Sommer (DWR), Mr. Teejay Orear (Dr. Moyle at UCD), Dr. Louise Conrad (Dr. Sih at UCD), and several personnel at DFG. Our best efforts to develop TFS broodstock for the last two years did not successfully generate viable embryos and larvae.

Since we have tried extensively but were unable to find any TFS for reasons beyond our control, funding for one more year will provide us with the resources to conduct additional studies to address the adverse effects of *Microcystis* on early life stages of TFS. Alternative techniques for spawning captive TFS at the Contra Costa Mosquito and Vector Control and acquiring embryos from the SFE are promising. Utilizing these techniques in the 2011 spawning season will allow us to acquire the embryos and larvae needed to conduct Tasks 2.1 and 2.2. Because larval development corresponds with peaks of cyanobacterial blooms in the SFE, investigating the effects of *Microcystis* on early life stages of TFS is critical to understanding the range of effects that can be determined under field and laboratory conditions.

Cost extension will also ensure further investigation on the distribution and abundance of toxic and nontoxic *Microcystis* in impacted sites at the SFE. Because toxic *Microcystis* were found to be relatively more abundant in Antioch in 2007, the underlying factors promoting the increased growth rates of toxic *Microcystis* in this habitat need to be addressed. Importantly, the potential links between the general health of TFS and other pelagic species and zooplankton laden with *Microcystis* remain poorly understood. The existing tools that we developed (qPCR/PCR, immunohistochemistry, in situ hybridization) will help elucidate the potential relationships between shifts in *Microcystis* abundance and toxicity, MC concentrations, and toxic effects to zooplankton and fish in controlled laboratory and ecosystem scales.

Proposed Work to Address Future Challenges

Study 1: Comprehensive characterization of key cyanobacterial toxin producers and green algae in the SFE using DNA microarray and specific qPCR

Objective: Identify and quantify the dominant "bad algae" (toxin-producing cyanobacteria) and "good algae" (nutritious green algae) comprised within and outside of the bloom season in the SFE.

Approach: We will extend previous PCR/qPCR methods for *Microcystis* to develop DNA microarray and specific qPCR assays to provide a qualitative (DNA array) and quantitative (qPCR) estimation of other key cyanobacteria (based on 16S rDNA and *mcy*D toxin gene sequences) and nutritious green algae (16S rDNA sequences) in the SFE.

Rationale: Toxicity of recurring blooms in the SFE has been mainly attributed to *M. aeruginosa* producing various microcystin congeners that may have potential links to declining numbers of pelagic organisms (i.e. POD). Bloom toxicity in the SFE may not be due solely to *M. aeruginosa* but may also arise through the association of other toxin-producing cyanobacterial strains as demonstrated in our pilot study (see page 39). By developing standard PCR and qPCR techniques specific to *Microcystis* found in the SFE, we were able to demonstrate a tiered analysis on the identification and quantification of toxin-producing versus nontoxin-producing *Microcystis* from cyanobacterial tissues and water samples during the bloom season in 2007. Following initial cultures of local *Microcystis* assemblages in our laboratory, the number of toxin producing cells was attributed to not only *Microcystis* but to potentially other co-existing cyanobacterial species such as *Aphanizomenon* and other filamentous cyanobacteria. For this reason, a qualitative (DNA microarray) and quantitative (qPCR) assessment of toxin producing cyanobacteria (in addition to *Microcystis*) including nutritious green algae in the delta will provide critical information on the contribution of other cyanobacteria to bloom toxicity and the relevance of green algae as food sources to larval species. Furthermore, these techniques can monitor the composition and abundance of nutritious green algae before and after the occurrence of harmful algal blooms.

Study 2: Estimating the presence and abundance of *Eurytemora affinis* and *Pseudodiaptomus forbesi* in ambient surface waters and in gut contents of larval species in the SFE

Objective: Evaluate the relevance of the key copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* as food sources to larval species in the SFE.

Approach: Develop specific qPCR assays to identify and quantify the copepods by utilizing their 18S rDNA sequences.

Rationale: Calanoid copepods, particularly *E. affinis* and *P. forbesi*, are the principal food sources of many endangered larval and pelagic fishes in the SFE. Copepod abundance varies spatially and seasonally in certain habitats of the upper SFE. Copepods have similarly declined to unprecedented numbers along with other pelagic organisms such as fish and nutritious phytoplankton. Because they contain essential fatty acids, the calanoid copepods provide greater nutritional quality to primary consumers compared to others (Mueller-Solger et al. 2006). As such, developing specific and sensitive techniques such as qPCR to demonstrate the potential link between the presence and abundance of these copepods in certain habitats and the growth and survival of larval species that rely upon the zooplankton as food sources is essential.

Study 3: Field studies to evaluate the potential impacts of exposure to contaminants (metals, pesticides) and *Microcystis* on fish health

Objective: Determine the general health status of key pelagic fish species due to the additive effects of exposures to contaminants and *Microcystis* in the SFE

Approach: Fish health will be evaluated and established by linking four measurements of biological effects: 1) Condition factor and organo-somatic indices, 2) Biochemical and histopathological biomarker responses as indicators of contaminant exposure and effects, 3) Isolation and identification of pathogens/diseases using specific cell lines for virus isolation, and 4) Proximate analyses to determine fish nutritional status.

Rationale: Baseline information on the health of native and threatened fish species in the upper San Francisco Estuary is currently lacking. Indicator species such as the threadfin shad (*Dorosoma petenense*) and the endemic Sacramento splittail (*Pogonichthys macrolepidotus*) are exposed to contaminants and *Microcystis* during critical life stages (i.e. spawners and larvae). Linking biological effects and fish health is warranted because key stressors may ultimately determine fundamental fish functions such as growth and reproduction. Baseline health status is essential for assessing the health of the targeted species over time that may be altered due to emerging risk factors affecting the Delta and its fishery resources.

Study 4: Laboratory studies to evaluate the potential relationship between exposures to environmental contaminants (metals, pesticides) including *Microcystis* and health of key pelagic and other economically important fish species

Objective: Determine how exposure to contaminants and *Microcystis* may impact the general health of critical life stages of at risk fish species (e.g. delta smelt, longfin smelt, Sacramento splittail, green sturgeon, and Chinook salmon)

Approach: Adverse effects of exposure to contaminants and *Microcystis* will be evaluated and established by 1) determining toxic biological effects on liver and gonad development, histopathology to assess lesion severity in embryo and larval stages, biochemistry including immunohistochemistry and in situ hybridization to assess evidence of contaminant exposure, 2) proximate analyses of major storage forms (glycogen, lipid, protein) to assess nutritional adequacy as a result of contaminant and *Microcystis* exposure, and 3) body burden analysis or Toxicity Identification and Evaluation (TIE) to identify chemical or chemical mixtures of interest.

Rationale: We have demonstrated that exposure to *Microcystis* in TFS (see **Task 2.3**) and to selenium and pesticides in Sacramento splittail (Teh et al. 2005, CALFED Final Report) can impair growth and reproduction. MC toxicity targets the liver, affects reproductive potential of adult species including direct toxicity to embryos and larvae. As most of the POD species have an average of two-year life span, concurrent exposures to certain contaminants and to *Microcystis* may render severe reproductive abnormalities that may ultimately affect growth and population condition (recruitment). Controlled laboratory studies will be conducted based on field data (Study 3) to determine the effect of the contaminant of interest and of *Microcystis* on fish including potential impacts on key zooplankton species (i.e. *Pseudodiaptomus forbesi* and *Eurytemora affinis*).

Literature Cited

Acuña S, Deng DF, Lehman P, Teh S. Dietary effects of *Microcystis* in Sacramento splittail, *Pogonichthys macrolepidotus*, in preparation

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: 342 – 349

Carbis CR, Rawlin GT, Mitchell GF, Anderson JW, McCauley I, 1996. The histopathology of carp, *Cyprinus carpio* L. exposed to MCs by gavage, immersion and intraperitoneal administration. Journal of Fish Diseases 19: 199 – 207

Deng DF, Zhang K, Teh FC, Lehman PW, Teh SJ, 2009. Toxic threshold of dietary microcystin (-LR) for quart medaka. Toxicon 4: 787 – 794

Feyrer F, Sommer T, Slater SB, 2009. Old school vs new school: Status of threadfin shad (*Dorosoma petenense*) decades after its introduction to the Sacramento-San Joaquin Delta. San Francisco Estuary and Watershed Science 7: 1 – 12

Ger KA, Teh SJ, Goldman CR, 2009a. Microcystin-LR toxicity on dominant copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* of the upper San Francisco Estuary. Science of the Total Environment 407: 4852 – 4857

Ger KA, Teh SJ, Baxa DV, Lesmeister S, Goldman CR, 2009b. The effects of dietary *Microcystis aeruginosa* and microcystin on the copepods of the upper San Francisco Estuary. Freshwater Biology 55: 1548 – 1559

Kaebernick M, Dittmann E, Börner T, Neilan BA, 2002. Multiple alternate transcripts direct the biosynthesis of microcystin, a cyanobacterial nonribosomal peptide. Applied and Environmental Microbiology 68: 449– 455

Kaneko T and 22 other co-authors, 2007. Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843. DNA Research 14: 247 – 256

Lakeman MB, Dassow PV, Cattolico RA, 2009. The strain concept in phytoplankton ecology. Harmful Algae 8: 746 – 758

Lehman PW, Waller S. 2003. *Microcystis* blooms in the Delta. Interagency Ecological Program for the San Francisco Estuary Newsletter 16: 18 – 19

Lehman, P.W., Boyer, G., Hall, C., Waller, S., Gehrts, K., 2005. Distribution and toxicity of a new colonial *Microcystis aeruginosa* bloom in the San Francisco Bay Estuary, California. Hydrobiologia 541: 87 – 99

Lehman, P.W., Boyer, G., Satchwell, M., Waller, S., 2008. The influence of environmental conditions on the seasonal variation of *Microcystis* cell density and microcystins concentration in San Francisco Estuary. Hydrobiologia 600: 187 – 204

Lehman PW, Teh SJ, Boyer GL, Nobriga ML, Bass E, Hogle C, 2010. Initial impacts of *Microcystis aeruginosa* blooms on the aquatic food web in the San Francisco Estuary Hydrobiologia, 637: 229 – 248

Mezhoud K, Bauchet AL, Chateau-Joubert S, Praseuth D, Marie A, Francois JC, Fontaine JJ, Jaeg JP, Cravedi JP, Puiseux-Dao S, Edery M, 2008. Proteomic and phosphoproteomic analysis of cellular responses in medaka fish (*Oryzias latipes*) following oral gavage with microcystin-LR. Toxicon 51: 1431 – 1439

Moyle PB 2002. Inland Fishes of California; University of California Press: Berkeley, CA,

Mueller-Solger A, Hall C, Jassby A, Goldman C, 2006. Final Report: Food resources for zooplankton in the Sacramento-San Joaquin River Delta. CALFED

Rinta-Kanto JM, Ouellette AJ, Boyer GL, Twiss MR, Bridgeman TB, Wilhelm SW, 2005. Quantification of toxic *Microcystis* spp. during the 2003 and 2004 blooms in western Lake Erie using quantitative real-time PCR. Environmental Science and Technology 39: 4198 – 4205

Sommer T and 13 other co-authors, 2007. The collapse of pelagic fishes in the upper San Franciso Estuary. Fisheries 32: 270 – 277

Tencalla FG, Dietrich DR, Schlatter, C. 1994. Toxicity of *Microcystis aeruginosa* peptide toxin to yearling rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 30: 215 – 224

World Health Organization (WHO), 1999. Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management. Routledge: London and New York.

Zurawell RW, Chen H, Burke JM, Prepas EE, 2005. Hepatoxic cyanobacteria: A review of the biological importance of microcystins in freshwater environments. Journal of Toxicology and Environmental Health 8: 1 – 37

Publications that were generated from the TFS project

Note: PDFs are included in the appendix

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: 342 – 349

2. Ger KA, Arneson P, Goldman CR, Teh SJ, 2010. Species specific differences in the ingestion of *Microcystis* cells by the calanoid copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi*. Journal of Plankton Research 32: 1479 – 1484

3. Deng DF, Zhang K, Teh FC, Lehman PW, Teh SJ, 2009. Toxic threshold of dietary microcystin (-LR) for quart medaka. Toxicon 4: 787 – 794

4. Ger KA, Teh SJ, Goldman CR, 2009a. Microcystin-LR toxicity on dominant copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* of the upper San Francisco Estuary. Science of the Total Environment 407: 4852 – 4857

5. Ger KA, Teh SJ, Baxa DV, Lesmeister S, Goldman CR, 2009b. The effects of dietary *Microcystis aeruginosa* and microcystin on the copepods of the upper San Francisco Estuary. Freshwater Biology 55: 1548 – 1559

6. Acuña S, Deng DF, Lehman P, Teh S. Dietary effects of *Microcystis* in Sacramento splittail, *Pogonichthys macrolepidotus*, in progress

Author's personal copy

Harmful Algae 9 (2010) 342–349

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Estimating the abundance of toxic *Microcystis* in the San Francisco Estuary using quantitative real-time PCR

Dolores V. Baxa [a,*], Tomofumi Kurobe [a], Kemal A. Ger [a], Peggy W. Lehman [b], Swee J. Teh [a]

[a] School of Veterinary Medicine, Department of Anatomy, Physiology and Cell Biology, University of California, One Shields Avenue, Davis, CA 95616, USA
[b] Division of Environmental Sciences, Department of Water Resources, 3500 Industrial Blvd, West Sacramento, CA 95691, USA

ARTICLE INFO

Article history:
Received 4 August 2009
Received in revised form 17 November 2009
Accepted 4 January 2010

Keywords:
*mcy*D gene
Microcystis
Microcystin
qPCR
San Francisco Estuary
Toxic strains

ABSTRACT

Developing an effective and rapid method to identify and estimate the abundance of *Microcystis* is warranted in the San Francisco Estuary (SFE) in view of expanding cyanobacterial blooms dominated by *Microcystis* spp. Blooms that occurred in the estuary from July to September 2007 were initially assessed using a standard polymerase chain reaction (PCR) employing primers designed for the conserved *Microcystis*-specific 16S ribosomal DNA (rDNA) region. The presence of microcystin-producing (MC+) toxic *Microcystis* was observed in cyanobacterial and water samples as shown by the amplification of the MC toxin synthetase genes *mcy*B and *mcy*D by standard PCR. The goal of this study was to develop 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. 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. Total *Microcystis* was also abundant (7.7×10^4 to 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 to 2.2×10^7 cells L^{-1}) indicated a large variation in the ratio of toxic *Microcystis* among total *Microcystis* (0.01–27%). Differences in the proportion of toxic and nontoxic *Microcystis*, as deduced from the cell equivalents of total *Microcystis*, were observed across the sampling locations and seasons where concentrations of total MCs (0.007–10.81 μg/L) also varied. By revealing trends in the sources and magnitude of toxic and nontoxic *Microcystis*, qPCR can contribute to rapid risk assessment and prediction of strategies designed to manage the adverse effects of cyanobacterial blooms in the SFE.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Cyanobacteria, also known as blue-green algae can form blooms that produce toxins, cause oxygen depletion, alter food webs, and threaten freshwater bodies worldwide that are utilized for drinking, agriculture, fishing, and recreation (Paerl and Huisman, 2009). Toxic blooms in temperate freshwater environments are commonly dominated by *Microcystis* which produce the hepatotoxin, microcystin (Carmichael, 1996; Chorus and Bartram, 1999; Paerl, 2008). Microcystins (MCs) are cyclic heptapeptides produced mainly by cyanobacterial species belonging to the genera *Microcystis*, *Anabaena*, *Nostoc*, and *Oscillatoria* (Sivonen and Jones, 1999; Nishizawa et al., 2000). The peptide synthetase gene cluster is a bidirectional complex of 10 genes, *mcy* (A–J), which control the synthesis of polyketide and peptide synthetases associated with MC synthesis (Tillett et al., 2000; Pearson et al., 2004). Only MC producing (MC+) cyanobacteria carry the *mcy* genes, and genetic differences within this gene cluster determine the production of amounts and congeners of MCs (Pearson et al., 2004). Chronic exposure to cyanotoxins such as MCs causes widespread and serious health problems in animals and humans including liver, digestive and skin diseases, neurological impairment, and death (Carmichael, 2001; Cox et al., 2003). Although the worldwide occurrence of cyanobacterial blooms has increased in recent decades (Chorus and Bartram, 1999; Hudnell and Dortch, 2008), the underlying factors associated with their toxicity and the impact to ecosystems are poorly understood.

Since their initial detection in 1999, cyanobacterial blooms occur annually between June and October in the upper San Francisco Estuary (SFE) across a wide range of salinities (0.1–18 ppt), from the low-flow waters of the central SFE to the western reaches including the Sacramento River (Lehman et al., 2005, 2008). The blooms are composed primarily of colonial forms of *Microcystis aeruginosa* although single cells are also present. Total

* Corresponding author. Tel.: +1 530 754 8020; fax: +1 530 752 7690.
E-mail address: dvbaxa@ucdavis.edu (D.V. Baxa).

1568-9883/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.hal.2010.01.001

Author's personal copy

MC concentrations in the estuary 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). Various MC congeners are present in the SFE such as LR, LA, and WR that vary in dominance spatially and temporally (Lehman et al., 2005, 2008).

One way *Microcystis* blooms may be detrimental to the SFE is their potential role in the decline of pelagic organisms referred to locally as POD (Sommer et al., 2007; IEP, 2008). Increased levels of MCs during seasonal blooms are hypothesized to cause adverse effects in food resources of pelagic species through direct toxicity or indirectly through impacts on the food web (IEP, 2008; Lehman et al., 2008), which may lead to bioaccumulation of toxins at higher trophic levels particularly fish (Malbrouck and Kestemont, 2006). Variations in the abundance of MC+ *Microcystis* and MC concentrations among locations in the SFE have important ecological implications. For example, liver lesions suggestive of exposure to MCs were observed in striped bass *Morone saxatilis* collected at Antioch in 2005 and 2007 (Lehman et al., 2010). While the potential mechanism of toxin exposure to the striped bass is unknown, fish in the estuary may ingest MC+ phytoplankton and/or zooplankton directly or accidentally while feeding (Vanderploeg et al., 2001; Sedmak and Elersek, 2005; Malbrouck and Kestemont, 2006). In addition, *Microcystis* is an unsuitable food to zooplankton due to its nutritional inadequacy, toxicity, and colonial aggregation (Carmichael, 1996; Hessen et al., 2006; Wilson et al., 2006) that can negatively affect feeding, growth, and reproduction (Kirk and Gilbert, 1992). Survival of dominant zooplankton species in the SFE was reduced following experimental exposures to dissolved MCs and dietary *Microcystis* (Ger et al., 2009, in press). Preliminary *in situ* hybridization analysis also showed the localization of *Microcystis* DNA in the gut and body surface of the zooplankton (Baxa, unpublished data). These findings indicate the need for rapid and accurate diagnosis of *Microcystis* abundance, toxicity and species-specific food web interactions in the SFE using advances in DNA-based molecular tools (Ouellette and Wilhelm, 2003; Nejstgaard et al., 2008).

Although MC concentrations are known to be produced mainly from *Microcystis* populations in the SFE (Lehman et al., 2005, 2008), the proportion of *Microcystis* and other cyanobacterial species that produce the toxin is unknown. It is also unknown how environmental conditions influence MC production in the estuary. The presence of nutrients, light, water temperature and flow, and zooplankton grazing can influence the growth of algae, the onset and development of blooms and the production of MCs (Sivonen and Jones, 1999; Jacoby et al., 2000; Zurawell et al., 2005; Paerl, 2008). For the SFE, research suggests that low water flow is strongly correlated with the production of higher cellular MC concentration (Lehman et al., 2008). Although environmental parameters may affect toxicity by an order of magnitude, the predominance of toxic strains can affect toxicity to 1000 fold (Zurawell et al., 2005). As the proportion of toxic genotypes may determine the overall toxicity of blooms (Kardinaal et al., 2007a), predicting bloom toxicity requires the ability to determine the occurrence and often co-existence of toxic and nontoxic strains of the same species within a genus that are morphologically and taxonomically indistinguishable (Otsuka et al., 1999; Kurmayer et al., 2002).

Real-time quantitative PCR has been successfully applied in toxicity assessments of recurring phytoplankton blooms (Kurmayer et al., 2002; Vaitomaa et al., 2003; Hotto et al., 2008; Pearson and Neilan, 2008; Rinta-Kanto et al., 2005, 2009). While molecular analysis of cyanobacteria in the SFE using the toxin synthetase *mcy*A gene has identified toxic species of *Microcystis* (Moisander et al., 2009), neither the abundance nor the dominance of MC+ *Microcystis* has been quantified in the SFE. The occurrence of cells carrying specific gene targets for cyanobacteria particularly *Microcystis* cells carrying the genes associated with the production of MC toxin was assessed using conventional PCR. Our study focused on developing a qPCR assay to estimate the ratio of MC+ *Microcystis* based on the proportion of *Microcystis* with *mcy*D genes and the abundance of total *Microcystis* to provide an overview of the toxicity of blooms that occurred in the SFE from July to September 2007.

2. Materials and methods

2.1. Study sites and collection of samples

In collaboration with the CA Department of Water Resources, cyanobacterial samples were collected from 7 stations in the SFE including Brannan Island and Chipps Island in the Sacramento River; Antioch, Mildred Island, Old River, San Joaquin, and Venice Cut in the San Joaquin River (Fig. 1). Samples were collected intermittently from these sites between July 24 and September 18, 2007 when *Microcystis* blooms were abundant across the estuary.

Cyanobacterial samples were collected using diagonal tows with a plankton net (153 μm mesh). This collection procedure provided a representative example of large *Microcystis* colonies that may be dispersed in the water column. To include colonies and single cells from ambient surface waters which can escape the plankton net, samples were collected by dipping 2 L amber HDPE bottle (Fisher, PA, USA) on the water surface. Plankton samples from tow nets were stored in acid washed containers while the surface samples were retained in their containers (acid washed). Samples were kept at 4 °C and filtered within 4 h onto a 0.5-μm GF/F Whatman filter (Whatman, Maidstone, UK). For cellular MC analysis, plankton samples from the net tows were filtered onto 0.5-μm GF/F glass fiber filters (Whatman). Filtered plankton samples were wrapped in aluminum foil and frozen at −80 °C until used for DNA extraction and total MC analysis.

2.2. Microcystin (MC) analysis

Toxins from cyanobacterial samples were extracted from the filter by sonication (3 times, 20 s bursts on ice) with 10 ml of 50%

Fig. 1. Map of San Francisco Estuary showing the locations for collecting cyanobacterial samples during the bloom season in 2007.

Author's personal copy

methanol containing 1% acetic acid, and the extract clarified by centrifugation and used for MC analysis. Recovery of MCs (e.g., LR, RR) using this procedure is greater than 90% as determined using duplicate samples. Concentrations of total MCs in cyanobacterial samples were determined using the protein phosphatase inhibition assay, PPIA (Carmichael and An, 1999). Briefly, assays were run in 96-well plates containing 0.1 mU recombinant phosphatase 1A catalytic subunit (Roche Applied Science, Indianapolis, IN), 1.05 mg para-nitrophenyl phosphate (Sigma, St. Louis, MO) and 10 μl of sample or *Microcystis aeruginosa* MC-LR (Sigma) as previously described (Lehman et al., 2008).

2.3. Extraction of genomic DNA

Cyanobacterial biomass showed an even distribution on the filters following filtration of samples from net tows and surface waters. Biomass from the net tows was generally denser of which only half filter was used for genomic DNA extraction. In contrast, cyanobacteria from surface waters were relatively rare in which the entire filter was used for DNA extraction. In both cases, the number of filtered biomass fractions and the volume of filtered water were factored in estimating the DNA concentration. After thawing the frozen filters at room temperature, plankton biomass was removed aseptically from the filter using forceps and processed for genomic DNA extraction using a standard phenol–chloroform procedure (Sambrook and Russell, 2001) combined with a phenol extraction method used in qPCR analysis of plankton (Rinta-Kanto et al., 2005). Briefly, plankton cells were suspended in 100 μl lysis buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.2% sodium dodecyl sulfate), and added proteinase K (Qiagen Inc., Valencia, CA) to a final concentration of 50 μg/ml. The volumes of lysis buffer and proteinase K were doubled in dense plankton biomass from the net tows. The suspension was placed overnight in a shaking incubator at 56 °C. Genomic DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma), following to ethanol precipitation. DNA was dissolved in 100 μl of Tris HCl pH 8.0–EDTA (TE) buffer and the concentration was measured with a spectrophotometer (BioPhotometer plus, Eppendorf, NY). Some of the DNA samples were added TE buffer to obtain ≤400 ng/μl of concentrations.

2.4. Conventional PCR

2.4.1. Detection of cyanobacteria, Microcystis and MC synthetase genes

Genomic DNA of cyanobacterial samples were initially examined by conventional PCR to demonstrate the presence of cyanobacteria and *Microcystis* using specific PCR primer sets targeting the 16S rDNA (Neilan et al., 1997; Urbach et al., 2001). To establish cyanobacterial toxicity, primer sets designed for the MC synthetase genes, *mcy*B and *mcy*D, were used to detect MC+ *Microcystis* (Kaebernick et al., 2000; Ouellette et al., 2006). The targets, primer sequences, amplicon size and references are listed in Table 1. The volume of the PCR cocktail was 50 μl containing 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 40 pmol of each primer, 1.5 unit Platinum Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA) and 10× buffer at 1/10 the volume of the reaction. The PCR conditions for the conventional PCR are as follows: initial denaturation step of 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s, followed by a final extension step at 72 °C for 5 min and then held at 4 °C. The PCR product was separated on 2% agarose gels and observed by a transilluminator after staining with 1% ethidium bromide solution for 20 min. Genomic DNA extracted from *M. aeruginosa* producing MC toxin, MC+ (MC-LR UTEX 2385) and non-MC producing *M. aeruginosa*, MC− (MC-LR UTEX 2386) (University of Texas Culture Collection, Austin, TX) were used as controls for the initial screening of mixed cyanobacterial samples to detect the target genes by conventional PCR. Both type strains of *M. aeruginosa* were grown in axenic batch cultures in our laboratory using a modified ASM-1 medium maintained at 22 °C and 16:8 L:D light regime (Ger et al., in press).

2.4.2. Cloning, sequencing and sequence analysis of PCR amplicons

The amplicons were ligated into pGEM-T Easy vector (Promega BioSciences, San Luis Obispo, CA) that was used to transform *Escherichia coli* DH5α competent cells (Invitrogen Corp., Carlsbad, CA). The plasmid containing the inserted DNA fragment was extracted using QIAprep Spin Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The sequence of the insert was confirmed using M13 forward and reverse primers by fluorescently labeled dideoxy terminator sequencing using an ABI 377 automated DNA sequencer (Applied Biosciences, Foster City, CA). Gene sequences were determined from the plasmid DNA of 10 plankton samples from the 7 locations in the SFE. The sequences were aligned and compared to available sequences of *Microcystis*, gene clusters encoding the biosynthetic enzymes *mcy* (microcystin) and other peptide synthetase genes in GenBank database using Clustal W Program Version 1.83 (Thompson et al., 1994).

2.5. Real-time quantitative PCR (qPCR)

2.5.1. Plasmid preparation for qPCR

The plasmid DNA carrying the target sequence for cyanobacteria, *Microcystis* 16S rDNA, and *mcy*D were prepared as follows: the partial fragment of the 16S rDNA from cyanobacteria (1200 bp), the *Microcystis* 16S rDNA fragment (230 bp), the *mcy*B (320 bp) and *mcy*D (298 bp) synthetase gene fragments were amplified with forward and reverse primer sets listed in Table 1. The genomic DNA of 10 plankton samples from the 7 locations in the SFE was used as template DNA while MC+ *M. aeruginosa* UTEX 2385 and MC− *M. aeruginosa* UTEX 2386 were used as control DNA template. The PCR assay and conditions were conducted as described in Section 2.4.1, the amplicons were cloned into a vector, and the plasmid DNA was extracted as described above. The obtained sequences were deposited in GenBank database (Accession nos. GQ426031, GQ426032). The copy number of the plasmid DNA was calculated according to its molecular weight and concentration, and then converted into the copy number based

Table 1
Primers used for conventional PCR to amplify cyanobacteria, *Microcystis* and microcystin synthetase genes, *mcy*B and *mcy*D, from cyanobacteria in the San Francisco Estuary.

Target	Primer	Sequence (5′–3′)	Target size	Reference
Cyanobacteria 16S rRNA	CYA 108 F	ACG GGT GAG TAA CRC GTR A	1200 bp	Urbach et al. (2001)
	CYA16SCYR	CTT CAY GYA GGC GAG TTG CAG C		
Microcystis 16S rRNA	MIC 184 F	GCC GCR AGG TGA AAM CTA A	230 bp	Neilan et al. (1997)
	MIC 431 R	AAT CCA AAR ACCT TCC TCC C		
*Microcystis mcy*B	mcyB 2959 F	TGG GAA GAT GTT CTT CAG GTA TCC AA	320 bp	Nonneman and Zimba (2002)
	mcyB 3278 R	AGA GTG GAA ACA ATA TGA TAA GCTA C		
Microcystis	mcyD F2 F	GGT TCG CCT GGT CAA AGT AA	298 bp	Kaebernick et al. (2000)
	*mcy*D R2 R	CCT CGC TAA AGA AGG GTT GA		

Author's personal copy

on Avogadro's number (1 mol = 6.022×10^{23} molecules). Serial dilutions of the plasmid DNA (1×10^6 through 10 copies/5 μl) were prepared with molecular grade water and used for generation of the standard curve for cyanobacteria, *Microcystis*, and *mcy*D qPCR assays.

2.5.2. Development of real-time quantitative PCR (qPCR) for Microcystis

The TaqMan probe and forward and reverse primers for *Microcystis* were designed with the Primer Express 3.0 software (Applied Biosystems) using the 16S rDNA sequences obtained from cyanobacterial samples from the SFE. These sequences were compared against the unique region of *Microcystis* based on alignments with other cyanobacterial species such as *Anabaena* sp. (AJ133160) and *Nostoc* sp. (AB187508). The TaqMan probe was labeled with the fluorescent reporter FAM at the 5′ end and with the quencher minor groove binding (MGB) at the 3′ end. The PCR contained 400 nM of each primer, 80 nM of the TaqMan probe, 2× TaqMan Universal Master Mix with UNG (Applied Biosystems, Foster City, CA), and 5 μl of the extracted DNA samples in a final volume of 12 μl. The samples and plasmid DNA standards were placed in MicroAmp fast optical 48-well reaction plate and sealed with MicroAmp 48-well optical adhesive film (Applied Biosystems). The qPCR assays were run using a StepOne Real-Time PCR System (Applied Biosystems). The PCR program for cyanobacteria and *Microcystis*-specific 16S rDNA was conducted according to conditions suggested in the StepOne qPCR machine: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. For the *mcy*D assay, thermocycling steps were followed from Rinta-Kanto et al. (2005): 50 °C for 3 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 30 s, 61 °C for 1 min, and 72 °C for 20 s.

Calculations of threshold cycle (*C*t) were conducted using the StepOne Version 2.0 Software (Applied Biosystems), which automatically determined the highest possible correlation coefficient (r^2) for the standard curve. *Microcystis* has two copies of 16S rDNA in its genome (Kaneko et al., 2007) therefore the gene copy number obtained by qPCR based on the plasmid standard curve was divided by 2 to determine the total *Microcystis* MC-LR cell equivalents. Reactions were run in triplicate and results are reported as mean ± SD. The number of MC− *Microcystis* cells were estimated by subtracting the cell number of MC+ *Microcystis* (*mcy*D carriers) from the total number of *Microcystis* cells (MC+ and MC− strains) (Rinta-Kanto et al., 2005).

For samples used in the current study, dense plankton biomass were collected from the tow nets rendering prolonged filtration, from which colonial forms were randomly picked in some samples. As water volumes were not used from these net tow samples, the qPCR values were expressed only as gene copies/μg DNA. In contrast, surface water samples represent ambient concentrations of colonial and single cells with less plankton biomass thus, the *Microcystis* cell density was estimated as the number of cell equivalents L^{-1} based on the volume of samples collected.

2.5.3. Specificity and sensitivity of TaqMan real time qPCR assay for Microcystis 16S rDNA and mcyD

The specificity of the qPCR assays was verified by running reactions using genomic DNA from pure cultures of cyanobacterial species including *Synechoccus* sp., *Planktothrix* sp., *Microcystis aeruginosa* type strains: MC+ MC-LR UTEX 2385 and PCC 7806 (from Dr. Steven Wilhelm, University of Tennessee), MC− *M. aeruginosa* (MC-LR UTEX 2386), as well as 10 different *Microcystis* spp. from California (provided by Dr. Pia Moisander, UC Santa Cruz). Approximately 50 ng of genomic DNA was used in the qPCR using procedures described above. The sensitivity of the assays was determined by comparing the standard curve generated from plasmid standards.

3. Results

Cyanobacteria and *Microcystis*-specific 16S rDNA and the MC synthetase genes *mcy*B and *mcy*D were amplified from cyanobacterial samples collected in the SFE (Fig. 2). Using pure cultures of MC+ *M. aeruginosa* (MC-LR UTEX 2385) and MC− *M. aeruginosa* (MC-LR UTEX 2386) as reference strains, *Microcystis* that amplified the *mcy*B and *mcy*D toxin genes was considered toxic and was discriminated from nontoxic *Microcystis* lacking these genes (Fig. 2). Specific primers and probes for the qPCR assay that were designed in this study (Table 2) were able to detect and quantify the 16S rDNA target genes for cyanobacteria and *Microcystis*, and the MC synthetase *mcy*D gene of high analytical sensitivity detecting as few as 10 copies of the target gene/reaction with high specificity against closely related cyanobacteria such as *Synechoccus* sp., *Planktothrix* sp. and 10 different strains of *Microcystis* spp. (data not shown). Analysis by qPCR quantified the gene copies of cyanobacteria, *Microcystis* spp. and *mcy*D from tow net (Table 3) and from ambient surface water samples (Table 5) from which gene copies were expressed as cell equivalents. As mentioned above, the lack of information on filtered biovolumes of some tow net samples precluded the conversion of gene copies to cell equivalents.

Using the qPCR assays developed in this study, variations in the cell equivalents of toxic *Microcystis* (*mcy*D gene carriers) and total *Microcystis* spp. were generally observed across the sites and sampling dates in surface water samples (Table 5). The tow net samples also showed variations in gene copies across sampling sites and dates (Table 3). Based on the estimated percentages of *Microcystis* 16S, cyanobacterial 16S, and *mcy*D gene copies, the

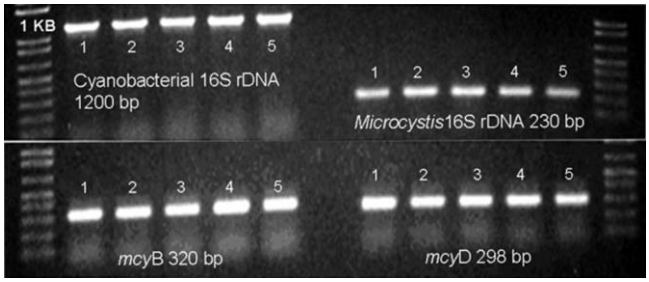

Fig. 2. Gel image of conventional PCR showing amplification of target genes and corresponding lengths (bp): cyanobacterial and *Microcystis* 16S rDNA fragments and the microcystin synthetase genes *mcy*B and *mcy*D from cyanobacterial samples in the San Francisco Estuary.

Table 2
Primers and probes for *Microcystis* spp. and cyanobacteria designed from the 16S rDNA sequences of *Microcystis* present in the San Francisco Estuary.

Target	Primer/probe	Sequence (5′ → 3′)
Microcystis 16S rRNA	MIC16S F	AAA GCG TGC TAC TGG GCT GTA
	MIC16S R	CCC TTT CGC TCC CCT AGC T
	MIC16S P[a]	CTG ACA CTC AGG GAC G
*Microcystis mcy*D	*mcy*D F	GGT TCG CCT GGT CAA AGT AA
	*mcy*D R	CCT CGC TAA AGA AGG GTT GA
	*mcy*D P[a]	ATG CTC TAA TGC AGC AAC GGC CAA A
Cyanobacteria 16S rRNA	CYA16S F	TCG CCC ATT GCG GAA A
	CYA16S R	AGA CAC GGC CCA GAC TCC TA
	CYA16S P[a]	TTC CCC ACT GCT GCC

[a] The probes were labeled with 6FAM and MGBNFQ as reporter and quencher, respectively. All primers and probes were developed in the current study except that of *mcy*D from Rinta-Kanto et al. (2005).

Author's personal copy

Table 3
Mean copy numbers of toxic *Microcystis* (*mcy*D), total *Microcystis* (16S rDNA) and cyanobacterial (16S rDNA) genes, and mean total microcystin concentrations in the San Francisco Estuary. Data refer to cyanobacteria collected by diagonal net tows.

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	$8.0 \pm 0.42 \times 10^4$	$1.6 \pm 0.05 \times 10^6$	$2.6 \pm 2.77x\ 10^6$
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.

proportion of MC+ *Microcystis* formed 0.4–20.2% of the total *Microcystis* spp., and from 0.16 to 12.5% of the total cyanobacterial community (as determined by the cyanobacterial 16S; Table 4). The abundance of MC+ *Microcystis* with *mcy*D genes was generally lower when estimated among cyanobacterial 16S rDNA genes. The mean proportion of *Microcystis* spp. with *mcy*D genes over total *Microcystis* spp. was about 2–20 times higher in Antioch, Brannan Island, Chipps Island, and Mildred Island compared to Old River, San Joaquin, and Venice Cut. Based on the calculated percentages of *Microcystis* and cyanobacterial 16S of plankton samples, *Microcystis* dominated (>50%) the cyanobacterial population with the exception of Old River (28.6%, 8/22/07), Venice Cut (40.0%, 7/25/07), Mildred Island (41.7%, 9/04/07), and Antioch (36.1%, 9/05/07) (Table 4).

In addition to colony forming cyanobacteria throughout the water column, single cells were sampled in the surface layer. The estimated cell equivalents carrying the gene targets in surface water showed that MC+ *Microcystis* and total *Microcystis* varied across the sites and phases of the bloom development (Table 5). On the average, the lowest cell equivalents of toxic *Microcystis* spp. was observed at Chipps Island ($8.5 \pm 6.7 \times 10^3$ cells L^{-1}), Brannan Island ($3.04 \pm 5.1 \times 10^4$ cells L^{-1}), and San Joaquin ($3.4 \pm 3.3 \times 10^4$ cells L^{-1}). The mean highest cell equivalents of toxic *Microcystis* spp. occurred in Antioch ($4.8 \pm 9.5 \times 10^6$ cells L^{-1}) and Franks Tract (mean $5.1 \pm 7.1 \times 10^6$ cells L^{-1}) with the rest of the sites at one magnitude lower (Table 5). While the cell equivalents of total *Microcystis* cells was generally high in all sampling sites and dates

Table 4
Percentages of gene copy numbers of toxic *Microcystis* (*mcy*D) in the San Francisco Estuary. Data refer to cyanobacteria collected by diagonal net tows.

Site / Date	Toxic *Microcystis* / Total *Microcystis*	Toxic *Microcystis* / Total cyanobacteria	Total *Microcystis* / Total cyanobacteria
AT-7/24/07	11.49 ± 4.0	6.96 ± 0.7	64.27 ± 16.9
AT-8/07/07	3.12 ± 2.6	1.76 ± 1.4	55.02 ± 3.2
AT-8/21/07	3.58 ± 0.6	2.05 ± 0.2	57.86 ± 7.4
AT-9/05/07	1.24 ± 0.1	0.31 ± 0.2	36.19 ± 2.1
AT-9/18/07	17.87 ± 3.6	12.56 ± 0.6	72.12 ± 13.9
BI-8/07/07	17.89 ± 16.4	5.51 ± 1.8	56.17 ± 29.1
BI-8/21/07	2.48 ± 0.1	2.07 ± 0.3	75.76 ± 0.01
BI-9/05/07	6.60 ± 0.7	6.32 ± 0.4	96.17 ± 5.1
CI-7/25/07	15.93 ± 14.1	8.64 ± 5.9	72.49 ± 21.9
MI-7/25/07	3.93 ± 0.9	2.14 ± 0.4	55.11 ± 8.8
MI-8/08/07	5.05 ± 1.2	2.87 ± 0.3	66.11 ± 12.1
MI-8/22/07	3.56 ± 1.6	3.38 ± 0.04	73.31 ± 11.6
MI-9/04/07	1.89 ± 0.1	0.78 ± 0.3	41.76 ± 19.7
MI-9/18/07	20.25 ± 1.2	12.02 ± 1.1	59.32 ± 3.2
OR-8/08/07	2.49 ± 0.1	1.35 ± 0.1	54.32 ± 1.7
OR-8/22/07	6.37 ± 1.3	1.83 ± 0.4	28.60 ± 0.9
OR-9/04/07	3.81 ± 1.3	2.38 ± 0.3	64.49 ± 12.2
SJ-7/25/07	0.59 ± 0.1	0.57 ± 0.09	93.28 ± 2.7
VC-7/25/07	0.37 ± 0.1	0.16 ± 0.02	40.00 ± 6.1

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

Table 5
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})[a]	Total *Microcystis* (Cell equivalents L^{-1})	Toxic *Microcystis* / Total *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.
[a]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.

Author's personal copy

($7.7 \pm 0.02 \times 10^4$ to $9.9 \pm 0.16 \times 10^7$ cells L^{-1}), the proportion of toxic among total *Microcystis* spp. was highest on the average in Antioch (10.37%), Franks Tract (9.67%), and Venice Cut (11.97%) and generally lower (mean ranges of 0.49–2.09%) in Brannan Island, Chipps Island, San Joaquin, Mildred Island, and Old River (Table 5).

Total MC concentrations from the tow net samples varied across locations and sampling times (0.007–10.8 μg/L) with the highest level in one sampling date in Venice Cut on 7/25/07 (10.8 μg/L), followed by Brannan Island (mean 1.08 μg/L) and Antioch (mean 0.51 μg/L) (Table 3). The estimated mean cell equivalents of *Microcystis* with *mcy*D genes in plankton samples from these sites were 1.4×10^5, 1.4×10^6, and 2.2×10^6 gene copies/μg DNA for Venice Cut, Brannan Island, and Antioch, respectively (Table 3). In ambient surface waters, the estimated mean cell equivalents of *Microcystis* with *mcy*D genes were 8.2×10^5, 3.0×10^4, and 4.8×10^6 cells L^{-1}, respectively for Venice Cut, Brannan Island and Antioch (Table 5).

4. Discussion

Microcystis was dominant among cyanobacterial populations collected in the SFE with variations in cell equivalents of toxic *Microcystis* carrying *mcy*D genes. The proportion of toxic and nontoxic *Microcystis*, which represent the difference in the frequency of toxic and total number of *Microcystis* cells (Rinta-Kanto et al., 2005), varied between sites and phases of the bloom development. Toxic *Microcystis* with *mcy*D genes formed up to 27% of the total *Microcystis* population in the SFE (cell equivalents of ambient surface water), which co-existed with approximately 73% nontoxic (MC−) *Microcystis*. Our findings concur with previous investigations in Lake Erie where toxic genotypes are generally lower than the abundance of total *Microcystis* in natural algal populations (Rinta-Kanto et al., 2009). In other cyanobacterial-prone bodies of water, the percentage of MC+ among nontoxic *Microcystis* assemblages ranged from 1.7 to 71% in Lake Wannsee, Germany (Kurmayer and Kutzenberger, 2003) and from 0.5 to 35% in Lake Mikata, Japan (Yoshida et al., 2007).

A combination of factors may influence variations in the abundance of MC+ and MC− *Microcystis* in the SFE. Streamflow was shown to affect the spread of plankton blooms across the estuary (Lehman et al., 2008). Alternately, wind and tide can enhance the aggregation of *Microcystis* biomass in shallow or low-flow water reaches such as Brannan Island and Antioch. Mixing processes in the ecosystem can affect the development and distribution of blooms (Huisman et al., 2004; Jöhnk et al., 2008) in addition to water temperature, nutrient loading, and light (Zurawell et al., 2005). Furthermore, the role of climate change has been suggested as a potent catalyst for the further expansion of cyanobacterial blooms in eutrophic waters on a global scale (Paerl and Huisman, 2009). How variations in water temperature can affect the magnitude of recurrence and toxicity of plankton blooms in the SFE is unknown and is an enormous task that remains to be determined.

The presence or absence of MC− and MC+ *Microcystis* was established in the current study using the *Microcystis*-specific 16S rRNA gene (16S rDNA) and the *mcyD* gene. The *Microcystis* 16S rRNA gene is specific to the *Microcystis* genus, which was used to estimate total *Microcystis* cell equivalents. As the MC biosynthesis gene cluster, *mcy*, is present only in toxic cyanobacterial (MC+) strains (Tillett et al., 2000), the ability to detect and quantify the *mcy*D gene among mixed populations of *Microcystis* spp. provided an evidence of the relative abundance and frequency of MC+ *Microcystis* strains in the SFE. Estimating the proportion of cells carrying *Microcystis*-specific 16S rRNA and *mcy*D gene targets in the current study provided, to the best of our knowledge, the first description on the abundance of MC+ *Microcystis* strains among cyanobacterial populations in the SFE. Our results enhance recent research on the description of toxic *Microcystis* in the estuary based on the *mcy*A gene, which partly encodes the peptide synthetase cluster for MC production (Moisander et al., 2009).

The genomic DNA of MC+ strain of *M. aeruginosa* (MC-LR UTEX 2385) was used to estimate the cell equivalents of *Microcystis* in the SFE carrying the specific 16S rDNA target genes. Although a local *Microcystis* strain would have been an ideal standard, the MC-LR is one of the dominant toxin congeners in the estuary (Lehman et al., 2005) making this strain an appropriate standard surrogate in the current study. Furthermore, the 16S rDNA gene has two copies in each *Microcystis* genome (Kaneko et al., 2007) that would account for the different *Microcystis* spp. producing different MC+ congeners in the SFE.

Difficulties have been encountered in the development of qPCR for cyanobacterial diagnostics in field samples due to variations in the copy number of 16S rDNA genes that may affect estimates of cell density (Rinta-Kanto et al., 2005). In our study, two copies of 16S rDNA genes per genome (Kaneko et al., 2007) and one copy of *mcy*D gene per genome (Kaebernick et al., 2002) were used to estimate the number of cell equivalents. This ratio was effectively used to determine the percentage of toxic *Microcystis* and the total *Microcystis* cell equivalents in the cyanobacterial samples. In some surface water samples however, the cell equivalents were lower for cyanobacteria compared to *Microcystis* (data not shown). While the cause of this anomalous result is unknown in the current study, previous studies attributed this finding to using the 16S rDNA as a target for quantifying both cyanobacteria and *Microcystis* with variable copy number of 16S rDNA operons (Rinta-Kanto et al., 2009), to sequence heterogeneity in natural populations of cyanobacterial cells (Crosby and Criddle, 2003), or an error in estimating the genome size of reference strains (Kardinaal and Visser, 2005). Although estimates of total cyanobacterial cell equivalents were not accurate in some samples in the current study, assessing the genome ratio of the *Microcystis* 16S rDNA and *mcy*D genes of plankton and surface waters demonstrated the variations in the frequency and distribution of MC+ and MC− *Microcystis* during the bloom development in the SFE, which was the main focus of this investigation.

Microcystin concentrations did not correspond to the frequency of toxic *Microcystis* cell equivalents in our study. One possible explanation is the potential occurrence of other MC+ genera, in addition to *Microcystis*, that may contribute to bloom toxicity in the SFE (Lehman et al., 2010). Other types of MC toxin congeners with varying toxicity were probably present in the estuary at the time of the study (Lehman et al., 2005, 2008) that potentially amplified the variations of total MC concentrations. In addition, the MC+ MC-LR UTEX strain used as a standard in the qPCR assay specifically targets MC+ (MC-LR) *Microcystis* precluding the different MC composition present in the SFE. Although the presence of other MC+ cyanobacteria (e.g., *Anabaena*, *Planktothrix* sp.) was not detected from samples examined in the current study using *mcy*A and E primers using conventional PCR assays (Hotto et al., 2008), other toxin-producing cyanobacterial species have been previously reported from the estuary including *Planktothrix* sp., *Anabaena* sp., and *Cylindrospermopsis* sp. (phytoplankton data files, www.iep.water.ca.gov). Microscopic analysis of samples was not conducted in the current study to verify the presence of other species of MC+ cyanobacteria.

Some studies indicate that MC levels do not necessarily correlate to the frequency of MC+ *Microcystis*. For example, qPCR analysis targeting the toxin synthetase *mcy*E gene in *Microcystis* and *Anabaena* from most locations examined showed relatively weak to no correlation between MC concentrations and *Microcystis* *mcy*E gene copies due to seasonal differences in *Microcystis* genome copies and toxin concentrations (Vaitomaa et al., 2003). Further

Author's personal copy

investigation is needed to elucidate the potential gap between the proportion of toxic *Microcystis* including other MC+ cyanobacteria and MC levels during bloom development in the SFE. Lastly, the few number of samples tested in a geographically large and dynamic ecosystem such as the SFE may have resulted in the lack of correlation between MC concentrations and qPCR-based cell equivalents of MC+ *Microcystis*.

Factors that promote or exacerbate the growth and survival of MC+ and MC− strains of *Microcystis* spp. in the SFE are unknown. The role of nutrients (e.g., iron or phosphorus) in altering *Microcystis* toxicity as shown in other watersheds (Sevilla et al., 2008; Davis et al., 2009), has only been initially examined in the SFE (Lehman, unpublished data). Environmental parameters including high water temperature, eutrophication, nutrients, and light affect the ability of MC+ genotypes to produce MCs (Kardinaal et al., 2007b; Davis et al., 2009). The length of spring–summer period and light availability can alter interactions between toxic and nontoxic strains (Kardinaal et al., 2007b) including the seasonal succession of different *Microcystis* genotypes as a key mechanism affecting MC concentrations in *Microcystis*-dominated waters (Via-Ordorika et al., 2004; Kardinaal et al., 2007a; Welker et al., 2007).

The management implications of this study demonstrate that the qPCR technique can facilitate rapid and specific determination of the relative proportion of MC+ strains among the total cyanobacterial population within the SFE based on the quantity of target genes from mixed cyanobacterial samples. The qPCR technique will be useful alone or in combination with other currently used methods such as the enzyme-linked immunosorbent assay (ELISA) or the protein phosphatase inhibition assay (PPIA) (Lehman et al., 2005, 2008) to enhance the analysis and interpretation of *Microcystis* toxicity in the SFE. While ELISA can demonstrate the presence and abundance of MCs, it cannot quantify the relative abundance of MC+ cells within a cyanobacterial sample. The PPIA can detect relative toxicity due to enzymatic activity but it cannot identify the MCs involved in the toxicity. While both ELISA and PPIA have their advantages, they do not offer any information on the species composition of a cyanobacterial bloom. In contrast, qPCR can quickly identify and quantify the presence and proportion of cyanobacterial species, particularly *Microcystis*, that are toxic (MC+) or nontoxic (MC−). Quantifying the relationship between total *Microcystis* biomass and MC+ *Microcystis* biomass 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 (Lehman et al., 2005, 2008). As measurements such as cell density do not correlate well with toxicity (Sivonen and Jones, 1999; Baker et al., 2002; Ouellette and Wilhelm, 2003), generally as well as in the SFE (Lehman et al., 2005, 2008), 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. As such, qPCR can contribute to rapid risk assessment and prediction of strategies designed to manage the adverse effects of blooms in this ecosystem, which provides drinking water to 22 million people in California (IEP, 2008).

In conclusion, the qPCR for *Microcystis* described in our study provided a specific and rapid approach for estimating the spatial and temporal variations in the proportion of toxic *Microcystis* strains among mixed cyanobacterial populations that co-exist in blooms in the SFE. As local *Microcysis* strains were recently shown to be unique in the estuary (Moisander et al., 2009), qPCR will be an invaluable method to further analyze the relationship between environmental factors and MC synthesis, and the potential threats of blooms to the food web by using cultures of local *Microcystis* strains currently being established in our laboratory.

Acknowledgements

The authors extend their gratitude to the CA Department of Water Resources and their staff for their generous support on sampling logistics in the San Francisco Estuary. We also thank Dr. Steven Wilhelm (University of Tennessee) and Dr. Pia Moisander (UC Santa Cruz) for providing the *Microcystis* and other cyanobacterial strains used as controls in the qPCR assays, and Dr. Greg Boyer (State University of New York, College of Environmental Science and Forestry) for his perspective on microcystin analysis. The invaluable contribution of Shawn Acunã and Kevin Marr in obtaining field samples and for coordinating field sampling events is greatly appreciated. Funding support for this study was provided, in part, by Dr. Swee Teh's Aquatic Toxicology Program research grants and the California Department of Water Resources Contract Nos. 4600007499 and 4600008137.[SS]

References

Baker, J., Entsch, B., Neilan, B., McKay, D., 2002. Monitoring changing toxigenicity of a cyanobacterial bloom by molecular methods. Applied and Environmental Microbiology 68, 6070–6076.

Carmichael, W.W., 1996. Toxic microcystis and the environment. In: Watanabe, M.F., Harada, K., Carmichael, W.W., Fujiki, H. (Eds.), Toxic Microcystis. CRC Press, USA, pp. 1–12.

Carmichael, W.W., 2001. Health effects of toxin producing cyanobacteria: the 'CyanoHABS'. Human and Ecological Risk Assessment 7, 1393–1407.

Carmichael, W.W., An, J., 1999. Using an enzyme linked immunosorbent assay (ELISA) and protein phosphatase inhibition assay (PPIA) for the detection of microcystins and nodularins. Natural Toxins 7, 377–385.

Chorus, I., Bartram, J., 1999. Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management. Spon Press, London, UK.

Cox, P.A., Banack, S.A., Murch, S.J., 2003. Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam. Proceedings of the National Academy of Sciences, USA 100, 13380–13383.

Crosby, L.D., Criddle, C.S., 2003. Understanding bias in microbial community analysis techniques due to rrn operon copy number heterogeneity. Biotechniques 34, 790.

Davis, T.W., Berry, D.L., Boyer, G.L., Gobler, C.J., 2009. The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms. Harmful Algae 8, 715–725.

Ger, K.A., Teh, S.J., Goldman, C.R., 2009. Microcystin-LR toxicity on dominant copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* of the upper San Francisco Estuary. The Science of the Total Environment 407, 4852–4857.

Ger, K.A., Teh, S.J., Baxa, D.V., Lesmeister S., Goldman, C.R., in press. The effects of dietary *Microcystis aeruginosa* and microcystin on the copepods of the upper San Francisco Estuary. Freshwater Biology.

Hessen, D.O., Faafeng, B.A., Brettum, P., Andersen, T., 2006. Nutrient enrichment and planktonic biomass ratios in lakes. Ecosystems 9, 516–527.

Hotto, A.M., Satchwell, M.F., Berry, D.L., Gobler, C.J., Boyer, G.L., 2008. Spatial and temporal diversity of microcystins and microcystin-producing genotypes in Oneida Lake, NY. Harmful Algae 7, 671–681.

Hudnell, K.H., Dortch, Q., 2008. A synopsis of research needs identified at the interagency, international symposium on cyanobacterial harmful algal blooms (ISOC-HAB). In: Hudnell, K.H. (Ed.), Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs Series: Advances in Experimental Medicine and Biology, vol. 619. pp. 614–5950.

Huisman, J., Sharples, J., Stroom, J., Visser, P.M., Kardinaal, W.E.A., Verspagen, J.M.H., Sommeijer, B., 2004. Changes in turbulent mixing shift competition for light between phytoplankton species. Ecology 85, 2960–2970.

Interagency Ecological Program (IEP), 2008. Work Plan to Evaluate the Decline of Pelagic Species in the Upper San Francisco Estuary. Baxter, R., Breuer, R., Brown, L., Chotkowski, M., Feyrer, F., Herbold, B., Hrodey, P., Mueller-Solger, A., Nobriga, M., Sommer, T., Souza, K., http://www.science.calwater.ca.gov/pdf/workshops/POD/2008_IEP-POD_Workplan_060208.pdf.

Jacoby, J.M., Collier, D.C., Welch, E.B., Hardy, F.J., Crayton, M., 2000. Environmental factors associated with bloom of *Microcystis aeruginosa*. Canadian Journal of Fisheries and Aquatic Sciences 57, 231–240.

Jöhnk, K.D., Huisman, J., Sharples, J., Sommeijer, B., Visser, P.M., Stroom, J.M., 2008. Summer heatwaves promote blooms of harmful cyanobacteria. Global Change Biology 14, 495–512.

Author's personal copy

Kaebernick, M., Neilan, B.A., Börner, T., Dittmann, E., 2000. Light and the transcriptional response of the microcystin biosynthesis gene cluster. Applied and Environmental Microbiology 66, 3387–3392.

Kaebernick, M., Dittmann, E., Börner, T., Neilan, B.A., 2002. Multiple alternate transcripts direct the biosynthesis of microcystin, a cyanobacterial nonribosomal peptide. Applied and Environmental Microbiology 68, 449–455.

Kaneko, T., Nakajima, N., Okamoto, S., Suzuki, I., Tanabe, Y., Tamaoki, M., Nakamura, Y., Kasai, F., Watanabe, A., Kawashima, K., Kishida, Y., Ono, A., Shimizu, Y., Takahashi, C., Minami, C., Fujishiro, T., Kohara, M., Katoh, M., Nakazaki, N., Nakayama, S., Yamada, M., Tabata, S., Watanabe, M.M., 2007. Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843. DNA Research 14, 247–256.

Kardinaal, W.E.A., Visser, P.M., 2005. Dynamics of cyanobacterial toxins. In: Huisman, J., Matthijs, H.C.P., Visser, P.M. (Eds.), Harmful Cyanobacteria. Aquatic Ecology Series. Springer, pp. 41–63.

Kardinaal, W.E.A., Janse, I., Kamst-van Agterveld, M., Meima, M., Snoek, J., Mur, L.R., 2007a. *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes. Aquatic Microbial Ecology 48, 1–12.

Kardinaal, W.E.A., Tonk, L., Janse, I., Hol, S., Slot, P., Huisman, J., Visser, P.M., 2007b. Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. Applied and Environmental Microbiology 73, 2939–2946.

Kirk, K., Gilbert, J., 1992. Variation in herbivore responses to chemical defenses zooplankton foraging on toxic cyanobacteria. Ecology 73, 2208–2217.

Kurmayer, R., Kutzenberger, T., 2003. Application of real time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium *Microcystis* sp. Applied and Environmental Microbiology 69, 6723–6730.

Kurmayer, R., Dittmann, E., Fastner, J., Chorus, I., 2002. Diversity of microcystin genes within a population of the toxic cyanobacterium *Microcystis* spp. in Lake Wannsee (Berlin, Germany). Microbial Ecology 43, 107–118.

Lehman, P.W., Boyer, G., Hall, C., Waller, S., Gehrts, K., 2005. Distribution and toxicity of a new colonial *Microcystis aeruginosa* bloom in the San Francisco Bay Estuary, California. Hydrobiologia 541, 87–99.

Lehman, P.W., Boyer, G., Satchwell, M., Waller, S., 2008. The influence of environmental conditions on the seasonal variation of *Microcystis* cell density and microcystins concentration in San Francisco Estuary. Hydrobiologia 600, 187–204.

Lehman, P.W., Teh, S.J., Boyer, G.L., Nobriga, M., Bass, E., Hogle, C., 2010. Initial impacts of *Microcystis* on the aquatic food web in the San Francisco Estuary. Hydrobiologia 637, 229–248.

Malbrouck, C., Kestemont, P., 2006. Effects of microcystins on fish. Environmental Toxicology and Chemistry 25, 72–86.

Moisander, P.H., Lehman, P.W., Ochiai, M., Corum, S., 2009. Diversity of the toxic cyanobacterium *Microcystis aeruginosa* in the Klamath River and San Francisco Bay Delta, California. Aquatic Microbial Ecology 57, 19–31.

Neilan, B.A., Jacobs, D., DelDot, T., Blackall, L.L., Hawkins, P.R., Cox, P.T., Goodman, P.T., 1997. rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. International Journal of Systematic Bacteriology 47, 693–697.

Nejstgaard, J.C., Frischer, M.E., Simanelli, P., Troedsson, C., Brakel, M., Adiyaman, F., Sazhin, A.F., Artigas, L.F., 2008. Quantitative PCR to estimate copepod feeding. Marine Biology 153, 565–577.

Nishizawa, T., Ueda, A., Asayama, M., Fujii, K., Harada, K.I., Ochi, K., Shirai, M., 2000. Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. Journal of Biochemistry 127, 779–789.

Nonneman, D., Zimba, P.V., 2002. A PCR-based test to assess the potential for microcystin occurrence in channel catfish production ponds. Journal of Phycology 38, 230–233.

Otsuka, S., Suda, S., Li, R.H., Watanabe, M., Oyaizu, H., Matsumoto, S., Watanabe, M.M., 1999. Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence. FEMS Microbiology Letters 172, 15–21.

Ouellette, A.J., Wilhelm, S., 2003. Toxic cyanobacteria: the evolving molecular toolbox. Frontiers in Ecology and Environment 1, 359–366.

Ouellette, A.J., Handy, S.M., Wilhelm, S.W., 2006. Toxic *Microcystis* is widespread in Lake Erie: PCR detection of toxin genes and molecular characterization of associated cyanobacterial communities. Microbial Ecology 51, 154–165.

Paerl, H.W., 2008. Nutrient and other environmental controls of harmful cyanobacterial blooms along the freshwater marine continuum. Advances in Experimental Medicine and Biology 619, 216–241.

Paerl, H.W., Huisman, J., 2009. Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. Environmental Microbiology Reports 1, 27–37.

Pearson, L.A., Neilan, B.A., 2008. The molecular genetics of cyanobacterial toxicity as a basis for monitoring water quality and public health risk. Current Opinion in Biotechnology 19, 281–288.

Pearson, L.A., Hisbergues, M., Börner, T., Dittmann, E., Neilan, B.A., 2004. Inactivation of an ABC transporter gene, *mcy*H, results in loss of microcystin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Applied and Environmental Microbiology 70, 6370–6378.

Rinta-Kanto, J.M., Ouellette, A.J.A., Boyer, G.L., Twiss, M.R., Bridgeman, T.B., Wilhelm, S.W., 2005. Quantification of toxic *Microcystis* spp. during the 2003 and 2004 blooms in western Lake Erie using quantitative real-time PCR. Environmental Science and Technology 39, 4198–4205.

Rinta-Kanto, J.M., Konopko, E., DeBruyn, J., Bourbonniere, R.A., Boyer, G.L., Wilhelm, S.W., 2009. Lake Erie *Microcystis*: relationship between microcystin production, dynamics of genotypes and environmental parameters in a large lake. Harmful Algae 8, 665–673.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sedmak, B., Elersek, T., 2005. Microcystins induce morphological and physiological changes in selected representative phytoplanktons. Microbial Ecology 51, 508–515.

Sevilla, E., Martin-Luna, B., Vela, L., Bes, M.T., Fillat, M.F., Peleato, M.L., 2008. Iron availability affects mcyD expression and microcystin-LR synthesis in *Microcystis aeruginosa* PCC7806. Environmental Microbiology 10, 2476–2483.

Sivonen, K., Jones, G., 1999. Cyanobacterial toxins. In: Chorus, I., Bartram, J. (Eds.), Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. Spon Press, London, UK, pp. 41–111.

Sommer, T., Armor, C., Baxter, R., Breuer, R., Brown, L., Chotkowski, M., Culberson, S., Feyrer, F., Gingras, M., Herbold, B., Kimmere,r, W., Mueller-Solger, A., Nobriga, M., Souza, K., 2007. The collapse of pelagic fishes in the upper San Franciso Estuary. Fisheries 32, 270–277.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 11, 4673–4680.

Tillett, D., Dittman, E., Erhard, M., von Döhren, H., Börner, T., Neilan, B., 2000. Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC 7806: an integrated peptidepolyketide synthetase system. Chemistry and Biology 7, 753–764.

Urbach, E., Vergin, K.L., Young, L., Morse, A., Larson, G.L., Giovannoni, S.J., 2001. Unusual bacterioplankton community structure in the ultra-oligotrophic Crater Lake. Limnology and Oceanography 46, 5557–5572.

Vaitomaa, J., Rantala, A., Halinen, K., Rouhiainen, L., Tallberg, P., Mokelke, L., Sivonen, K., 2003. Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Applied and Environmental Microbiology 69, 7289–7297.

Vanderploeg, H.A., Liebig, J.R., Carmichael, W.W., Agy, M.A., Johengen, T.H., Fahnenstiel, G.L., Nalepa, T.F., 2001. Zebra mussel (*Dreissena polymorpha*) selective filtration promoted toxic *Microcystis* blooms in Saginaw Bay (Lake Huron) and Lake Erie. Canadian Journal of Fisheries and Aquatic Sciences 58, 1208–1221.

Via-Ordorika, L., Fastner, J., Hisbergues, M., Dittmann, E., Erhard, M., Komárek, J., 2004. Distribution of microcystin-producing and non-microcystin producing *Microcystis* in European freshwater bodies: detection of microcystins and mcy genes in single colonies. Systematic and Applied Microbiology 27, 592–602.

Welker, M., Šejnohová, S., Némethová, D., von Döhren, H., Jarkovský, J., Maršálek, B., 2007. Seasonal shifts in chemotype composition of *Microcystis* sp. communities in the pelagial and the sediment of a shallow reservoir. Limnology and Oceanography 52, 609–619.

Wilson, A.E., Sarnelle, O., Tillmanns, A.R., 2006. Effects of cyanobacterial toxicity and morphology on the population growth of freshwater zooplankton: Meta-analyses of laboratory experiments. Limnology and Oceanography 51, 1915–1924.

World Health Organization (WHO), 1999. Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management. Routledge, London and New York.

Yoshida, M., Yoshida, T., Takashima, Y., Hosoda, N., Hiroishi, S., 2007. Dynamics of microcystin-producing and non-microcystin-producing *Microcystis* populations is correlated with nitrate concentration in a Japanese lake. FEMS Microbiology Letters 266, 49–53.

Zurawell, R.W., Chen, H., Burke, J.M., Prepas, E.E., 2005. Hepatotoxic cyanobacteria: a review of the biological importance of microcystins in freshwater environments. Journal of Toxicology and Environmental Health B 8, 1–37.

JPR Advance Access published June 18, 2010

SHORT COMMUNICATION

Species specific differences in the ingestion of *Microcystis* cells by the calanoid copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi*

KEMAL ALI GER [1], PATRICIA ARNESON [1], CHARLES REMINGTON GOLDMAN [1] AND SWEE JOO TEH [2]*

[1]DEPARTMENT OF ENVIRONMENTAL SCIENCE AND POLICY, UNIVERSITY OF CALIFORNIA, DAVIS, CA 95616, USA AND [2]AQUATIC TOXICOLOGY PROGRAM, DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND CELL BIOLOGY, SCHOOL OF VETERINARY MEDICINE, DEPARTMENT OF ANATOMY, PHYSIOLOGY AND CELL BIOLOGY, UNIVERSITY OF CALIFORNIA, 1321 HARING HALL, ONE SHIELDS AVENUE, DAVIS, CA 95616, USA

*CORRESPONDING AUTHOR: sjteh@ucdavis.edu

Received April 6, 2010; accepted in principle May 20, 2010; accepted for publication May 24, 2010

Corresponding editor: John Dolan

Copepod species showed large differences in the ingestion of *Microcystis* cells, but no difference among microcystin producing (MC+) or lacking (MC−) strains in a short feeding experiment. Differences in selective feeding may allow some copepods to better tolerate *Microcystis*.

KEYWORDS: microcystis; copepod; ingestion; zooplankton; cyanobacteria

Ingestion of *Microcystis* cells by zooplankton causes lethal and sub-lethal effects including toxicity, nutritional inadequacy and feeding suppression (Fulton and Paerl, 1987; DeMott and Moxter, 1991). Zooplankton can minimize these negative impacts by feeding adaptations to avoid ingestion or through tolerance to ingested toxins (Engstrom *et al.*, 2000; Hansson *et al.*, 2007). Hence, species-specific differences among ingestion rates on cyanobacteria can have major consequences for zooplankton community composition as well as the potential for managing blooms (Paerl, 1988; DeMott *et al.*, 1991; Kirk and Gilbert, 1992; Wang *et al.*, 2010).

Although copepods frequently dominate zooplankton and can co-exist with cyanobacteria blooms, studies on copepod-feeding behavior related to *Microcystis* are rare (Bouvy *et al.*, 2001; Panosso *et al.*, 2003; Work and Havens, 2003; Wilson *et al.*, 2006). Tolerance for cyanobacteria varies among copepods, partly because species rely on different chemosensory cues for avoiding cyanobacterial food (Kurmayer and Juttner, 1999; Engstrom *et al.*, 2000). *Microcystis* contains several toxic metabolites including microcystin (MC), microviridin, lipopolysaccharides and unidentified lipophylic compounds, which may act as cues for zooplankton to avoid ingestion (Kurmayer and Juttner, 1999; Rohrlack *et al.*, 2004; Wiegand and Pflugmacher, 2005). Comparing ingestion rates on *Microcystis* strains of varying toxicity but similar morphology has been an effective method to show how some zooplankton tolerate *Microcystis* more than others (Rohrlack *et al.*, 1999; Lurling, 2003; Ger *et al.*, 2010).

doi:10.1093/plankt/fbq071, available online at www.plankt.oxfordjournals.org.
© The Author 2010. Published by Oxford University Press. All rights reserved. For permissions, please email: journals.permissions@oxfordjournals.org

Downloaded from http://plankt.oxfordjournals.org at University of California, Davis on June 19, 2010

Copepods, especially *Eurytemora affinis* and *Pseudodiaptomus forbesi*, are the dominant zooplankton and the main food source for endangered fish in the freshwater portion of the San Francisco Estuary, where annual blooms of *Microcystis aeruginosa* raise concern for the food limited zooplankton (Muller-Solger *et al.*, 2002; Sommer *et al.*, 2007; Lehman *et al.*, 2008). In a previous laboratory study, *Microcystis* (MC+ or MC−) was toxic to both *E. affinis* and *P. forbesi*, but the latter was able to co-exist especially with the MC+ strain, most likely because it minimized *Microcystis* ingestion (Ger *et al.*, 2010). Our objective was to verify differences in the ingestion rates of *E. affinis* and *P. forbesi* on *Microcystis*, and to test the role of cellular MC as a possible copepod cue to avoid and thereby tolerate *Microcystis*. We hypothesized that in a mixed diet, *E. affinis* would ingest more *Microcystis* (MC+ or MC−) than *P. forbesi*, and that *P. forbesi* ingestion of MC+ *Microcystis* would be less than the MC− strain.

Ingestion experiments used identical organisms, culturing conditions and treatment diets as in the survival experiments detailed in Ger *et al.* (Ger *et al.*, 2010). Axenic batch cultures of MC+ (UTEX 2385) and MC− (UTEX 2386) *Microcystis* were maintained in the exponential growth phase in a modified ASM-1 medium. We assumed that the only difference between the two strains used was MC content, and that each strain had a comparable nutritional profile and digestibility. Both strains were previously verified by a conventional PCR targeting the *MIC* and *mcyB* genes to assure no cross contamination, and the MC production was measured using a commercially available ELISA (Envirologix, USA). The mean cell bound concentration of the MC+ strain during the experiment was 348 $\mu g\ L^{-1}$ (± 49, $n = 8$) MC−LR, which corresponds to an estimated 4.87 $\mu g\ mg\ C^{-1}$ (± 0.98, $n = 7$) of MC−LR per *Microcystis* biomass. Copepods were collected from ongoing cultures that have been under controlled laboratory conditions for over 1 year. An equal biovolume of *Nannochloropsis* (2 μm cell diameter, Eustigmatophyceae) and *Pavlova* (4 μm cell diameter, Chrysophyceae) (Instant Algae, USA), IA for short, was given as food at 400 and 500 $\mu g\ C\ L^{-1}\ day^{-1}$ for *E. affinis* and *P. forbesi*, respectively. Only CV-stage copepodites and adults were used in the ingestion experiment.

Each *Microcystis* strain (MC+ and MC−) was subsampled (150 mL) from exponentially growing cultures described above and transferred to 300 mL glass flasks, diluted with 100 mL culture medium and spiked with 2 mL of 24.39 μCi/mL $NaH^{14}CO_3$ (Oak Ridge National Laboratory, USA). Flasks were capped with sterile cotton balls, swirled three times a day and incubated for 48 h, which was previously determined as adequate for the uniform uptake of the radioactive label. Cell density and exponential growth were verified by changes in absorbance at 800 nm.

Ingestion of *Microcystis* was quantified by feeding copepods a mixed diet containing ^{14}C labeled *Microcystis* during a 30 min ingestion experiment. The treatment diets consisted of a *Microcystis*−IA mixture, with the proportion of *Microcystis* at 10, 25, 50 or 100% of total food (by carbon), using either the MC+ or MC− strain of *Microcystis*, plus IA, to a total food concentration of 400 (*E. affinis*) and 500 (*P. forbesi*) $\mu g\ C\ L^{-1}$, and given in triplicates. For each replicate, about 100 copepods were transferred from the batch cultures to a 2 L glass beaker, in clean culture medium, and starved for 4 h prior to addition of labeled food. This allows sufficient time to evacuate gut contents (W. Kimmerer, San Francisco, personal communication). All experiments took place at 22°C (± 1) and other conditions were identical to batch cultures. Copepods were acclimated to this temperature 24 h prior to starvation.

Labeled treatment diets were added at appropriate amounts at the beginning of the experiment. Copepods were allowed to feed for 30 min, then collected on a 150 um mesh screen and anesthetized with carbonated water to prevent loss of fecal matter (DeMott and Moxter, 1991). Copepods were flushed and rinsed three times with carbonated water to wash off any external *Microcystis* cells, and placed in a petri dish with clean carbonated water for each replicate. From each petri dish, 10, 20 and 30 copepods were selected individually with pipettes, and filtered on a 25 mm HA filter (Millipore, USA) for radioactivity analysis measured via gas proportional counting using a Tennelec LB 5100 Series III system (Canberra Industries, USA) as described in Goldman (Goldman, 1961). Hence, each replicate consisted of 60 copepods divided on three filters.

The average per copepod ingestion rate (cells $copepod^{-1}\ h^{-1}$) was calculated by comparing the specific activity of copepods (μCi/animal) with that of *Microcystis* (μCi/cell *Microcystis*). Per copepod activity was measured by taking the average of three subsamples for each replicate. The activity of *Microcystis* was estimated by filtering 1 mL of culture (in replicates) on a 25 mm diameter HA filter (Millipore, USA) and comparing total filter activity to the *Microcystis* cell density during the experiment. The following formula was used to calculate ingestion rates:

$$\text{Ingestion} = (^{14}C/\text{copepod}) \times (^{14}C/\text{cell})^{-1} \times h^{-1}$$

Differences in ingestion rates between the treatments were analyzed using a two-way ANOVA (JMP 7.0). The

Downloaded from http://plankt.oxfordjournals.org at University of California, Davis on June 19, 2010

effect of diet and copepod species on the ingestion rate was calculated. The diet, whose attributes were further broken down to strain (MC+ or MC−) and ratio of *Microcystis* (% *Microcystis*), was analyzed for differences in the effect of these parameters on ingestion. Only significant differences at the $P = 0.05$ level are mentioned. The specific ingestion rate was calculated by dividing the ingestion rate with the biomass per copepod, which was previously measured as 1.75 and 2.54 μg C for *E. affinis* and *P. forbesi*, respectively (Bouley and Kimmerer, 2006).

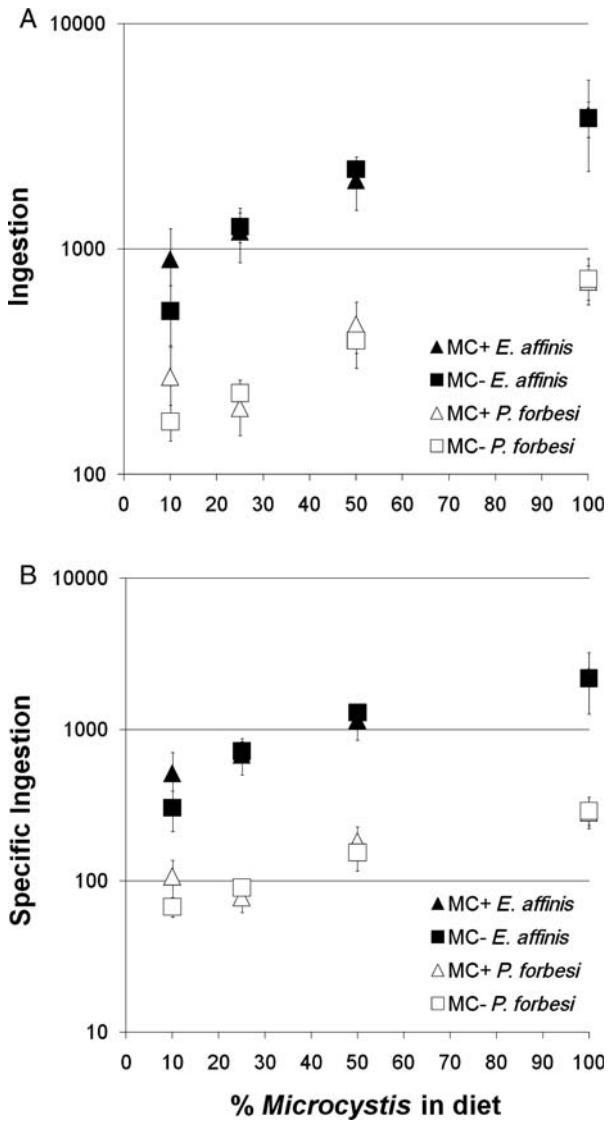

Fig. 1. (**A** and **B**) Ingestion rates of the copepods E. affinis and P. forbesi on Microcystis (MC+ or MC− strains) when provided as different proportions in a mixed diet containing Microcystis and IA. Comparing the ingestion rate per copepod [cell × (copepod h)−1] (A) with the specific ingestion rate relative to copepod biomass [cell × (μg C copepod h)−1] (B) corrects for the effect of copepod size on ingestion rates. Note the logarithmic scale on the y-axis. Bars indicate SE at the P = 95% level.

Copepods ingested *Microcystis* in all treatments, though there were significant differences between species and their grazing rates on *Microcystis* (Fig. 1A). Ingestion rates varied between 500 and 4000 *Microcystis* cells copepod^{-1} h^{-1} for *E. affinis* and 170−720 *Microcystis* cells copepod^{-1} h^{-1} for *P. forbesi*, and were linearly proportional to the ratio of *Microcystis* in the diet ($r^2 = 0.66$ for *E. affinis* and 0.61 for *P. forbesi*, $P < 0.001$). There were strong differences between the two species grazing response to *Microcystis*. *Eurytemora affinis* ingestion was an order of magnitude higher compared with *P. forbesi*, for either strain and across all diets except the 10% *Microcystis* diet ($P < 0.001$). A 10-fold increase in the proportion of *Microcystis* resulted in a 3.47-fold increase for *P. forbesi* ingestion, compared to 5.76-fold increase in *E. affinis*. The slope for *E. affinis* ingestion was higher than that of *P. forbesi* (by a factor of 6.06, $P < 0.001$). Since the copepod species had comparable biomass, the specific ingestion rates showed similar trends, and the differences in ingestion rates were not due to differences in copepod size (Fig. 1B).

Results showed that the copepod species and the diet had a significant effect on the ingestion rate, independently and as an interaction term (Table Ia). Further, differences in the ratio of *Microcystis* and the way each species responded to this ratio (interaction) had a significant effect on ingestion (Table Ib). In contrast, both copepods grazed on either strain of *Microcystis* (MC+ or MC−) at similar rates (Table Ib).

Table I: Summary results of a factorial analysis of variance on the Microcystis ingestion rate of copepods E. affinis and P. forbesi in relation to overall diet (a) and in relation to the strain (MC+ or MC−) and proportion of Microcystis in diet (%) (b)

Parameter	D.F.	*F*	*P*
(a)			
Species	1	44.246	<0.001
Diet	7	4.975	<0.001
Species × diet	7	2.950	0.036
(b)			
MC	1	0.024	0.876
%	3	11.511	<0.001
Species × MC	1	0.001	0.973
Species × %	3	5.752	0.003

Results show differences in the effect of copepod species, and specific attributes of the diet, such as presence of MC and the ratio of dietary Microcystis on copepod ingestion rates. The interaction term shows differences in how each copepod species responds to the dietary attributes.

Downloaded from http://plankt.oxfordjournals.org at University of California, Davis on June 19, 2010

Although *Microcystis* was ingested by both copepods, results indicate that *E. affinis* is less efficient at avoiding *Microcystis*, especially as its proportion in the diet increases. A similar comparison also found that *E. affinis* was relatively inefficient in avoiding *Nodularia* (cyanobacteria) when compared with a raptorial feeding copepod (Engstrom *et al.*, 2000). In our study, both copepods were filter-feeding calanoids, and lower *Microcystis* ingestion by *P. forbesi* is likely due to more effective selective feeding. *Microcystis* is typically the least preferred food for copepods and is ingested when alternative food becomes scarce (DeMott and Moxter, 1991; Burns and Hegarty, 1994; Kumar, 2003).

Zooplankton can co-exist with cyanobacteria through a species-specific combination of physiological tolerance to toxins and the rate of ingestion (Fulton and Paerl, 1987; Kurmayer and Juttner, 1999; Koski *et al.*, 2002). Selective feeding zooplankton that avoid cyanobacteria tend to have lower physiological tolerance to their toxins (Demott *et al.*, 1991; Kozlowski-Suzuki *et al.*, 2003; Gustaffson and Hansson, 2004; Sarnelle and Wilson, 2005). Compared to *E. affinis*, *P. forbesi* is less tolerant to dissolved MC, but more tolerant to the presence of *Microcystis* in the diet, suggesting that improved selective feeding (and not physiological tolerance) allows higher tolerance (Ger *et al.*, 2009, 2010).

Here, *P. forbesi* maintained a relatively low grazing rate even as the proportion of *Microcystis* increased in the diet. Considering also that this copepod survived over 11 days despite the presence of *Microcystis* (Ger *et al.*, 2010), such low *Microcystis* ingestion provides evidence that *P. forbesi* is indeed more efficient at avoiding harmful food. Since both copepods had similar optimal diet concentrations, it is likely that they ingest comparable levels of IA when *Microcystis* is not present. Yet, it is not possible to calculate the selective feeding efficiency without knowing the ingestion of the IA cells in addition to *Microcystis*. This information will be critical in future studies to compare selective feeding among copepods exposed to cyanobacteria. Thus, while the results do not prove it, they do provide further evidence that *P. forbesi* is more efficient at avoiding *Microcystis* compared with *E. affinis*.

Previously, *P. forbesi* survival was higher on a MC+ diet, indicating lower ingestion on this strain (Ger *et al.*, 2010). Contrary to expectation, *P. forbesi* ingested both strains similarly, at least in the short term. Since some copepods avoid cyanobacteria species regardless of the strain and others ingest only strains that lack certain metabolites, it is possible that both copepods in this study responded to a general *Microcystis* metabolite rather than MC (Kurmayer and Juttner, 1999; Engstrom *et al.*, 2000). However, simply looking at the initial response to a 30 min *Microcystis* exposure may be misleading because previous exposure to *Microcystis* can improve zooplankton tolerance through changes in feeding behavior (Gustaffson and Hansson, 2004; Sarnelle and Wilson, 2005). Indeed, *P. forbesi* tolerance to *Microcystis* and the strain-specific effects (MC+ vs. MC−) emerged after 5 days of being exposed to the diet in the earlier survival experiment (Ger *et al.*, 2010).

When this is viewed in light of the current ingestion results, the negative relationship between ingestion and copepod survival as well as the importance of acclimation to *Microcystis* is highlighted. The results show that *P. forbesi* can avoid *Microcystis* better than *E. affinis* even during the initial response without any acclimation to the cyanobacteria. However, we know that *P. forbesi* tolerance to *Microcystis* increases after 5 days of exposure (Ger *et al.*, 2010). We also know that following this acclimation period, *P. forbesi* survival is higher when fed the MC+ *Microcystis*, most likely because it uses MC as a cue to avoid this strain (Ger *et al.*, 2010). Yet, *P. forbesi* ingested both strains (MC+ and MC−) of *Microcystis* at comparable rates in this short-term exposure. This is most likely because *P. forbesi* needs an acclimation period to further decrease the ingestion of *Microcystis*, and particularly the MC+ strain. As such, we predict that acclimation is a significant factor increasing the efficiency of *P. forbesi* feeding selectivity, and it may be a critical process for this copepod to detect different strains using MC as a potential cue to avoid ingestion. Accordingly, comparing survival with ingestion before and after exposure to *Microcystis* would clarify why some zooplankton can improve tolerance to cyanobacteria over the short term (within lifetime). This would also reveal if copepods and especially *P. forbesi* develop strain-specific responses to *Microcystis* after several days of exposure.

Laboratory-based studies can provide mechanisms that are useful but may not represent natural conditions. Using single-celled *Microcystis* to measure zooplankton ingestion is a common limitation that can overestimate what happens in nature (Wilson *et al.*, 2006). *Microcystis* typically exists as large colonies during blooms, which increases efficiency of feeding selectivity in copepods (Tackx *et al.*, 2003; Wilson *et al.*, 2006; Tillmans *et al.*, 2008). For this reason and because of the possible effects of previous exposure explained above, copepods are expected to ingest less *Microcystis* during natural blooms. Finally, the use of non-living IA as "good" food may have caused the copepods to ingest more *Microcystis* compared to a control diet with live algae, resulting in an overestimation of its ingestion. Thus, our results likely represent an upper limit for the ingestion of *Microcystis* by the copepods *E. affinis* and *P. forbesi*.

Downloaded from http://plankt.oxfordjournals.org at University of California, Davis on June 19, 2010

The results support conclusions of the previous survival experiment that *P. forbesi* can tolerate *Microcystis* better than *E. affinis* due to its superior ability to avoid *Microcystis* while most likely feeding selectively on alternative food sources. Selective grazing can promote *Microcystis* by eliminating phytoplankton competitors, and *Microcystis* can further shift the zooplankton community to the dominance of selective feeding or smaller zooplankton, creating a more stable plankton assemblage (Fulton and Paerl, 1987; Hansson *et al.*, 2007; Wang *et al.*, 2010). We found that copepods ingest *Microcystis* at different rates, which may have significant effects on both the phytoplankton and zooplankton community in the San Francisco Estuary. Specifically, *P. forbesi* is more likely to co-exist with and may promote blooms of *Microcystis* via highly selective feeding on competing phytoplankton species.

ACKNOWLEDGEMENTS

We greatly appreciate Ida Flores and Sarah Lesmeister for assistance with culturing copepods and the ingestion experiments.

FUNDING

This work was supported by Dr Swee Teh's Aquatic Toxicology Program fund and partially by the California Interagency Ecological Program Pelagic Organisms Decline (project No. PO685515); and California Department of Water Resources (Contract Nos 4600007499, 4600008137).

REFERENCES

Bouley, P. and Kimmerer, W. J. (2006) Ecology of a highly abundant, introduced cyclopoid copepod in a temperate estuary. *Mar. Ecol. Prog. Ser.*, **324**, 219–228.

Bouvy, M., Pagano, M. and Troussellier, M. (2001) Effects of cyanobacterial bloom (*Cylindrospermopsis raciborskii*) on bacteria and zooplankton communities in Ingazeira reservoir (northeast Brazil). *Aquat. Microbial. Ecol.*, **25**, 215–227.

Burns, C. W. and Hegarty, B. (1994) Diet selection by copepods in the presence of cyanobacteria. *J. Plankton Res.*, **16**, 1671–1690.

DeMott, W. R. and Moxter, F. (1991) Foraging on cyanobacteria by copepods: responses to chemical defenses and resource abundance? *Ecology*, **75**, 1820–1834.

DeMott, W. R., Zhang, Q. X. and Carmichael, W. (1991) Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. *Limnol. Oceanogr.*, **36**, 1346–1357.

Engstrom, J., Koski, M., Viitasolo, M. *et al.* (2000) Feeding interactions of the copepods *Eurytemora affinis* and *Acartia bifilosa* with the cyanobacteria Nodularia. *J. Plankton Res.*, **22**, 1403–1409.

Fulton, R. S. and Paerl, H. W. (1987) Toxic and inhibitory effects of the blue-green alga *Microcystis aeruginosa* on herbivorous zooplankton. *J. Plankton Res.*, **9**, 837–855.

Ger, K. A., Teh, S. J. and Goldman, C. R. (2009) Microcystin-LR toxicity on dominant copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* of the upper San Francisco Estuary. *Sci. Total Environ.*, **407**, 4852–4857.

Ger, K. A., Teh, S. J., Baxa, D. *et al.* (2010) The Role of Microcystis and microcystin in the survival of estuarine copepods. *Freshwater Biol.* (in press).

Goldman, C. R. (1961) The contribution of alder trees (Alnus tenuifolia) to the primary productivity of Castle Lake, California. *Ecology*, **42**, 282–288.

Gustafsson, S. and Hansson, L-A. (2004) Development of tolerance against toxic cyanobacteria in Daphnia. *Aquat. Ecol.*, **38**, 37–44.

Hansson, L-A., Gustaffson, S., Rengefors, K. *et al.* (2007) Cyanobacterial chemical warfare affects zooplankton community composition. *Freshwater Biol.*, **52**, 1290–1301.

Kirk, K. L. and Gilbert, J. J. (1992) Variation in herbivore response to chemical defenses: zooplankton foraging on toxic cyanobacteria. *Ecology*, **73**, 2208–2217.

Koski, M., Schmidt, K., Engström-Öst, J. *et al.* (2002). Calanoid copepods feed and produce eggs in the presence of toxic cyanobacteria *Nodularia spumigena*. *Limnol. Oceanogr.*, **43**, 878–885.

Kozlowski-Suzuki, B., Karjalainen, M., Lehtiniemi, M. *et al.* (2003) Feeding, reproduction and toxin accumulation by the copepods *Acartia bifilosa* and *Eurytemora affinis* in the presence of the toxic cyanobacterium Nodularia spumigena. *Mar. Ecol. Prog. Ser.*, **249**, 237–249.

Kumar, R. (2003) Effect of different food types on developmental rates and demographic parameters of *Phyllodiaptomus blanci* (Copepoda:Calanoida). *Arch. Hydrobiol.*, **157**, 351–377.

Kurmayer, R. and Juttner, F. (1999) Strategies for the co-existence of zooplankton with the toxic cyanobacterium *Planktothrix rubescens* in Lake Zurich. *J. Plankton Res.*, **21**, 659–683.

Lehman, P. W., Boyer, G., Satchwell, M. *et al.* (2008) The influence of environmental conditions on the seasonal variation of *Microcystis* cell density and microcystins concentration in San Francisco Estuary. *Hydrobiologia*, **600**, 187–204.

Lurling, M. (2003) *Daphnia* growth on microcystin-producing and microcystin-free *Microcystis aeruginosa* in different mixtures with the green alga *Scenedesmus obliqus*. *Limnol. Oceanogr.*, **48**, 2214–2220.

Muller-Solger, A., Jassby, A. D. and Muller-Navarra, D. (2002) Nutritional quality of food resources for zooplankton (*Daphnia*) in a tidal freshwater system (Sacramento-San Joaquin River Delta). *Limnol. Oceanogr.*, **47**, 1468–1476.

Paerl, H. W. (1988) Nuisance phytoplankton blooms in coastal, estuarine, and inland waters. *Limnol. Oceanogr.*, **33**, 823–847.

Panosso, R., Carlsson, P., Kozlowsky-Suzuki, B. *et al.* (2003) Effect of grazing by a neotropical copepod *Notodiaptomus*, on a natural cyanobacterial assemblage of toxic and non-toxic cyanobacterial strains. *J. Plankton Res.*, **25**, 1169–1175.

Rohrlack, T., Dittman, E., Borner, T. *et al.* (1999) Effects of cell-bound Microcystins on survival and feeding of Daphnia spp. *Appl. Environ. Microbiol.*, **67**, 3523–3529.

Downloaded from http://plankt.oxfordjournals.org at University of California, Davis on June 19, 2010

Rohrlack, T., Christoffersen, K., Kaerbernick, M. *et al.* (2004) Cyanobacterial protease inhibitor Microviridin J causes a lethal molting disruption in *Daphnia pulicaria*. *Appl. Environ. Microbiol.*, **70**, 5047–5050.

Sarnelle, O. and Wilson, A. E. (2005) Local adaptation of Daphnia pulicaria to toxic cyanobacteria. *Limnol. Oceanogr.*, **50**, 284–289.

Sommer, T., Armor, C., Baxter, R. *et al.* (2007) The collapse of pelagic fishes in the upper San Franciso Estuary. *Fisheries*, **32**, 270–277.

Tackx, M. L. M., Herman, P. J. M., Gasparini, S. *et al.* (2003) Selective feeding of *Eurytemora affinis* (Copepoda, Calanoida) in temperate estuaries: model and field observations. *Estuarine Coastal Shelf Sci.*, **56**, 305–311.

Tillmans, A. R., Wilson, A. E., Pick, F. R. *et al.* (2008) Meta-analysis of cyanobacterial effects on zooplankton population growth rate: species specific responses. *Fundam. Appl. Limnol.*, **171**, 285–295.

Wang, X., Boquiang, Q., Gao, G. *et al.* (2010) Nutrient enrichment and selective predation by zooplankton promote Microcystis (cyanobacteria) blooms. *J. Plankton Res.* (in press).

Wiegand, C. and Pflugmacher, S. (2005) Ecotoxicological effects of selected cyanobacterial metabolites a short review. *Toxicol. Appl. Pharmacol.*, **203**, 201–218.

Wilson, A. E., Sarnelle, O. and Tillmanns, A. R. (2006) Effects of cyanobacterial toxicity and morphology on the population growth of freshwater zooplankton: meta-analyses of laboratory experiments. *Limnol. Oceanogr.*, **51**, 1915–1924.

Work, K. A. and Havens, K. A. (2003) Zooplankton grazing on bacteria and cyanobacteria in a eutrophic lake. *J. Plankton Res.*, **25**, 1301–1307.

Downloaded from http://plankt.oxfordjournals.org at University of California, Davis on June 19, 2010

Toxicon 55 (2010) 787–794

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Toxic threshold of dietary microcystin (-LR) for quart medaka

Dong-Fang Deng [a], Keke Zheng [b], Foo-Ching Teh [a], Peggy W. Lehman [c], Swee J. Teh [a,*]

[a] Center for Health and the Environment and Aquatic Toxicology Program, School of Veterinary Medicine, University of California, 1321 Haring Hall, One Shield Ave, Davis, CA 95616, USA
[b] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, Shandong Province 266071, China
[c] Department of Water Resources, Division of Environmental Services, Sacramento, CA 95691, USA

ARTICLE INFO

Article history:
Received 12 August 2009
Received in revised form 6 November 2009
Accepted 12 November 2009
Available online 23 November 2009

Keywords:
Apoptosis
Microcystin
Medaka
Protein phosphatase
Reproduction
Stress protein

ABSTRACT

This study was designed to estimate the toxic threshold of male and female fish to microcystins based on different biomarkers. Japanese medaka (*Oryzias latipes*) were fed dietary Microcystin-LR (0, 0.46, 0.85, 2.01 and 3.93 μg MC-LR/g dry diet for 8 weeks at 25 °C. The results revealed that dietary MC-LR inhibited growth at the end of 8 weeks. The survival of embryos and the RNA/DNA ratio of whole fish decreased significantly ($P < 0.05$) in fish fed 3.93 μg MC-LR/g dry diet. Heat shock protein (Hsp60) expression was induced in the liver of female and male fish fed diets containing ≥0.85 and 0.46 μg MC-LR/g diet, respectively. The activity of liver caspase 3/7 was significantly higher in female fish fed 3.93 μg MC-LR/g diet and in males fed 2.01 MC-LR μg/g dry diet than fish fed the control diet. The threshold for inhibition of liver protein phosphatase expression was lower in female (2.01 μg/g diet) than that in male fish (3.93 μg/g diet). Histopathological examination showed significant single-cell necrosis in female and male medaka fed diets containing 0.85 and 3.93 μg MC-LR/g diet, respectively. Based on different biomarkers, this study demonstrated that dietary MC-LR is toxic to Medaka and the effects are gender dependent.

Published by Elsevier Ltd.

1. Introduction

Cyanobacteria (*Microcystis aeruginosa*) blooms are known to cause deleterious effects in aquatic ecosystems including zooplankton, fish, waterfowl, mammals, and humans. The most common and well-studied cyanotoxin is the hepatotoxin microcystin-LR (MC-LR), which has been on the rise in abundance and distribution in the upstream portion of the San Francisco Estuary since 1999 (Lehman et al., 2005). Of the 70+ isoforms of microcystins identified, MC-LR is the most studied, toxic and common (Zurawell et al., 2005). Although MC-LR specifically targets liver (Carmichael, 1995), it also impairs the function of other organs such as kidney, gills and the gastrointestinal tract (Rabergh et al., 1991; Kotak et al., 1996; Carbis et al., 1997). Several studies have reported the impact of MC-LR on the reproductive system in mice (Ding et al., 2006), rat (Li et al., 2008; Xiong et al., 2009), and fish (Baganz et al., 1998). Furthermore, field investigations on aquatic invertebrates and fish have strongly implicated the adverse effect of MC-LR on reproductive organs (Chen and Xie, 2005; Zhang et al., 2009).

MC-LR toxicity is the result of inhibition of phosphatase (PP1/PP2A) activity (Runnegar et al., 1993) and destruction of the cytoskeleton, which ultimately leads to cytotoxicity, interruption of cell division, and tumor-promoting activity (Carmichael, 1994; Humpage and Falconer, 1999; Fischer et al., 2000). Microcystin toxicity is also due to oxidative stress that causes apoptosis or necrosis depending on exposure concentration and duration (Ding and Ong, 2003; Li et al., 2005, 2007; Morena et al., 2005; Cazenave et al., 2006).

Most investigations on microcystin toxicity were based on aqueous (Tencalla et al., 1994), one time force-feeding (Tencalla and Dietrich, 1997), short term dietary exposure bioassays (Juhel et al., 2006) that determine the acute effects of microcystins on fish (Sun et al., 2008). Only

* Corresponding author. Fax: +1 530 752 7690.
E-mail address: sjteh@ucdavis.edu (S.J. Teh).

0041-0101/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.toxicon.2009.11.012

limited information is available on the chronic dietary effect of microcystin on fish (Xie et al., 2004; Zhao et al., 2006) which was shown to be the major route of microcystin toxicity for fish in the natural environment (Zhang et al., 2009). In addition, there has been growing evidence to indicate that a suite of environmental chemicals, both anthropogenic and those occurring naturally, have the potential to alter endocrine-mediated sexual development resulting in disruption of gonadal sex differentiation and gametogenesis (Shutt, 1976; Bergeron et al., 1994; White et al., 1994; Kelce and Wilson, 1997; Gray et al., 2006). Medaka (*Oryzias latipes*) is a well-studied, highly-responsive fish model that has been used successfully to characterize acute and chronic toxicity in fish. Recent studies have also demonstrated that medaka is an appropriate model for studying toxic effects of cyanobacteria (Jacquet et al., 2004; Huynh-Delerme et al., 2005; Escoffiera et al., 2007; Mezhoud et al., 2008). To our knowledge, there is no information on gender effects of MC-LR on fish. The purpose of this study was to determine the dietary toxic threshold of MC-LR on male and female medaka based on integrated biomarkers. We hypothesized that 1) MC-LR affects reproduction performance in fish and 2) the sensitivity to the toxic effect of MC-LR is gender dependent.

2. Materials and methods

2.1. Experimental diets

Five test diets were prepared to contain graded levels of microcystin-LR (MC-LR), Dietary levels were: 0, 0.46, 0.85, 2.01 and 3.93 μg/g dry diet, respectively, and the levels of MC-LR were analyzed based on the method described by Hu et al. (2008). MC-LR (*M. aeruginosa*, $C_{49}H_{74}N_{10}O_{12}$) was purchased from EMD Biosciences Inc. (San Diego, CA, USA). The control diet was formulated without supplementation of MC-LR. The basal diet contained (g/kg): vitamin free casein, 310; wheat gluten, 150; dextrin, 272; egg albumin, 40; soy lecithin, 52; non nutritive bulk, 36; cod liver oil, 50; corn oil, 20; vitamin premix, 40; and mineral premix, 30. Except for the vitamin and mineral premixes, which were purchased from ICN (Biomedical, Inc., Irvine, CA), all other ingredients were obtained from U.S. Biochemical Corporation (Cleveland, OH, USA). The dry ingredients were thoroughly mixed before the oil was added. Double distilled water containing different concentrations of MC-LR (previously dissolved in methanol) was added to make wet dough. Pellets were prepared, freeze-dried and stored in the dark at −20 °C until use (Deng et al., 2008).

2.2. Dietary exposure of MC-LR

Embryos of Japanese medaka (*O. latipes*) were collected from our medaka culture system and separated by gender within 4 days post fertilization based on sex-linked coloration (Wada et al., 1998). After hatching (usually 8–10 days post-fertilization), larvae were cultured in a recirculation system with 20 fiberglass tanks (20 L per tank) and fed three times daily with the basal purified diet until used for the exposure study. Water flow-rate and temperature was 0.9 L/min and 25 ± 1 °C, respectively. Water quality including dissolved oxygen (8.3 mg L^{-1}), pH (7.8), water hardness (120 mg L^{-1}), and ammonium (not detectable) were monitored weekly.

The dietary exposure of MC-LR was conducted using 7-week old medaka. The initial body weight of fish was 82 ± 2 mg. Four tanks were randomly assigned to each dietary treatment with 2 tanks per gender and 100 fish per tank. Fish were fed twice per day (0900 and 1500 h) based on 5% of body weight daily. Water flow was stopped during feeding to prevent contamination of the recirculation system with dissolved MC-LR. The waste, uneaten feed and 50% of the water were siphoned from each tank 30 min after each feeding. To ensure that dissolved MC-LR from the diets did not contribute to any significant health effect to the fish, charcoal filters were changed weekly and 100% of the water in the recirculation system was replaced each day. Care, maintenance, handling, and tissue sampling of the fish followed the protocols approved by the University of California-Davis Animal Care and Use Committee.

2.3. Growth and reproduction

Fish were weighed at the end of 2, 4 and 8 weeks of feeding to estimate fish growth. In addition, at the end of 4 weeks of feeding, 30 females and 20 males fed the same dietary MC-LR concentration were mixed and allowed to breed to estimate reproductive performance. The fecundity (egg production per female) and survival of embryos were monitored each morning.

2.4. Sampling

At the end of 8 weeks, fish were killed by an overdose of MS-222 (tricaine methane sulfonate, Argent Chemical Laboratories Inc., Redmond, WA). Fish were weighed and measured for length, then separated into three groups. Group 1): 5 females and 5 males from each replicate tank were fixed in 10% neutral buffered formalin for histopathological examination by the method described by Teh et al. (2004). Group 2): 5 females and 5 males from each replicate tank were dissected to remove liver and ovary tissues. Liver and ovary tissues were weighed to estimate liver and ovarian somatic indices. Tissues were frozen in liquid nitrogen and stored at −80 °C until used for stress protein, protein phosphatase analysis and enzyme assay. Group 3): 5 females and 5 males from each replicate tank were frozen in liquid nitrogen and stored at −80 °C until pulverized with liquid nitrogen using a Freezer/Mill (SPEX SamplePrep, L.L.C., Metuchen, NJ, USA) and used for RNA/DNA analysis to estimate fish growth and recent feeding status.

2.5. Sample analysis

Fixed samples for histopathology were dehydrated in a graded ethanol series and embedded with both surgically cut sections face down in paraffin. Serial longitudinal sections (3 μm) were stained with hematoxylin and eosin (H&E), and lateral views of liver, kidney and gonads were screened for a variety of histopathological features and lesions. Livers were analyzed for lesions of glycogen depletion (GD), lipidosis (LIP), and single-cell necrosis

(SCN) and scored on an ordinal ranking system of 0 = none/minimal, 1 = mild, 2 = moderate, and 3 = severe using a BH-2 Olympus microscope as described by Teh et al. (2004). Briefly, Glycogen depletion (GD) is characterized by decreased size of hepatocytes, loss of the "lacy", irregular, and poorly demarcated cytoplasmic vacuolation typical of glycogen and increased cytoplasmic basophilia (i.e., blue coloration). Fatty vacuolar degeneration or lipidosis (LIP) is characterized by excess lipid appears as clear, round, and well demarcated cytoplasmic vacuoles. Single cell necrosis (SCN) is characterized by cells having eosinophilic (i.e., pink coloration) cytoplasm with nuclear pyknosis and karyorrhexis.

For stress protein and enzyme assays, frozen liver samples were extracted for protein according to the method described by Deng et al. (2009) using an ice-cold T-PER tissue protein extraction reagent (Pierce, Rockford, IL). Protein concentration of the supernatant was determined by the improved Lowry method (Bio-Rad, DC Protein Assay kit). Supernatant protein was used for analysis of stress protein, caspase and protein phosphatase activity.

Expression of Hsp60 and protein phosphatase in liver was analyzed by western block technique as described by Deng et al. (2009) except that 10% Tris–HCl precasted gels were used in the current study. The primary and second antibodies for PP1 and PP2A were purchased from Santa Cruz Biotechnology, Inc (CA, USA). The antibodies of stress proteins were purchased from Assay Designs Inc. (Ann Arbor, MI, and Santa Cruz Biotechnology, Inc, CA, USA). Equal amounts of protein (25 μg) were loaded onto 10% Tris–HCl precasted gels and separated by one-dimensional SDS-PAGE gel. Western blot and ECL (enhanced chemiluminescence) detection were performed by the methods of Hemre et al. (2004). Protein bands were quantified by a GS-710 imaging densitometer (Bio-Rad, Hercules, CA, USA). Protein standard (Assay Designs Inc., Ann Arbor, MI, and Santa Cruz Biotechnology, Inc, CA, USA) and molecular weight markers (Amersham Biosciences Corp, Piscataway, PA, USA) were loaded with samples in each gel to confirm the molecular mass of bands. The relative band density was calculated by comparing the band density of sample to that of standard.

Caspase-3/7 was determined by Apo-One Homogeneous caspase-3/7 Assay kit (Promega Corporation, Madison, WI, USA). Fluorescence activity was determined at an excitation wavelength of 485 nm and an emission wavelength of 530 nm by a Spectra Max M2 micro plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). The caspase activity was expressed as fluorescence/mg protein. Nucleic acids were measured by an ethidium bromide fluorometric technique (Caldarone et al., 2001) and quality control of analysis followed the same protocol as Deng et al. (2009).

2.6. Statistics

Data are presented as means ± standard errors and tested for homogeneity of variance before being analyzed by STATISTITC 6.0 software. All data were subjected to two-way analysis of variance to determine treatment and their interaction effects. Significant differences between the effect of dietary microcystin × gender were estimated by a Fisher LSD test ($P < 0.05$).

3. Results

3.1. Growth and reproduction performances

During the first 4 weeks of feeding, dietary microcystin did not affect fish growth or condition factor (CF), an index of fish fitness (Table 1). There was no difference in body weight (BW) between gender. The value of CF, however, was higher ($P < 0.05$) in female than in male fish after 4 weeks of feeding. At the end of 8 weeks of feeding, female fish fed the diet containing 0.85 μg MC-LR/g diet and male fish fed the 0.46 μg MC-LR/g diet had significantly lower BW than fish fed the control diet (Table 2). The BW and HSI were also higher in female fish than in male fish.

Dietary MC-LR did not show any adverse effect on gonadosomatic Index (GSI = 100 × gonad weight (g)/body weight (g)), fecundity or embryo weight (Table 3). Survival was significantly decreased in embryos collected from fish fed the 3.93 μg MC-LR/g diet. Gender differences in the RNA/DNA ratio were not observed in fish fed similar dietary treatments. However, female and male medaka fed the 3.93 and 2.01 μg MC-LR/g diet had significantly lower RNA/DNA ratios compared to control fish (Fig. 1).

3.2. Molecular biomarkers and histopathology

The levels of hsp60 were significantly induced in female and male medaka fed 0.85 and 0.46 μg MC-LR/g diet, respectively (Fig. 2). Dietary MC-LR did not affect the levels of hsp70 in liver (data not shown). Measurement of apoptosis, i.e., caspase 3/7 activities increased significantly in female fish fed the 3.93 μg MC-LR/g diet and in male fish fed ≥2.01 μg MC-LR/g diet (Fig. 3). The levels of PP1 in liver decreased significantly in females fed ≥2.01 μg MC-LR/g diets but no changes were observed in males. The PP2A levels in liver of female and male fish were both significantly inhibited when fed the 2.01 and 3.93 μg MC-LR/g diet, respectively. Histopathological examination showed that prevalence of lesions and the liver lesion score were generally increased with increased concentrations of dietary MC-LR (Tables 4–6). Female fish fed the diets containing 0.85 and 3.93 MC-LR showed significant higher lesion scores for single-cell necrosis compared to controls (Table 4). Male fish fed the diet containing 3.93 MC-LR had the highest lesion scores for lipidosis compared to that in fish fed the control diet (Table 5).

4. Discussion

Growth inhibition has been observed in different species of fish exposed to microcystin toxins. For example, depressed growth was observed in carp (*Cyprinus carpio* L.) after oral feeding of microcystis for 28 days at a dose of 50 μg microcystin/kg body weight (Li et al., 2004). The growth of brown trout (*Salmo trutta* L.) was retarded by exposure of fish to water contaminated with MC-LR (Bury et al., 1995). The current study reveals that the effect of dietary MC-LR is both dose and time dependent. Our results indicate that 4 weeks of dietary exposure was too short to impair growth of medaka. The lower BW and CF in medaka fed dietary MC-LR for 8 weeks may be the result of shifting

Table 1
Growth performance of medaka exposed to test diets for 2 and 4 weeks.

MC-LR (ug/g)	BW_2 (mg)		CF_2		BW_4 (mg)		CF_4	
	Female	Male	Female	Male	Female	Male	Female	Male
0	119.3 ± 2.6	143.0 ± 7.2	0.85 ± 0.01	0.88 ± 0.02	169.0 ± 7.3	168.2 ± 5.9	0.88 ± 0.04^{x}	0.80 ± 0.01^{y}
0.46	119.9 ± 9.1	133.1 ± 3.6	0.87 ± 0.00	0.84 ± 0.02	170.2 ± 13.8	165.9 ± 7.4	0.87 ± 0.02^{x}	0.80 ± 0.02^{y}
0.85	110.1 ± 6.4	125.6 ± 2.4	0.86 ± 0.01	0.86 ± 0.05	172.8 ± 9.3	167.8 ± 3.5	0.85 ± 0.02^{x}	0.77 ± 0.02^{y}
2.01	128.7 ± 1.6	138.7 ± 2.7	0.85 ± 0.01	0.86 ± 0.03	166.3 ± 10.4	164.1 ± 5.1	0.90 ± 0.01^{x}	0.86 ± 0.03^{y}
3.93	123.3 ± 5.2	136.2 ± 7.0	0.82 ± 0.00	0.80 ± 0.02	164.9 ± 7.0	159.3 ± 9.3	0.88 ± 0.01^{x}	0.81 ± 0.01^{y}

Data are presenting as Mean ± SE. Initial weight of medaka was 81–84 mg. BW_2 and BW_4: Body weight of week 2 and week 4, respectively; CF_2 and CF_4: Condition factor of week 2 and week 4, respectively. Letter x and y indicate significant difference between male and female fish fed the same diet ($P < 0.05$). Condition factor (CF) = 100 × body weight (g)/body length $(cm)^3$.

Table 2
Growth performance of medaka exposed to test diets for 8 weeks.

MC-LR (ug/g)	BW (mg)		CF		HSI (%)	
	Female	Male	Female	Male	Female	Male
0	290.7 ± 12.2 ^{a}	273.4 ± 12.7 ^{a}	1.07 ± 0.04 ^{a,x}	0.99 ± 0.05 ^{a,y}	2.78 ± 0.18 ^{x}	1.63 ± 0.13 ^{y}
0.46	271.9 ± 12.8 ^{ab}	245.9 ± 7.3 ^{b}	0.95 ± 0.02 ^{b,x}	0.85 ± 0.01 ^{b,y}	2.96 ± 0.18 ^{x}	1.52 ± 0.12 ^{y}
0.85	256.2 ± 11.3 ^{b}	232.5 ± 7.9 ^{b}	0.93 ± 0.02 ^{b,x}	0.84 ± 0.02 ^{b,y}	2.95 ± 0.19 ^{x}	1.64 ± 0.08 ^{y}
2.01	271.8 ± 9.8 ^{ab}	248.9 ± 5.7 ^{ab}	0.97 ± 0.01^{b,x}	0.84 ± 0.03 ^{b,y}	2.73 ± 0.22 ^{x}	1.76 ± 0.10 ^{y}
3.93	257.8 ± 9.3 ^{b}	248.5 ± 6.6 ^{ab}	0.92 ± 0.02 ^{b,x}	0.81 ± 0.01 ^{b,y}	2.60 ± 0.17 ^{x}	1.63 ± 0.12 ^{y}

Data are presenting as Mean ± SE. BL: Body length; BW: Body weight; CF: Condition factor; HSI: Hepatosomatic index. Different letters indicate significantly difference among different dietary treatments ($P < 0.05$). Different letters within the same column (a, b) indicate significant difference among dietary treatments and the letters within the same row (x, y) indicate significant difference between gender fed the same diet ($P < 0.05$). Hepatosomatic Index (HSI) = 100 × liver weight/body weight (g).

Table 3
Reproduction performance of medaka exposed to test diets for 8 weeks.

MC-LR (ug/g)	Ovarian ratio (%)	Fecundity (egg number/female)	Embryo weight (mg)	Survival of embryo (%)
0	6.31 ± 0.38	4.1 ± 0.3	1.12 ± 0.01	62.3 ± 2.0[a]
0.46	5.18 ± 0.40	3.5 ± 0.3	1.13 ± 0.01	62.7 ± 1.8[a]
0.85	6.21 ± 0.36	3.6 ± 0.1	1.11 ± 0.01	68.1 ± 2.4[a]
2.01	5.63 ± 0.37	3.9 ± 0.5	1.14 ± 0.01	63.8 ± 2.8[a]
3.93	7.04 ± 0.98	3.7 ± 0.2	1.10 ± 0.01	54.9 ± 2.6[b]

Data are presenting as Mean ± SE. Different letters (a, b) indicate significantly difference among different dietary treatments ($P < 0.05$).

energy in the fish from supporting growth to handling chronic stress due to the toxin.

Dietary MC-LR at the level of 3.93 μg MC-LR/g diet significantly impaired reproductive performance of medaka based on embryo survival in this study. The low survival of embryos is likely due to the effect of MC-LR on embryo quality, which may have affected egg or sperm quality and fertilization process or other unknown factors that were not investigated in this study. Jacquet et al. (2004) found that medaka embryos had low survival and earlier hatching when exposed to MC-LR by microinjection. Oberemm et al. (1997) also found that MC-LR in water (5–50 μg/L) decreased embryo development and retarded larval growth of zebra fish. Similarly, exposure of southern catfish fertilized eggs to crude extracts of MCs (10–100 mg MC-LR eq L^{-1}) delayed egg and larval development, reduced hatching rate, increased malformation rate and hepatocytes damage in larvae (Zhang et al., 2008). Although the mechanism of MC-LR on reproduction is not well understood, oxidative stress as well as an increased energy demand for detoxification in adult fish or the trophic transfer of MC-LR to the embryo may cause the deleterious effects of microcystins on fish earlier life stages when organogenesis is not completed (Wiegand et al., 1999). The current study presents strong evidence that dietary *Microcystis* reduces fish reproduction. This effect of MC-LR on embryo survival or hatching could be a contributing factor to the decline of pelagic fish populations exposed to microcystins through their food web in the San Francisco estuary.

The RNA/DNA ratio, an indicator of recent growth or nutritional condition of fish (Clemmesen et al., 1997; Buckley et al., 2004), decreased in medaka fed ≥2.01 μg MC-LR/g diets suggests that MC-LR inhibited protein synthesis and thus growth. This further supports our assumption that decreased food intake or shifting of energy from growth to toxin stress and is in agreement with the reduction in growth by dietary MC-LR as discussed above. The lower threshold of MC-LR inhibiting protein synthesis (RNA/DNA ratio) in females than males indicated that the growth of females is more sensitive than males to the effects of MC-LR.

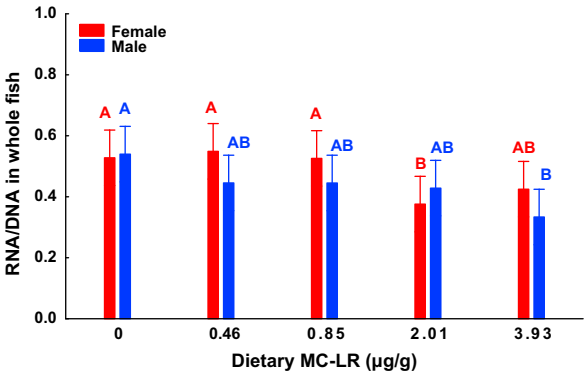

Fig. 1. The ratios of RNA/DNA in whole fish fed different levels of dietary MC-LR for 8 weeks. Data are presented as Mean ± SE. Different letters indicate significant difference among dietary treatments within the same gender ($P < 0.05$).

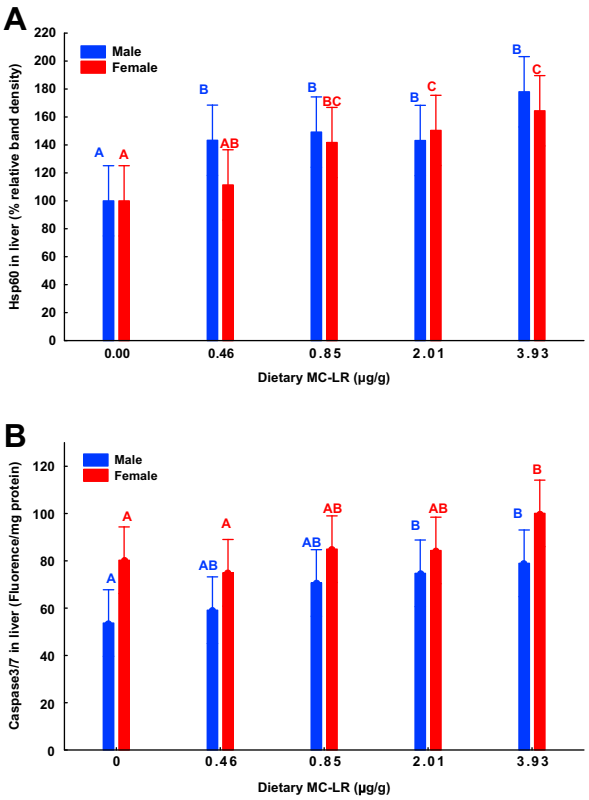

Fig. 2. The Hsp60 expression (A) and activity of caspase 3/7 (B) in liver of medaka fed different levels of dietary MC-LR for 8 weeks. Data are presented as Mean ± SE. Different letters indicate significant difference among dietary treatments within the same gender ($P < 0.05$).

Heat shock proteins (Hsp), also called stress protein, are a group of structurally conserved proteins present at a relative low level under normal physiological conditions (Basu et al., 2002). The level of Hsp, however, can be induced via a wide variety of stressors such as temperature change (Deng et al., 2009), contaminant exposure (Sanders, 1993; Werner and Nagel, 1997) or feeding (Cara et al., 2005; Deng et al., 2009). Heat shock protein is also involved in apoptotic processes through their role as chaperones. Hsp60 is mainly localized in the mitochondrial matrix (Werner and Nagel, 1997). The increased level of Hsp60 in Medaka liver tissue for this study suggests that fish turn on their earlier defense mechanism when exposed to diets containing MC-LR as low as 0.46–0.85 μg MC-LR/g diet. Increasing levels of dietary MC-LR, however, may surpass the defense capacity of stress protein to deal with the folding or degradation proteins and eventually damage the

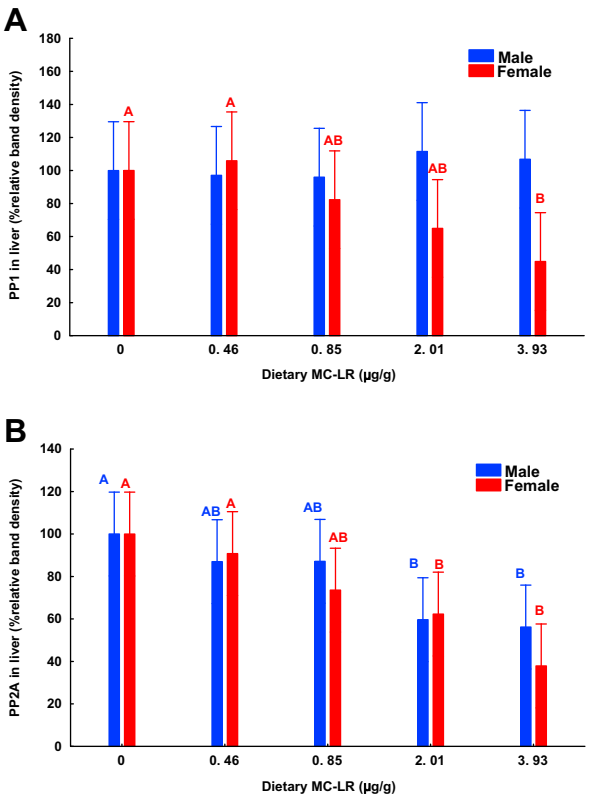

Fig. 3. The protein phosphatase PP1 (A) and PP2A (B) expression in liver of medaka fed different levels of dietary MC-LR for 8 weeks. Data are presented as Mean ± SE. Different letters indicate significant difference among dietary treatments with the same gender ($P < 0.05$).

Table 4
Summary of histopathology scores in female medaka fed different diets.

Dietary MC-LR (μg/g)	Parameters	Fish number of different lesion score				Average score	Standard error
		0	1	2	3		
0	GD	11	4	3	2	0.80[a]	0.46
0.46	GD	10	4	5	1	0.85[a]	0.40
0.85	GD	8	3	8	1	1.10[a]	0.33
2.01	GD	11	2	2	5	1.20[a]	0.69
3.93	GD	7	2	4	7	1.55[a]	0.57
0	SCN	14	6	0	0	0.25[a]	0.05
0.46	SCN	12	8	0	0	0.40[ab]	0.08
0.85	SCN	7	13	0	0	0.65[b]	0.10
2.01	SCN	12	8	0	0	0.40[ab]	0.14
3.93	SCN	9	10	1	0	0.60[b]	0.14
0	LIP	14	5	1	0	0.30[a]	0.24
0.46	LIP	13	5	2	0	0.45[a]	0.17
0.85	LIP	12	8	0	0	0.40[a]	0.14
2.01	LIP	14	4	1	1	0.45[a]	0.26
3.93	LIP	9	5	2	4	0.90[a]	0.42

GD: glycogen depletion; SCN: single-cell necrosis or piecemeal necrosis; Lip: lipidosis or hepatocellular vacuolation. Scores represent ordinal effects: 0 = non/minimal. 1 = mild, 2 = moderate, and 3 = severe. Different letters indicate significant difference among dietary treatments ($P < 0.05$). Twenty fish from each dietary treatment were evaluated for histopathology.

Table 5
Summary of histopathology scores in male medaka fed different diets.

Dietary MC-LR (μg/g)	Parameters	Fish number of different lesion score				Average score	Standard error
		0	1	2	3		
0	GD	14	5	1	0	0.35[a]	0.17
0.46	GD	12	6	2	0	0.50[a]	0.13
0.85	GD	7	8	3	2	1.00[a]	0.38
2.01	GD	8	4	6	2	1.10[a]	0.48
3.93	GD	5	2	9	4	1.50[a]	0.53
0	SCN	17	3	0	0	0.15[a]	0.10
0.46	SCN	19	1	0	0	0.05[a]	0.05
0.85	SCN	14	6	0	0	0.30[a]	0.13
2.01	SCN	18	2	0	0	0.10[a]	0.10
3.93	SCN	12	8	0	0	0.40[a]	0.22
0	LIP	18	2	0	0	0.10[a]	0.06
0.46	LIP	14	4	2	0	0.40[ab]	0.14
0.85	LIP	9	8	1	2	0.80[ab]	0.18
2.01	LIP	12	8	0	0	0.35[ab]	0.10
3.93	LIP	10	3	5	2	0.95[b]	0.35

GD: glycogen depletion; SCN: single-cell necrosis or piecemeal necrosis; Lip: lipidosis or hepatocellular vacuolation. Scores represent ordinal effects: 0 = non/minimal. 1 = mild, 2 = moderate, and 3 = severe. Different letters indicate significant difference among dietary treatments ($P < 0.05$). Twenty fish from each dietary treatment were evaluated for histopathology.

cell. As a consequence, more defense or protection mechanisms will be needed to prevent further damage. Apoptosis is a physiological process involving caspase as one of the executers that eliminate damaged or unwanted DNA from the cell. This prevents cells from further deleterious effects damaged proteins or cells. Therefore, the increased caspase activity in fish fed high dietary MC-LR (2.01–3.93 μg/g diet) suggests that an apoptosis pathway was involved in the protection of medaka from toxic effects at high dietary concentrations of MC-LR. The threshold based on Hsp60 expression and caspase activity was lower for males than females, suggesting that the defense mechanism in male medaka is more responsive to dietary MC-LR than in females. This sensitive or efficient protection mechanism may have protected the male fish from further harmful effects by the MC-LR toxin. This is further supported by the less significant inhibition of protein phosphatase expression (PP1 and PP2A) in males than in females. The threshold based on protein phosphatase inhibition was generally lower in females (2.01–3.93 μg/g diet) than in males (≥3.93 μg/g diet). The current study also

Table 6
The toxic threshold of dietary MC-LR for medaka based on different biomarkers.

Parameters	Effective concentration (μg MC-LR/g diet)	
	Female	Male
Body weight and CF	0.46–0.85	0.46
Stress protein	0.85	0.46
RNA/DNA	2.01	3.93
Embryo survival	3.93	
Apoptosis (caspase3/7)	3.93	2.01
Protein phosphatase	2.01–3.93	2.01
Histopathology	0.85–3.93	3.93

demonstrated that MC-LR was not only inhibiting the activity of protein phosphatase as have been found in previous study (Runnegar et al., 1993 but also decreasing the enzyme levels in liver in this study. The long term exposure of high dose of MC-LR may have resulted in necrosis of cell and thus protein (enzyme) synthesis was decreased. The mechanism for MC-LR inhibiting the enzyme expression, however, is not studied in this experiment and will be needed for future research.

The dietary MC-LR (0.85 μg/g diet) led to single-cell necrosis in the liver of female but not male Medaka also suggested that females are more sensitive to this toxin. Gender difference in tumor incidence has been observed in medaka exposed to diethylnitrosamine, with higher incidence and faster development in foci and tumors in females than in males (Teh and Hinton, 1998). The mechanism is not clear but steroid hormones may play an important role in these differences between genders.

In summary, our results demonstrated that the toxic threshold of dietary MC-LR for medaka is different between males and females (Table 6). Male medaka appears to have a more sensitive defense mechanism than female medaka but females are more sensitive to the toxic effect of MC-LR. As a consequence, the effective toxic thresholds such as RNA/DNA ratio, inhibition of protein phosphatase and occurrence of cell necrosis were lower in mature females (2.01 μg MC-LR/g diet) than in males (3.93 μg MC-LR/g diet). The decreased survival of embryos by dietary MC-LR suggests a possible reason for the reported decline in fish population, especially pelagic species of fish, in habitats where toxic algal blooms naturally occur.

Acknowledgements

Funding of this study is supported by Dr. Swee Teh's Aquatic Toxicology Program fund and partially by the California Interagency Ecological Program Pelagic Organisms Decline (IEP-POD), CALFED (Project# PO685515), and California Department of Water Resources (Contract # 4600007499). We would also like to thank Dr. Robert P. Wilson for his help in editing and reviewing this manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Baganz, D., Staaks, G., Steinberg, C., 1998. Impact of the cyanobacteria toxin, microcystin-LR on behaviour of zebrafish, *Danio rerio*. Water Res. 32, 948–952.

Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M., Iwama, G.K., 2002. Heat shock protein genes and their functional significance in fish. Gene 295, 173–183.

Bergeron, J.M., Crews, D., McLachlan, J.A., 1994. PCB's as environmental estrogens: turtle sex determination as a biomarker of environmental contamination. Environ. Health Perspect. 102, 780–781.

Buckley, L.J., Caldarone, E.M., Lough, G., 2004. Optimum temperature and food-limited growth of larval Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) on Georges Bank. Fish. Oceanogr. 13, 134–140.

Bury, N.R., Eddy, F.B., Codd, G.A., 1995. The effects of the cyanobacterium *Microcystis aeruginosa*, the cyanobacterial toxin microcystin-LR and ammonia on growth rate and ionic regulation of brown trout (*Salmo trutta* L.). J. Fish. Biol. 46, 1042–1054.

Caldarone, E.M., Wagner, M., St. Onge-Burns, J., Buckley, L.J., 2001. Protocol and guide for estimating nucleic acids in larval fish using a fluorescence microplate reader, Northeast Fish. Sci. Cent. Ref. Doc. 01-11 (2001) 22 pp. National Marine Fisheries Service, 166 Water Street, Woods Hole, MA 02543-1026.

Cara, J.B., Aluru, N., Moyano, F.J., Vijayan, M.M., 2005. Food-deprivation induces HSP70 and HSP90 protein expression in larval gilthead sea bream and rainbow trout. Comp. Biochem. Physiol. 142B, 426–431.

Carbis, C.R., Rawlin, G.T., Grant, P., Mitchell, G.F., Anderson, J.W., McCauley, I., 1997. A study of feral carp, *Cyprinus carpio* L., exposed to *Microcystis aeruginosa* at Lake Mokoan, Australia, and possible implications for fish health. J. Fish. Dis. 20, 81–91.

Carmichael, W.W., 1994. The toxins of cyanobacteria. Sci. Am. 270, 78–86.

Carmichael, W.W., 1995. Toxic microcystis in the environment. In: Watanabe, M.F., Harada, K., Carmichael, W.W., Fujiki, H. (Eds.), Toxic Microcystis. CRC Press, New York, pp. 1–12.

Cazenave, J., de los Angeles Bistoni, M., Zwirnmann, E., Wunderlin, D.A., Wiegand, C., 2006. Attenuating effects of natural organic matter on microcystin toxicity in zebra fish (*Danio rerio*) embryos – benefits and costs of microcystin detoxication. Environ. Toxicol. 21, 22–32.

Chen, J., Xie, P., 2005. Seasonal dynamics of the hepatotoxic microcystins in various organs of four freshwater bivalves from the large eutrophic Lake Taihu of subtropical China and the risk to human consumption. Environ. Toxicol. 20, 572–584.

Clemmesen, C., Sanchez, R., Wongtschowski, C., 1997. A regional comparison of the nutritional condition of SW Atlantic anchovy larvae, *Engraulis anchoita*, based on RNA/DNA ratios. Arch. Fish. Mar. Res. 45, 17–43.

Deng, D.F., Teh, F.C., Teh, S.J., 2008. Effect of dietary methylmercury and seleno-methionine on Sacramento splittail larvae. Sci. Total Environ. 407, 197–203.

Deng, D.F., Wang, C.F., Lee, S.H., Bai, S.C., Hung, S.S.O., 2009. Feeding rates affect heat shock protein levels in liver of larval white sturgeon (*Acipenser transmontanus*). Aquaculture 287, 223–226.

Ding, W.-X., Ong, C.N., 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. FEMS Microbiol. Lett. 2 (20), 1–7.

Ding, X.S., Li, X.Y., Duan, H.Y., Chung, I.K., Lee, J.A., 2006. Toxic effects of *Microcystis* cell extracts on the reproductive system of male mice. Toxicon 48, 973–979.

Escoffiera, N., Gaudina, J., Mezhouda, K., Huetb, H., Chateau-Joubertb, S., Turquetc, J., Crespeaub, F., Edery, M., 2007. Toxicity to medaka fish embryo development of okadaic acid and crude extracts of *Prorocentrum* dinoflagellates. Toxicon 49, 1182–1192.

Fischer, W.J., Hitzfield, B.C., Tencalla, F., Eriksson, J.E., Mikhailov, A., Dietrich, D.R., 2000. Microcystin-LR toxicodynamics, induced pathology, and immunohistochemical localization in livers of blue-green algae exposed rainbow trout (*Oncorhynchus mykiss*). Toxicol. Sci. 54, 365–373.

Gray Jr., L.E., Wilson, V.S., Stoker, T., Lambright, C., Furr, J., Noriega, N., Howdeshell, K., Ankley, G.T., Guillette, L., 2006. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. Int. J. Androl. 29, 96–104.

Hemre, G., Deng, D., Wilson, R.P., Berntssen, M.H., 2004. Vitamin A metabolism and early biological responses in juvenile sunshine bass (*Morone chrysops x M. saxatilis*) fed graded levels of vitamin A. Aquaculture 235 (1–4), 645–658.

Hu, C.L., Gan, N.Q., He, Z.K., Song, L.R., 2008. A novel chemiluminescent immunoassay for microcystin (MC) detection based on gold nanoparticles label and its application to MC analysis in aquatic environmental samples. Int. J. Environ. Anal. Chem. 88, 267–277.

Humpage, A.R., Falconer, I.R., 1999. Microcystin-LR and liver tumor promotion: effects on cytokinesis, ploidy, and apoptosis in cultured hepatocytes. Environ. Toxicol. 14, 61–75.

Huynh-Delerme, C., Edery, M., Huet, H., Puiseux-Dao, S., Bernard, C., Fontaine, J.-J., Crespeau, F., de Luze, A., 2005. Microcystin-LR and embryo-larval development of medaka fish, *Oryzias latipes*. I. Effects on the digestive tract and associated systems. Toxicon 46, 16–23.

Jacquet, C., Thermes, V., Luze, A.D., Puiseux-Dao, S., Bernard, C., Joly, J.S., Bourrat, F., Edery, M., 2004. Effects of microcystin-LR on development of medaka fish embryo (*Oryzias latipes*). Toxicon 43, 141–147.

Juhel, G., Davenport, J., O'Halloran, J., Culloty, S.C., O'Riordan, R.M., James, K.F., Furey, A., Allis, O., 2006. Impacts of microcystins on the feeding behaviour and energy balance of zebra mussels, *Dreissena*

polymorpha: a bioenergetics approach. Aquat. Toxicol. (Amsterdam) 79, 391–400.

Kelce, W.R., Wilson, E.M., 1997. Environmental antiandrogens: developmental effects, molecular mechanisms, and clinical implications. J. Mol. Med. 75, 198–207.

Kotak, B.G., Semalulu, S., Fritz, D.L., Prepas, E.E., Hrudey, S.E., Coppock, R. W., 1996. Hepatic and renal pathology of intraperitoneally administered microcystin-LR in rainbow trout (*Oncorhynchus mykiss*). Toxicon 34, 517–525.

Lehman, P.W., Boyer, G., Hall, C., Waller, S., Gehrts, K., 2005. Distribution and toxicity of a new colonial *Microcystis aeruginosa* bloom in the San Francisco Bay estuary, California. Hydrobiologia 541, 87–99.

Li, X.Y., Chung, I.K., Kim, J.I., Lee, J.A., 2004. Subchronic oral toxicity of microcystin in common carp (*Cyprinus carpio* L.) exposed to Microcystis under laboratory conditions. Toxicon 44, 821–827.

Li, X.Y., Chung, I.K., Kim, J.I., Lee, J.-A., 2005. Oral exposure to *Microcystis* increases activity-augmented antioxidant enzymes in the liver of loach (*Misgurnus mizolepis*) and has no effect on lipid peroxidation. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 141, 292–296.

Li, X.Y., Wang, J., Liang, J.B., Liu, Y.D., 2007. Toxicity of microcystins in the isolated hepatocytes of common carp (*Cyprinus carpio* L.). Ecotoxicol. Environ. Saf. 67, 447–451.

Li, Y., Sheng, J., Sha, J.H., Han, X.D., 2008. The toxic effects of microcystin-LR on the reproductive system of male rats in vivo and in vitro. Reprod. Toxicol. 26, 239–245.

Mezhoud, K., Bauchet, A.L., Chateau-Joubert, S., Praseuth, D., Marie, A., Francois, J.C., Fontaine, J.J., Jaeg, J.P., Cravedi, J.P., Puiseux-Dao, S., Edery, M., 2008. Proteomic and phosphoproteomic analysis of cellular responses in medaka fish (*Oryzias latipes*) following oral gavage with microcystin-LR. Toxicon 51, 1431–1439.

Morena, I., Pichardo, S., Jos, A., Gomez-Amores, L., Mate, A., Vazquez, C.M., Camean, A.M., 2005. Antioxidant enzyme activity and lipid peroxidation in liver and kidney of rats exposed to microcystin-LR administered intraperitoneally. Toxicon 45, 395–402.

Oberemm, A., Fastner, J., Steinberg, C.E.W., 1997. Effects of microcystin-LR and cyanobacterial crude extracts on embryo-larval development of zebrafish (*Danio rerio*). Water Res. 31, 2918–2921.

Rabergh, C.M.I., Bylund, G., Eriksson, J.E., 1991. Histopathological effects of microcystin-LR a cyclic peptide toxin from the cyanobacterium blue-green alga *Microcystis-aeruginosa* on common carp *Cyprinus-carpio* L. Aquat. Toxicol. 20, 131–146.

Runnegar, M.T., Kong, S., Berndt, N., 1993. Protein phosphates inhibition and in-vivo hepatotoxicity of microcystin. Am. Physiol. Soc. 265, G224–G231.

Sanders, B.M., 1993. Stress proteins in aquatic organisms: an environmental perspective. Crit. Rev. Toxicol. 23, 49–75.

Shutt, D.A., 1976. The effect of plant oestrogens an animal reproduction. Endeavour 35, 110–113.

Sun, Y., Tang, R., Li, D., Zhang, X., Fu, J., Xie, P., 2008. Acute effects of microcystins on the transcription of antioxidant enzyme genes in crucian carp *Carassius auratus*. Environ. Toxicol. 23, 145–152.

Teh, S.J., Deng, X., Deng, D.F., Teh, F.C., Hung, S.S.O., Fan, T.W.M., Liu, J., Higashi, R.M., 2004. Chronic effects of dietary selenium on juvenile Sacramento splittail (*Pogonichthys amcrolepidotus*). Environ. Sci. Technol. 38, 6085–6093.

Teh, S.J., Hinton, D.E., 1998. Gender-specific growth and hepatic neoplasia in medaka (*Oryzias latipes*). Aquat. Toxicol. 41, 141–159.

Tencalla, F., Dietrich, D., 1997. Biochemical characterization of microcystin toxicity in rainbow trout (*Oncorhynchus mykiss*). Toxicon 35, 583–595.

Tencalla, F.G., Dietrich, D.R., Schlatter, C., 1994. Toxicity of *Microcystis aeruginosa* peptide toxin to yearling rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 30, 215–224.

Wada, H., Shimada, A., Fukamachi, S., Naruse, K., Shima, A., 1998. Sex-linked inheritance of the lf locus in the medaka fish (*Oryzias latipes*). Zool. Sci. 15, 123–126.

Werner, I., Nagel, R., 1997. Stress proteins HSP60 and HSP70 in the species of amphipods exposed to cadmium, diazinon, dieldrin and fluoranthene. Environ. Toxicol. Chem. 16, 2393–2403.

White, R., Jobling, S., Hoare, S.A., Sumpter, J.P., Parker, M.G., 1994. Environmentally persistent alkyphenolic compounds are estrogenic. Endocrinology 135, 175–182.

Wiegand, C., Pflumacher, S., Oberemm, A., Meems, N., Beattie, K.A., Steinberg, C.E.W., Codd, G.A., 1999. Uptake and effects of microcystin-LR on detoxication enzymes of early life stages of the zebra fish (*Daniorerio*). Environ. Toxicol. 14, 89–96.

Xie, L., Xie, P., Ozawa, K., Honma, T., Yokoyama, A., Park, H.D., 2004. Dynamics of microcystins-LR and -RR in the planktivorous silver carp in a sub-chronic toxicity experiment. Environ. Poll 127, 431–439.

Xiong, Q., Xie, P., Li, Y., Hao, L., Li, G., Qiu, T., Liu, Y., 2009. Involvement of Fas/FasL system in apoptotic signaling in testicular germ cells of male Wistar rats injected i.v. with microcystins. Toxicon 54, 1–7.

Zhang, X.Z., Xie, P., Wang, W.M., Li, D.P., Li, L., Tang, R., Lei, H., Shi, Z.C., 2008. Dose-dependent effects of extracted microcystins on embryonic development, larvae growth and histopathological changes of southern catfish (*Silurus meridionalis*). Toxicon 51, 449–456.

Zhang, D.W., Xie, P., Liu, Y.Q., Qiu, T., 2009. Transfer, distribution and bioaccumulation of microcystins in the aquatic food web in Lake Taihu, China, with potential risks to human health. Sci. Total Environ. 407, 2191–2199.

Zhao, M., Xie, S., Zhu, X., Yang, Y., Gan, L., Song, L., 2006. Effect of inclusion of blue-green algae meal on growth and accumulation of microcystins in gibel carp (*Carassius auratus gibelio*). J. Appl. Ichthyol. 22, 72–78.

Zurawell, R.W., Chen, H., Burke, J.M., Prepas, E.E., 2005. Hepatotoxic cyanobacteria: a review of the biological importance of microcystins in freshwater environments. J. Toxicol. Environ. Health B Crit. Rev. 8, 1–37.

Science of the Total Environment 407 (2009) 4852–4857

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Microcystin-LR toxicity on dominant copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* of the upper San Francisco Estuary

Kemal A. Ger [a], Swee J. Teh [b,*], Charles R. Goldman [a]

[a] *Environmental Science and Policy, University of California, Davis, California 95616, USA*
[b] *Aquatic Toxicology Program, School of Veterinary Medicine, Department of Anatomy, Physiology, and Cell Biology, 1321 Haring Hall, One Shields Avenue, University of California, Davis, California 95616, USA*

ARTICLE INFO

Article history:
Received 19 March 2009
Received in revised form 22 May 2009
Accepted 26 May 2009
Available online 17 June 2009

Keywords:
Microcystis
Copepods
Microcystin
Harmful algal blooms
San Francisco Estuary

ABSTRACT

This study investigates the toxicity and post-exposure effects of dissolved microcystin (MC-LR) on the dominant copepods of the upper San Francisco Estuary (SFE), where blooms of the toxic cyanobacteria *Microcystis aeruginosa* coincide with record low levels in the abundance of pelagic organisms including phytoplankton, zooplankton, and fish. The potential negative impact of *Microcystis* on the copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* has raised concern for further depletion of high quality fish food. Response of copepods to MC-LR (MC) was determined using a 48-h standard static renewal method for acute toxicity testing. Following exposure, a life table test was performed to quantify any post-exposure impacts on survival and reproduction. The 48-h LC-50 and LC-10 values for MC were 1.55 and 0.14 mg/L for *E. affinis*; and 0.52 and 0.21 mg/L for *P. forbesi*. Copepod populations recovered once dissolved MC was removed and cultures returned to optimal conditions, suggesting no post-exposure effects of MC on copepod populations. Dissolved microcystin above 0.14 mg/L proved likely to have chronic effects on the survival of copepods in the SFE. Since such high concentrations are unlikely, toxicity from dissolved microcystin is not a direct threat to zooplankton of the SFE, and other mechanisms such as dietary exposure to *Microcystis* constitute a more severe risk.

Published by Elsevier B.V.

1. Introduction

Cyanobacterial toxins are a key issue in aquatic management for a variety of reasons that stem from toxin exposure to the foodweb and changes in trophic transfer of carbon (Chorus and Bartram, 1999; Hansson et al., 2007). *Microcystis aeruginosa* is one of the most common species of freshwater cyanobacteria in eutrophic waters and produces several secondary metabolites that are toxic to aquatic organisms including zooplankton (Rohrlack et al., 2003; Wilson et al., 2006).

In recent years there has been an increase in the abundance and distribution of toxic *Microcystis* blooms in the upstream portion of the San Francisco Estuary (SFE), where abundance of pelagic organisms have reached historically low levels (Lehman et al., 2005; Sommer et al., 2007). The calanoid copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* are among the dominant prey species for endangered fish in this system (Kimmerer, 2004). Despite trends of declining abundance, these copepods are the seasonally dominant zooplankton of the upper SFE (*E. affinis* — spring and *P. forbesi* — summer) (Kimmerer, 2004; Sommer et al., 2007). Among the management actions under consideration for reversing the declining fish populations are actions aimed at improving their food supply and especially the seasonal abundance of *E. affinis* and *P. forbesi* (CA Resources Agency, 2007). Thus, potential mechanisms through which *Microcystis* can impact copepod abundance are of both management and scientific concern in the SFE.

Although *Microcystis* produces a variety of secondary metabolites toxic to aquatic organisms, research has mainly focused on microcystins (MCs), which covalently bind to and inhibit protein phosphatases 1 and 2A, causing damage in a wide variety of animals, and can also negatively affect plants, algae, and protozoa (Dawson, 1998; Watanabe et al., 1996; Rohrlack et al., 2003; Zurawell et al., 2005; Gkelis et al., 2006). Of the 70+ different structural variants of MCs identified, MC-LR is one of the most toxic and common forms (WHO, Chorus and Bartram 1999; Best et al. 2003; Zurawell et al., 2005). MCs can have severe impacts on aquatic ecosystems ranging from acute toxicity to the disruption of trophic transfer of energy (Christoffersen, 1996). While MCs can transfer through the foodweb, their fate in the foodweb is not well understood, and it is not clear if MCs actually "bio-accumulate" in the proper sense of the term (Thostrup and Christoffersen, 1999; Ibelings et al., 2005; Xie et al., 2005; Ibelings and Chorus 2007). However, in a recent study MCs were found to transfer from lake water and aquatic animals to a chronically exposed human population with indication of hepatocellular damage (Chen et al., 2009). As an endotoxin, MC remains within healthy cells until they rupture at death when the toxin

* Corresponding author. Tel.: +1 530 754 8183; fax: +1 530 752 7690.
E-mail address: sjteh@ucdavis.edu (S.J. Teh).

0048-9697/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.scitotenv.2009.05.043

dissolves and degrades relatively quickly, in a matter of days to several weeks, though factors determining degradation rates are variable (Perez and Aga, 2005; Zurawell et al., 2005).

In general, *Microcystis* impacts on zooplankton are caused by exposure to toxic metabolites either from dissolved extracellular concentrations or from ingested algal cells (Rohrlack et al., 1999, Zurawell et al., 2005, Wilson et al., 2006). While most of the *Microcystis*–zooplankton literature uses daphnids, copepods are known to be more sensitive to dissolved MC exposure (DeMott et al., 1991; Reinikainen et al., 2002). Reinikainen (2002) found reduced survival of *E. affinis* at elevated levels of MC's but no effect on egg hatching, suggesting that at least some reproductive processes are not affected once the toxin is removed. However, because lethal concentrations of MC are several orders of magnitude higher than typical environmental concentrations, natural cases of acute zooplankton toxicity from dissolved MC are probably very rare (DeMott et al., 1991; Christoffersen, 1996; Park et al., 1998; Song et al., 2007). While the significance and toxicity of non-MC metabolites such as microviridin and other cyanopeptides have become increasingly obvious, the focus of this paper is limited to MC toxicity (Rohrlack et al., 2003; Welker and von Döhren, 2006).

The purpose of this paper is to determine: 1) the species sensitivity of copepods (*E. affinis* and *P. forbesi*) to MC-LR, and 2) whether a short term (48 h) exposure to dissolved MC-LR has any long term impacts on copepod populations. To our knowledge, there is no information regarding post-exposure effects of MCs on copepods. We hypothesize that 1) copepods will have a species specific response to dissolved MC-LR, 2) that lethal concentrations will be significantly above common environmental concentrations, and 3) that possible effects on copepod population growth following exposure to sub-lethal MC-LR concentrations will be detected.

2. Materials and methods

2.1. Copepod cultures

P. forbesi was collected in June 2006, and *E. affinis* in March 2006 from various locations between Rio Vista and Suisun Marsh in the San Francisco Estuary. These periods reflect dominance of these species in the zooplankton community. Double filtered Delta water (35 mm mesh followed by GF/C, Millipore Corp) collected at Rio Vista was used as a culture medium, and cultures were maintained in aerated 4-L beakers placed in an environmental water bath at 24 ± 1 °C for *P. forbesi*, 18 ± 1 °C for *E. affinis* reflecting temperatures during their respective dominance in zooplankton community. Water quality in beakers including dissolved oxygen (>8 mg/L; YSI instruments), pH (8.3 ± 0.1), water hardness (100 mg/L; Hach, USA), salinity (5.0 ppt) and ammonia (<1 mg/L Hach, USA) was monitored weekly. Copepods were given 500 μg C L^{-1} day^{-1} equivalent of an equal mixture of highly nutritious algae *Nannochloropsis* and *Pavlova* as the food source (Reed Mariculture, San Jose, CA). Algal carbon content was determined by filtering known bio-volumes of diluted algae and analyzing for total carbon using a Gas Chromatogram (Stable Isotope facility, UC Davis). 60% of the total culture medium was replaced with freshly filtered medium weekly. Copepod culture density was monitored weekly, maintained between 50 and 100 adults/L, and diluted as necessary in order to ensure exponential population growth and minimize overcrowding. The systems were maintained under a natural photoperiod (16L:8D) and covered with a semi transparent black tarp to reduce contact with outside disturbance. Actively swimming healthy adult copepods were randomly collected from the brood cultures, which were acclimated to laboratory conditions for a minimum of three months prior to experiment.

2.2. Acute toxicity test (LC-50, TL-50)

To determine mean lethal concentrations of MC-LR after 48 h of exposure, three replicates with 15 copepods each in a 20-mL glass tube were exposed to control (0 mg/L MC), vehicle control (methanol), and seven nominal treatment concentrations of purified MC-LR toxin (0.1, 0.25, 0.50, 0.75, 1.0, 1.25, and 1.5 mg/L for *P. forbesi*, and 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, and 1.75 mg/L for *E. affinis*). These concentration ranges were chosen following a pilot study which indicated that *E. affinis* was more tolerant to MC-LR than *P. forbesi*. To prepare a 500-mg/L stock solution, 0.5 mg of MC-LR (Calbiochem, USA) was dissolved in 1 mL of 50% methanol:50% water solution, as MC-LR is slightly hydrophobic and methanol is commonly used as a solvent (Watanabe et al., 1996; Perez and Aga, 2005). Treatment solutions were then prepared by adding appropriate volumes of stock solution to the 15-mL culture medium immediately prior to adding the copepods. Healthy adult copepods were obtained by gently pouring the brood cultures through a 147-μm mesh and rinsing them to a petri dish. From here, 15 active adult copepods were randomly selected and placed in a small beaker with the 5-mL culture medium and then gently added to the previously prepared treatment solutions to bring the total volume to 20 mL for each replicate vessel. Copepods were starved for 24 h prior to the experiment. Vehicle control included the same concentration of methanol found in the highest treatment group of each experiment to ensure observation of effects due to trace additions of methanol only (0.175% by volume). Methanol concentration varied between 0.01 and 0.175% (by volume) among treatments, as added from the MC-LR stock solution, and 0% for the true control.

Two steps were taken to minimize the loss of MC-LR during the 48-h exposure period. First, inert glass tubes were presoaked overnight with the appropriate corresponding treatment concentration to minimize loss of dissolved MC-LR through glass surface binding during the exposure (Hyenstrand et al., 2001). Second, treatment solutions were replaced after 24 h with freshly prepared MC-LR spiked medium by gently siphoning through a 30-μm mesh with minimal disturbance to copepods.

Copepods were monitored between 4 and 6 h intervals for mortality by examining each 20-mL tube under a dissecting microscope. Dead copepods, defined as completely motionless after repeated disturbance (any twitching was still considered alive), were removed immediately. Mortality values were then used to calculate mean time to lethality (TL-50) and the 48-h LC-50. Percent mortality was calculated as the number of dead relative to total (15) in each replicate at a given time.

2.3. Post-exposure life table test

At the end of the 48-h exposure, all survivors were cleaned with three changes of culture medium and were transferred to optimal culture conditions for the observation of survival and reproduction.

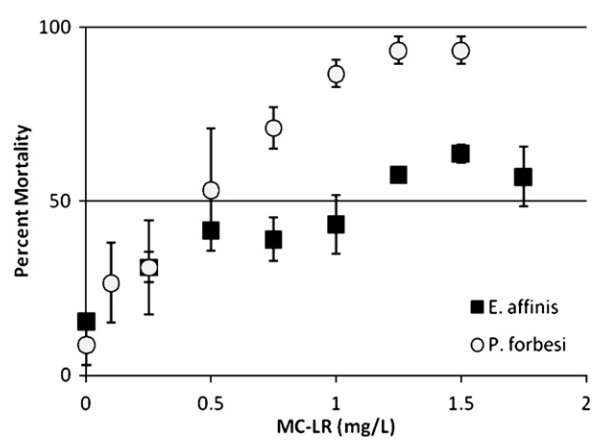

Fig. 1. Dose response of copepods to a 48-h exposure to increasing MC-LR concentrations. Mortality is significantly different between controls and all treatments for both species. Error bars = standard errors.

Table 1
LC values of dissolved MC-LR (mg/L) for *P. forbesi* and *E. affinis* obtained by PROBIT.

Copepod	LC-50 (95% CI)	LC-10 (95% CI)	Slope (95% CI)
P. forbesi	0.52 (0.38, 0.63)	0.21 (0.11, 0.31)	3.21 (2.19, 4.24)
E. affinis	1.55 (0.93, 3.59)	0.14 (0.03, 0.36)	1.24 (0.45, 2.03)

Immediately after the exposure (at $t = 48$ h; end of LC-50), this is defined as day 0. Because of high latent mortality between day 0 and day 4, population growth rates were calculated starting from day 4, when the mortality subsided in all treatments. Surviving copepods were transferred to fresh, MC-free filtered medium in 1-L aerated beakers and fed immediately following culture conditions above. A number of individual life stages (N_{1-5}, C_{1-6}) were monitored and recorded daily (by visual inspection and dissecting microscope as necessary), and maintained under brood culture conditions to evaluate post-exposure population growth rate and average development time for each treatment/replicate. Chronic effects were studied in treatments that were at or below the calculated LC-50 concentration because treatments above the LC-50 concentration did not have adequate copepods surviving for a reliable study. Population growth rate was quantified by population growth of adults over time (adult reproduction) for each treatment replicate (3) and averaged per treatment. The post-exposure experiment continued for 33 days after the 48-h LC-50 exposure ended. The following formula was used for net population growth rate (r).

$$r = ln(N_t / N_o) / t$$

where

N_t	number of adults at the end of experiment
N_o	number of adults at day 4 after the 48-h LC-50 ends
t	duration of experiment
r	per day population growth rate.

2.4. Data analysis

LC-50 results were analyzed using PROBIT software, where mortality is plotted against MC concentration and the log transformed dose response curve estimates percent mortality at a given toxin concentration. LC-50 concentration is by definition the estimated concentration that causes 50% mortality at a given time. LC-10 is calculated similarly, but for the concentration that results in 10% mortality at a given time. TL-50 was estimated using the linear regression for individual replicate mortality as a function of time. Each replicate was assigned a linear regression and the time to reach 50% mortality was calculated. The average of all three replicates was used to determine the TL-50 for a given treatment. Differences in the effect of MC-LR exposure and copepod species on the post-exposure population growth rate, as well as differences in each species response to MC-LR (interaction term) were analyzed with a factorial ANOVA. The growth rate for each treatment is based on the average from all replicates in the treatment.

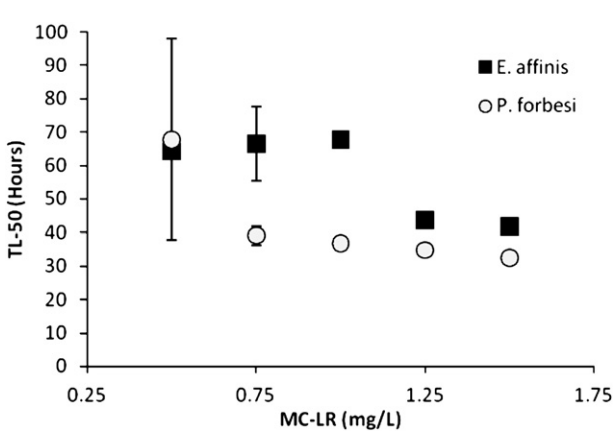

Fig. 2. Mean survival time for copepods exposed to MC-LR, as measured by TL-50, i.e. time to reach 50% mortality for both species. Error bars = standard errors.

Table 2
Population growth rate (r) of *P. forbesi* and *E. affinis* post 48-h exposure to MC-LR.

($n = 15$)	N_o range, mean, (SD)	N_{29} range, mean, (SD)	r range, mean, (SD)
E. affinis	3–6, 4.40 (0.82)	0–47, 22.4 (13.39)	0–0.09, 0.05 (0.02)
P. forbesi	2–7, 4.25 (2.15)	0–85, 29.87 (23.8)	0–0.09, 0.05 (0.03)

Range and mean values for number of copepods per replicate at day 4 (N_o) and final day (N_{29}). Values include all 15 replicates for each species, including those replicates with zero copepods at N_{29} (SD = standard deviation).

3. Results

3.1. Acute and latent mortality

Both species of copepods showed a clear dose response to increased concentrations of dissolved MC-LR over the 48-h exposure period. Mortality was significantly higher than controls in all treatments including the lowest MC-LR concentrations (Fig. 1). Vehicle control (w/ methanol) mortalities were 8.8% for *P. forbesi*, and 15.5% for *E. affinis*, respectively, during the 48-h exposure to MC-LR (Fig. 1). Comparison between the control and the methanol spiked control showed no significant difference (data not shown), and control hereafter refers to the vehicle (methanol) control. Species differences in sensitivity were observed in 48-h LC-50 values for MC-LR where *P. forbesi* (0.52 mg/L) was 3 folds more sensitive to MC-LR than *E. affinis* (1.55 mg/L) (Table 1). Mortality increased with treatment concentration for both species, though the slope of their response was significantly different, with *P. forbesi* having a more pronounced dose response. On average, *E. affinis* was less sensitive to MC-LR exposure, and its response to increasing concentrations was less pronounced, having almost a third of the slope compared to *P. forbesi* (Table 1, Fig. 1). The difference in slope resulted from the two copepods having similar mortality at MC-LR concentrations <0.5 mg/L, as their LC-10 concentrations were comparable (Table 1).

3.2. Mean survival time (TL-50)

TL-50 indicates how fast the toxin begins to cause mortality and is a temporal measure of the dose response by showing how long it takes a population to reach 50% mortality. While TL-50 of both copepods declined similarly to increasing MC-LR concentration, species specific mean survival time was consistently higher for *E. affinis* compared to *P. forbesi*

Table 3
Factorial ANOVA testing the effect of treatment variables on post-exposure population growth rates.

Source	DF	F ratio	p
Species	1	<0.001	0.98
MC-LR	1	0.14	0.71
Species × MC-LR	1	0.77	0.39

Variables are copepods species (species), MC-LR exposure concentration (MC-LR), and their interaction term (species × MC-LR: how previous MC-LR exposure affects each species population growth rate).

Table 4
Comparison of measured LC-50 concentrations for various zooplanktons exposed to MC-LR.

Zooplankton	48-h LC-50[a]	95% confidence interval	Source
Tetrahymena pyriformis (ciliate)	252[b]	(200–316)	Ward and Codd (1999)
Thamnocephalus platyurus nauplii (fairy shrimp)	0.46[c]	(0.38–0.54)	Keil et al. (2002)
Artemia salina (brine shrimp)	15–17[d]	–	Lahti et al. (1995)
Daphnia pulicaria	21.4	(16.8–29.2)	DeMott et al. (1991)
Daphnia hyalina	11.6	(7.0–15.6)	DeMott et al. (1991)
Daphnia pulex	9.6	(7.8–11.7)	DeMott et al. (1991)
Diaptomus birgei (copepod)	0.45	(0.29–0.58)	DeMott et al. (1991)
E. affinis (copepod)	0.27	(0.197–1.446)	Reinikainen et al. (2002)
E. affinis (copepod)	1.55	(0.93–3.59)	This study
P. forbesi (copepod)	0.52	(0.38–0.63)	This study

Values are in mg/L MC-LR and for a 48-h exposure unless otherwise noted.
[a] Unless otherwise noted.
[b] 24-h dissolved MC-LR exposure.
[c] Various congeners of MC.
[d] MC-LR and MC-RR mixture.

when MC-LR concentration >0.5 mg/L (Fig. 2). At lower concentrations, two species behaved similarly.

3.3. Post-exposure effects on copepod population growth

Once removed from the acute toxicity experiment, both species of copepods showed high mortality (up to 0.18 per day) for 4 days of the post-exposure period, regardless of MC-LR exposure. At the end of this 4-day period following exposure, the number of surviving copepods varied between 2 and 7 in each vessel, and did not depend on exposure history. Thereafter, mortality rate subsided for both species, and population growth started with the appearance of nauplii on day 4 for *E. affinis* and on day 7 for *P. forbesi* (data not shown). Gravid females were observed across all treatments within a few days following post-exposure conditions. More than 50% of the *E. affinis* treatments had gravid females by day 1, and *P. forbesi* by day 6. More than 50% of treatments in both species had copepodites by day 11, and more than 50% had new adults by day 21.

Post-exposure population growth rate ranged between 0 and 0.09 per day (Table 2), and was independent of both MC exposure history and copepod species (Table 3). Initial number of adult copepods at day 4 (N_o) predicting final number of adult copepods (N_{29}) was not related for *E. affinis*, and very weakly linear with *P. forbesi* ($R^2 = 0.03$ and 0.25 respectively).

While a few treatments had no population growth following exposure, the majority of vessels (80% for *P. forbesi* and 93% for *E. affinis*) showed positive growth rates at the end of the experiment with the emergence of new adults. A total of 3 vessels (out of 15) for *P. forbesi* and 1 vessel (out of 15) for *E. affinis* had a zero or negative population growth rate. We assumed these vessels had no population growth because they lacked both sexes due to latent mortality in the post-exposure period. These no growth vessels were included in the calculation of population growth rate for their respective treatments, though their exclusion does not change the results and lack of relationship (data not shown). Overall, there was no difference in average population growth between the control and exposed copepods, no effect of MC-LR exposure history, and no difference in post-exposure population growth between species (Table 3).

4. Discussion

The LC-50 values found for MC-LR in this study agree with the general consensus that calanoid copepods are more sensitive than cladocerans, by an order of magnitude (DeMott et al., 1991). Results demonstrate that calanoid copepods are some of the most sensitive zooplankton species to dissolved MC exposure (Table 4). While MC-LR is known to degrade relatively rapidly, the measures taken to minimize MC-LR loss such as presoaking and static renewal of treatment solutions should have prevented any MC-LR degradation strong enough to significantly change treatment concentrations (Perez and Aga, 2005). In a similar experiment Reinikainen et al. (2002) found no significant degradation of MC-LR over 48 h even though no measure was taken to prevent degradation.

MC-LR toxicity was similar for the two copepod species when concentrations were <0.5 mg/L. Above this, *P. forbesi* was more sensitive and its response to increased MC-LR was more pronounced than *E. affinis*. The difference at higher concentrations between two species could be due to species specific uptake via the integument and by inherent physiological tolerance and enzymatic detoxification, which may be controlled at larger scales through evolutionary adaptations (DeMott et al., 1991; Blaxter and Ten Hallers-Tjabbes, 1992; Chen et al., 2005).

In order to prevent protein phosphatase (PP) inhibition, *Daphnia* use enzymes of the anti-oxidant system (GST and glutathione) to detoxify MC (Pflugmacher et al., 1998; Chen et al., 2005). At low or initial doses of MC exposure, there is a decline in the anti-oxidant enzymes with no inhibition of PP enzymes; under extended MC exposure, the continuous detoxification can cause oxidative stress such as increased LDH, which eventually overwhelms detoxification and *Daphnia* die from PP inhibition (Chen et al., 2005). *E. affinis* is able to detoxify nodularin, another cyanobacterial hepatotoxin, showing that copepods have adapted to algal toxins and have detoxification mechanisms (Karjalainen et al., 2006).

Another factor that could explain why copepods here showed differences in sensitivity at higher concentrations is the different exposure temperature (18 °C for *E. affinis* versus 24 °C for *P. forbesi*), as higher temperature could increase sensitivity due to a faster metabolism. In another study for MC-LR, Reinikainen et al. (2002) found the 48-h LC-50 for *E. affinis* from the Baltic Sea to be significantly lower than the current study even at a lower exposure temperature (Table 4). However, comparing results of the two studies may not be that useful because unlike the current study, Reinikainen et al. (2002) fed copepods during MC exposure tests. Higher sensitivity in that study could be from both ingested and dissolved MC, since feeding during the exposure could increase ingestion of MC via adsorption to the food particles. Temperature effects on sensitivity within the context of this study may not be as vital as other factors such as geographical differentiation and previous exposure to MC.

Evidence is accumulating for geographically distinct populations of copepods from the same species having genetically distinct life history traits and tolerance to stress (Wyngaard, 1986). *E. affinis* is a common invasive species in Northern temperate estuaries and some lakes, and its success may be due to its ability to respond to natural selection (i.e. evolve tolerance), as opposed to an inherent broad range of plasticity and high tolerance to a wide range of conditions (Lee, 2002). Differences in stress tolerance among distinct populations of copepods, including MC toxicity for *E. affinis*, may be due to geographically isolated populations that have been adapting to local selective regimes (Lee, 2002).

Increased tolerance may also be related to previous exposure to *Microcystis*, which may improve dietary tolerance in some zooplankton, and could also mean decreased sensitivity to dissolved MC (Gustafsson and Hansson, 2004; Sarnelle and Wilson, 2005). Since copepods in this study were cultured with no contact to *Microcystis* or MC for several months, the possibility of any tolerance to MC from previous exposure can be omitted.

The difference in MC tolerance reported here could be due to gender biased effects as female copepods have higher tolerance to dissolved MC than males (Ojaveer et al., 2003). In this study, however, the chances for MC-LR toxic effects based on gender should be minor because 45 adult copepods per treatment were randomly selected from a pool of >200 copepods in a 4-L beaker and divided to 15 copepods per replicate.

The LC-10 concentrations, which represent the threshold for chronic effects, are at the highest of naturally observed MC concentrations (Song et al., 2007). This supports previous work showing that acute toxicity from dissolved MC-LR to copepods requires abnormally high concentrations to be a significant threat (McDermott et al., 1995; Park et al., 1998). Since their LC-10 concentration was comparable, MC-LR toxicity for *E. affinis* and *P. forbesi* should be similar at environmentally relevant concentrations. With regards to their sensitivity, calanoid copepods of the SFE are similar to other calanoids, and are among the most sensitive zooplankton to dissolved MC (Table 4).

While acute toxicity is not a direct threat expected to occur in the field, extended periods of low level dissolved MC can still present a potential impact to zooplankton through sub-lethal effects such as oxidative stress (Chen et al., 2005). Effects of longer MC exposure (>48 h) on copepods at sub-lethal concentrations are not known and may be significant in food limited systems such as the SFE. Monitoring of dissolved MC concentrations from the SFE was not available at the time of this study.

Results indicate no post-exposure effects on reproduction, and predict that short term (<48 h) spikes of sub-lethal MC will not have significant consequences on a population scale. Copepods can repopulate quickly if provided with good conditions such as clean water, high food quality, and optimal temperature under laboratory conditions. However such conditions do not usually exist in the SFE, where zooplanktons are limited by food, predation by invasive filter feeding bivalves, and possibly contaminants (Mueller-Solger et al., 2002; Kimmerer, 2004).

If exposure to sub-lethal MC concentrations had a significant effect on copepod fecundity and population growth, we would expect to see a negative relationship between MC exposure treatments and population growth rate. The number of initial females at N_o along with the individual fitness of each copepod will be a major determinant of the final number of copepods in each vessel population, and the current study would have benefited from knowing the number of females at the start of the post-exposure experiment. However, despite the lacking record for the initial female population per replicate, differences between individual population growth rates of replicates were completely random and not related to the number of initial copepods (N_o) or MC exposure concentration. Similarly, high latent mortality between the end of the exposure and beginning of the life table test (N_o) was not related to exposure history and was probably due to handling stress, including the 48-h starvation during the exposure period. The variation of population growth rate within each treatment was high, such that any potential effect of number of females is overwhelmed by the variation among replicates. For instance, *P. forbesi* replicates in the control, 0.25, and 0.5 mg/L MC-LR treatments each having only 2 copepods at N_o all had population growth rates that were among the highest (>0.08 per day). Thus, despite the difference in previous MC exposure, and regardless of the initial number of females, copepods can repopulate at variable but similar rates. Accordingly, the conclusion that a sub-lethal 48-h MC exposure does not affect post-exposure population growth in these copepods may still be extracted from the results.

While exposure to sub-lethal concentrations of dissolved MC had no effect on post-exposure reproduction in the laboratory, other stressors in the SFE could increase sensitivity of copepods to MC in the field. The ecosystem scale impact of dissolved MC to the SFE's zooplankton community is likely a function of its persistence, the extent and variability of dissolved MC, zooplankton adaptations, sub-lethal effects and other stressors in the environment. Taking a better look at several non-MC compounds which *Microcystis* produces has become necessary to realize the full impact of *Microcystis* on zooplankton (Rohrlack et al., 2003). More importantly, the potential threat from *Microcystis* to the dominant zooplankton of the SFE is probably regulated via ingestion and food quality related trophic interactions rather than dissolved MC. Research on copepod–*Microcystis* interactions should be directed to feeding studies, which have overwhelmingly focused on daphnids (Wilson et al., 2006).

Acknowledgements

We would like to acknowledge Nurulhafizah Samsudin and Robert E. Schroeter for their assistance with culturing and collection of copepods. Funding of this study is supported by Dr. Swee Teh's Aquatic Toxicology Program fund and partially by the California Interagency Ecological Program Pelagic Organisms Decline (IEP-POD), CALFED (project# PO685515), and California Department of Water Resources (contract# 4600007499).

References

Best JH, Eddy FB, Codd GA. Effects of *Microcystis* cells, cell extracts and lipopolysaccharide on drinking and liver function in rainbow trout *Oncorhynchus mykiss* Walbaum. Aquat Toxicol 2003;62:419–26.

Blaxter CCH, Ten Hallers-Tjabbes CC. The effects of pollutants on sensory systems and behavior of aquatic animals. Neth J Aquat Ecol 1992;26:43–58.

California Resources Agency Pelagic Fish Plan 2007. http://www.publicaffairs.water.ca.gov/newsreleases/2007/030507pod.pdf.

Chen W, Song L, Ou D, Gan N. Chronic toxicity and responses of several important enzymes in *Daphnia magna* on exposure to sub lethal microcystin-LR. Environ Toxicol 2005;20:323–30.

Chen J, Xie P, Li L, Xu J. First identification of the hepatotoxic microcystins in the serum of a chronically exposed human population together with indication of hepatocellular damage. Toxicol Sci 2009;108:81–9.

Chorus, I. and Bartram, J. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring, and management. E&FN Spon, on behalf of the World Health Organization, London and New York, 1999.

Christoffersen K. Ecological implications of cyanobacterial toxins in aquatic food webs. Phycologia 1996;35:42–50.

Dawson RM. The toxicology of microcystins. Toxicon 1998;36(7):953–62.

DeMott WR, Zhang Q-X, Carmichael WW. Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. Limnol Oceanogr 1991;36(7):1346–57.

Gkelis S, Lanaras T, Sivonen K. The presence of microcystins and other cyanobacterial bioactive peptides in aquatic fauna collected from Greek freshwaters. Aquat Toxicol 2006;78(1):32–41.

Gustafsson S, Hansson L-A. Development of tolerance against toxic cyanobacteria in *Daphnia*. Aquat Ecol 2004;38:37–44.

Hyenstrand P, Metcalf JS, Beattie KA, Codd GA. Effects of adsorption to plastics and solvent conditions in the analysis of the cyanobacterial toxin microcystin LR by high performance liquid chromatography. Water Res 2001;35(14):3508–5311.

Hansson LA, Gustaffson S, Rengefors K, Bomark L. Cyanobacterial chemical warfare affects zooplankton community composition. Freshw Biol 2007;52:1290–301.

Ibelings B, Chorus I. Accumulation of cyanobacterial toxins in freshwater "seafood" and its consequences for public health — a review. Environ Pol 2007;150:177–92.

Ibelings BW, Bruning K, Jonge J, Wolfstein K, Pires LM, Postma J, et al. Distribution of Microcystins in a lake foodweb: no evidence for biomagnification. Microb Ecol 2005;49:487–500.

Karjalainen M, Kozlowsky-Suzuki B, Lehtiniemi M, Engstrom-Ost J, Kankaanpaa H, Viitasalo M. Nodularin accumulation during cyanobacterial blooms and experimental depuration in zooplankton. Mar Biol 2006;148:683–91.

Keil C, Forchert A, Fastner J, Szewzyk U, Rotard W, Chorus I, et al. Toxicity and microcystin content of extracts from a *Planktothrix* bloom and two laboratory strains. Water Res 2002;36:2133–9.

Kimmerer W. Open water processes of the San Francisco Estuary: from physical forcing to biological responses. San Franc Est Water Sci 2004;2(1) Article 1. http://repositories.cdlib.org/jmie/sfews/vol2/iss1/art1.

Lahti K, Ahtlainen J, Rapala J, Sivonen K, Niemela SI. Assessment of rapid bioassays for detecting cyanobacterial toxicity. Lett Appl Microbiol 1995;21:109–14.

Lee CE. Rapid and repeated invasions of freshwater by the copepod *Eurytemora affinis*. Evolution 2002;55(5):1423–34.

Lehman PW, Boyer G, Hall C, Waller S, Gehrts K. Distribution and toxicity of a new colonial *Microcystis aeruginosa* in the San Francisco Bay Estuary, California. Hydrobiology 2005;541:87–99.

McDermott CM, Feola R, Plude J. Detection of cyanobacterial toxins (microcystins) in waters of Northeastern Wisconsin by a new immunoassay technique. Toxicon 1995;33(11):1433–42.

Mueller-Solger A, Jassby AD, Muller-Navarra D. Nutritional quality of food resources for zooplankton (*Daphnia*) in a tidal freshwater system (Sacramento–San Joaquin River Delta). Limnol Oceanogr 2002;47(5):1468–76.

Ojaveer E, Simm M, Balode M, Purina I, Suursaar U. Effect of *Microcystis aeruginosa* and *Nodularia spumigena* on survival of *Eurytemora affinis* and the embryonic and larval development of the Baltic Herring *Clupea harengus membras*. Environ Toxicol 2003;18(4):236–42.

Park HD, Iwami C, Watanabe MF, Harada K, Okino T, Hayashi H. Temporal variability in the concentrations of intra- and extracellular microcystin and toxic *Microcystis* species in a

hypertrophic lake, Lake Suwa, Japan (1991–1994). Environ Toxicol Water Qual 1998;13:61–72.
Perez S, Aga D. Recent advances in the sample preparation, liquid chromatography tandem mass spectrometric analysis and environmental fate of microcystins in water. Trend Anal Chem 2005;24(7):658–70.
Pflugmacher S, Wiegand C, Oberemm A, Beattie KA, Krause E, Codd G, et al. Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxification. Biochim Biophys Acta (BBA) — Gen Subj 1998;1425(3):527–33.
Reinikainen M, Lindvall F, Meriluoto JAO, Pepka S, Sivonen K, Spoof L, et al. Effects of dissolved cyanobacterial toxins on the survival and egg hatching of estuarine calanoid copepods. Mar Biol 2002;140:577–83.
Rohrlack T, Dittman E, Borner T, Christoffersen K. Effects of cell-bound Microcystins on survival and feeding of Daphnia spp. Applied and Environmental Microbiology 1999;67(8):3523–3529.
Rohrlack T, Christoffersen K, Hansen PE, Zhang W, Czarnecki O, Henning M, et al. Isolation, characterization, and quantitative analysis of microviridin J, a new *Microcystis* metabolite toxic to *Daphnia*. J Chem Ecol 2003;29(8):1757–70.
Sarnelle O, Wilson AE. Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. Limnol Oceanogr 2005;50:284–9.
Sommer T, Armor C, Baxter R, Breuer R, Brown L, Chotkowski M, et al. The collapse of pelagic fishes in the upper San Francisco Estuary. Fisheries 2007;32(6):270–7.
Song L, Chen W, Peng L, Wan N, Gan N, Zhang X. Distribution and bioaccumulation of microcystins in water columns: a systematic investigation into the environmental fate and the risks associated with microcystins in Meiliang Bay, Lake Taihu. Water Res 2007;41:2853–64.
Thostrup L, Christoffersen K. Accumulation of microcystin in *Daphnia magna* feeding on toxic *Microcystis*. Arch Hydrobiol 1999;145(4):447–67.
Ward CA, Codd GA. Comparative toxicology of four microcystins of different hydrophobicities to the protozoan, *Tetrahymena pyriformis*. J Appl Microbiol 1999;86:874–82.
Watanabe MF, Harada K, Carmichael W, Fujiki H, editors. Toxic *Microcystis*. New York: CRC Press; 1996.
Welker M, von Döhren H. Cyanobacterial peptides — nature's own combinational biosynthesis. FEMS Microbiol Rev 2006;30:530–63.
WHO (World Health Organization) Current edition of the WHO guidelines for drinking-water quality, chemical hazards in drinking water quality: MC-LR review document; 2003. http://www.who.int/water_sanitation_health/dwq/chemicals/microcystin/en/.
Wilson AE, Sarnelle O, Tillmanns AR. Effects of cyanobacterial toxicity and morphology on the population growth of freshwater zooplankton: meta-analyses of laboratory experiments. Limnol Oceanogr 2006;51(4):1915–24.
Wyngaard GA. Heritable life history variation in widely separated populations of *Mesocyclops edax* (Crustacae: Copepoda). Biol Bull 1986;170:296–304.
Xie LQ, Xie P, Guo LG, Li L, Miyabara Y, Park HD. Organ distribution and bioaccumulation of microcystins in freshwater fish at different trophic levels from the eutrophic Lake Chaohu, China. Environ Toxicol 2005;20(3):293–300.
Zurawell RW, Chen H, Burke JM, Prepas EE. Hepatotoxic cyanobacteria: a review of the biological importance of microcystins in freshwater environments. J Toxicol Environ Health 2005;B8:1–37.

Freshwater Biology

Freshwater Biology (2010) **55,** 1548–1559 doi:10.1111/j.1365-2427.2009.02367.x

The effects of dietary *Microcystis aeruginosa* and microcystin on the copepods of the upper San Francisco Estuary

KEMAL A. GER*, SWEE J. TEH†, DOLORES V. BAXA†, SARAH LESMEISTER† AND CHARLES R. GOLDMAN*

*Department of Environmental Science and Policy, University of California, Davis, CA, U.S.A.

†Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, Davis, CA, U.S.A.

SUMMARY

1. Increasing blooms of *Microcystis aeruginosa* have unknown impacts on the copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi*, which are the dominant zooplankters and key prey species for endangered larval fish in the upper San Francisco Estuary.
2. Laboratory feeding experiments were designed to measure the effect of *Microcystis* on copepod survival and to distinguish the effects of toxicity and nutrition. In a series of survival tests, copepods were fed a mixed diet of algae plus one of two strains of *Microcystis*, either producing (MC+) or lacking microcystin (MC−).
3. *Microcystis* significantly reduced survival even when it was a small proportion of the diet, indicating that toxicity was the major cause of mortality. Contrary to expectation, however, the MC+ strain did not result in higher mortality, suggesting that non-MC metabolites of *Microcystis* can be toxic to copepods.
4. Across treatments, survival of *P. forbesi* was greater than that of *E. affinis*, although the two copepods responded differently to both the ratio and the strain of *Microcystis* in their food. Survival of *P. forbesi* was greater on the MC+ strain and was inversely proportional to the ratio of dietary *Microcystis* (MC+ or MC−). In contrast, survival of *E. affinis* declined similarly across treatments and was not related to the proportion or strain of dietary *Microcystis*. Results indicate that the copepod *P. forbesi* can coexist with *Microcystis* while the other copepod *E. affinis* cannot.
5. Regardless of species, dietary *Microcystis* caused significant mortality to copepods, and it may cause adverse impacts to the potentially food-limited zooplankton community of the San Francisco Estuary. These impacts may not be related to the cellular MC concentration because *Microcystis* contains other metabolites that negatively affect copepods.

Keywords: copepod, harmful algal bloom, microcystin, *Microcystis*, San Francisco Estuary

Introduction

Global increases in the extent of bloom-forming cyanobacteria have attracted growing attention from both management and scientific interests because of potential negative impacts on water quality and food web structure (Kirk & Gilbert, 1992; Chorus &

Correspondence: Swee J. Teh, Department of Anatomy, Physiology, and Cell Biology, Aquatic Toxicology Program, School of Veterinary Medicine, 1321 Haring Hall, One Shields Avenue, University of California, Davis, CA 95616, U.S.A.
E-mail: sjteh@ucdavis.edu

© 2010 Blackwell Publishing Ltd

Bartram, 1999). *Microcystis* is a common bloom-forming cyanobacterium that can dominate nutrient-enriched freshwaters including upper reaches of estuaries, such as the San Francisco Estuary (Paerl, 1988; Lehman *et al*., 2008).

In blooms, *Microcystis* can change the food web because it is a poor quality food for zooplankton, reducing their fitness and restricting the trophic transfer of carbon (Paerl, 1988; Christoffersen, 1996; Chorus & Bartram, 1999). Negative impacts on zooplankton occur in response to a combination of factors including: (i) secondary metabolites with toxic effects, such as microcystins (MCs), (ii) nutritional inadequacy and (iii) poor digestibility or feeding inhibition (Fulton & Paerl, 1987; DeMott & Moxter, 1991; Lurling, 2003). However, not all zooplankton are affected equally (Christoffersen, 1996; Wilson, Sarnelle & Tillmanns, 2006). Species-specific adaptations in zooplankton lead to variation in the observed responses to *Microcystis* blooms (Kirk & Gilbert, 1992; Rohrlack *et al*., 1999; Gustafsson & Hansson, 2004). Although many studies have evaluated the interaction between zooplankton and *Microcystis*, most focus on cladocerans while impacts to copepods remain poorly understood (reviewed in Wilson *et al*., 2006).

Controlled manipulations of diets in the laboratory can provide valuable evidence of the mechanisms involved. A major problem in predicting the impacts of *Microcystis*, however, has been because of the use of oversimplified experimental conditions, such as pure or single strain diets (presence/absence experiments), a disproportionate focus on daphniids as the test organism, and species/strain-specific responses, preventing the interpretation of impacts on other zooplankton or under field conditions (reviewed in Wilson *et al*., 2006). As a result, the effect of *Microcystis* blooms on copepods and the dynamics of food webs in estuaries dominated by copepods are poorly understood.

Copepods are an important link between primary production and fish, and commonly dominate the mesozooplankton of estuaries (Turriff, Runge & Cembella, 1995; Sommer *et al*., 2007). Calanoid copepods, particularly *Eurytemora affinis* (Poppe, 1880) and *Pseudodiaptomus forbesi* (Poppe & Richard, 1890), are the principal food source of the endangered larval and pelagic fish in the San Francisco Estuary, where the abundance of pelagic organisms, including copepods and nutritious phytoplankton, has declined to values not seen before (Sommer *et al*., 2007). Increasing blooms of *Microcystis* in the freshwater and low salinity zone of the estuary raise concerns about the further depletion of high-quality fish food (Muller-Solger, Jassby & Muller-Navarra, 2002; Lehman *et al*., 2005, 2008). Understanding the impacts of *Microcystis* on the dominant copepods of the San Francisco Estuary has become highly significant in the management of its declining fish populations (Sommer *et al*., 2007).

While a pure diet of *Microcystis* reduces fitness and survival in zooplankton, it is not likely to occur in nature (DeMott, Zhang & Carmichael, 1991; Kumar, 2003; Wilson *et al*., 2006). Unlike daphniids, copepods can ingest nutritious particles selectively and avoid those that are unwanted or toxic (DeMott *et al*., 1991; Kleppel, 1993; Burns & Hegarty, 1994; Engstrom *et al*., 2000; Panosso *et al*., 2003). Even daphniids show different responses to *Microcystis* when offered in mixed versus pure diets (Rohrlack *et al*., 1999; Lurling, 2003). Therefore, in studying interactions between zooplankton and *Microcystis*, we need to experiment with diets consisting of mixtures of alternative, more palatable, food (Wilson *et al*., 2006).

Microcystis toxicity comes from well-known toxins, such as MC, but also from other, poorly understood and often unidentified, secondary metabolites (Lurling, 2003; Rohrlack *et al*., 2004; Wiegand & Pflugmacher, 2005; Wilson *et al*., 2006). Though the significance of metabolites other than MC is becoming clear, the relative toxicity to zooplankton of MC versus non-MC metabolites remains unresolved (DeMott *et al*., 1991; Kurmayer & Juttner, 1999; Lurling, 2003; Rohrlack *et al*., 2004). It is possible to quantify the effect of MC in the zooplankton diet using *Microcystis* strains of identical morphology (assuming similar nutritional profile) but different MC cellular content. This approach has been used successfully to distinguish the toxicity of MC versus non-MC metabolites in daphniids (Rohrlack *et al*., 1999; Lurling, 2003). When offered in a mixed diet, strains of *Microcystis* containing (MC+) and lacking (MC−) both caused mortality, showing the toxic effects of non-MC metabolites on daphniids (Lurling, 2003). The current study was designed to compare the effects of *Microcystis* and its toxic metabolites on the dominant copepods of the San Francisco Estuary.

Our objective was to evaluate the impact of *Microcystis* in the diet on the survival of the dominant

copepods. To distinguish between the effects of toxic metabolites and nutritional deficiency of *Microcystis* on copepods, mixed diets containing nutritious algae and *Microcystis* (MC+ or MC−) were offered to copepods in laboratory survival tests. Experiments were designed to compare the survival of *E. affinis* and *P. forbesi* as a function of dietary *Microcystis* (MC+ versus MC−) and food quantity. We hypothesised that (i) both strains of *Microcystis* would negatively affect survival, though the response might be species specific, (ii) survival with the MC+ strain in the diet would be lowest and (iii) survival would be inversely proportional to the proportion of *Microcystis* in the diet.

Methods

Microcystis aeruginosa strains

Cultures of two different strains of *M. aeruginosa* (Kutzing, 1846) were maintained for the feeding experiments in a modified ASM-1 medium (Reynolds & Jaworski, 1978). MC producing (MC+, UTEX 2385) and MC absent (MC−, UTEX 2386) strains of *Microcystis* were obtained from the University of Texas Culture Collection (Austin, TX, U.S.A.). Exponentially growing axenic batch cultures were maintained by dilution with freshly prepared cultured medium to keep cell density between 1×10^6 and 3×10^6 cells mL^{-1}, in gently bubbled 500-mL glass flasks, under a 16 : 8 L : D cycle under 30 μmol quanta m^{-2} s^{-1}, in a temperature-controlled room (22 ± 1 °C). Under these conditions, both strains were uni- and bi-cellular, the average growth rate of *Microcystis* was 0.32 (±0.04) day^{-1} and cell diameter was approximately 4 μm (±0.7) for both strains. We assumed that the only difference between the two strains used was MC content and that each strain had a comparable nutritional profile and digestibility.

Instant algae (IA)

Commercially available and highly nutritious pure phytoplankton food composed of *Nannochloropsis* and *Pavlova*, rich in fatty acids, were kept fresh at 4 °C (Reed Mariculture, Campbell, CA, U.S.A.). Because IA is in a fresh but non-living state, cells have a constant density. Cell diameter was approximately 2 μm for *Nannochloropsis* and 4 μm for *Pavlova*.

Copepod cultures

Copepods were collected from Rio Vista and Suisun Marsh in the San Francisco Estuary using a 174-μm zooplankton tow net. *Pseudodiaptomus forbesi* was collected in June 2007 and *E. affinis* in April 2007 (respective periods of dominance), and returned to the laboratory within 2 h of collection. Gravid females were selected and rinsed three times with salinity-adjusted culture medium before transferring them to the culture. We used reconstituted water (modified by NaCl to increase salinity) as the culture medium (Horning & Weber, 1985). Cultures were grown in aerated 4-L beakers placed in an environmental water bath, at 24 ± 1 °C for *P. forbesi* and 20 ± 1 °C for *E. affinis*. Water quality in beakers, including dissolved oxygen (>8 mg L^{-1}), pH (8.2 ± 0.1), water hardness (100 mg L^{-1}), salinity (5.0 ppt) and ammonia (<1 μg L^{-1}), was monitored weekly (Hach, Loveland, CO, U.S.A.). For optimal culture and population growth under laboratory conditions, a previously determined amount of the IA mixture (an equal biovolume of *Nannochloropsis* and *Pavlova*) was given as food at 400 and 500 μg C L^{-1} day^{-1} for *E. affinis* and *P. forbesi*, respectively. Approximately, 60% of the total culture medium was replaced weekly with aerated and temperature-adjusted medium. Copepod culture density was monitored weekly, maintained between 50 and 100 adults L^{-1}, and diluted as necessary to ensure exponential population growth and minimise overcrowding. The systems were maintained under a natural photoperiod (16L : 8D) and covered with a semitransparent black tarpaulin. The brood cultures were acclimated to these conditions for a minimum of 4 months before the feeding experiments.

Cell size, density and algal biomass determination

Algal biomass concentration (μg C mL^{-1}) for the IA mixture and *Microcystis* was determined by a calibration curve of total carbon and biovolume. A serial dilution of algal biovolumes were filtered on pre-combusted 25-mm GF/C (Whatman, Kent, U.K.), desiccated at 50 °C, and stored at −80 °C until the total carbon was measured by a gas chromatogram (UC Davis Stable Isotope Facility, U.S.A.). *Microcystis* cell size and density was determined using an inverted compound epifluorescent microscope (2007 Zeiss Axio Observer A1, Thornwood, NY, U.S.A), counted in a Haemocytometer

(Hausler Scientific, Horsham, PA, U.S.A.) and calibrated against light absorbance at 800 nm.

Survival test

We used a series of survival tests in which dietary treatments included *Microcystis* while controls did not. The treatment diets consisted of a *Microcystis* – IA mixture, with the proportion of *Microcystis* at 0, 10, 25, 50 or 100% of total food (by carbon), using either the MC+ or MC− strain of *Microcystis*, plus IA, to a total food concentration of 400 (*E. affinis*) and 500 (*P. forbesi*) μg C L^{-1} day^{-1}. The control diets were comprised of IA only at the same amount as in the associated treatment of IA and *Microcystis* mixture, which corresponded to 100, 90, 75, 50 and 0% (starvation) of the total food. This design allowed the distinction of impacts because of food quantity (total nutrition available) versus quality (MC and non-MC toxicity) by comparing survival over a range of a given diets with and without *Microcystis*. This design also enabled the assessment of the impact of alternative food sources when *Microcystis* was present in food.

Experiments took place in triplicates over 11 days in 1-L glass beakers, starting with 10 copepods per beaker and maintained under identical conditions described for the brood cultures. Animals were obtained by growing a new cohort of adults from nauplii in the brood cultures. Experimental copepods were then collected from the pool of >400 copepods in the new cohort by gently filtering in a Petri dish for each set of experiments. To minimise the chance of age-related mortality and stress over the 11 days, only active C_5 copepodites and young adults were randomly selected. Survival and activity were monitored daily using a light table and a dissecting microscope when necessary. Copepods were considered dead only if they showed no sign of movement after disturbance under a dissecting microscope; dead copepods were removed as soon as recorded.

MC verification of strains and treatments

Microcystis cultures were sampled for MC analysis during the experiment, to ensure the MC+ and MC− strains were uncontaminated and to measure MC concentration of the MC+ strain culture. In addition, the MC+ experimental beakers were sub-sampled at day 7 of the survival test for total and dissolved MC analysis to ensure that mortality was not because of any dissolved MCs. A commercially available MC – specific competitive ELISA assay with a detection range of 0.16–2.5 μg L^{-1} MC-LR was used for MC analysis (Envirologix, Portland, ME, U.S.A.). *Microcystis* cultures were sampled and kept frozen until analysis, upon which they were thawed to room temperature, sonicated, diluted and measured for MC concentration using a microplate reader at 450 nm. Dissolved MC in treatments was measured 7 days during the experiment, from whole water samples by separating algal cells on a GF/F glass fibre filter (Whatman Corp) and comparing MC concentration in the filtrate with an unfiltered sample. Additionally, a standard polymerase chain reaction (PCR) using published primers designed for the conserved *Microcystis*-specific 16S ribosomal DNA (rDNA) region and amplification of the MIC and MC synthetase genes *mcy*B and *mcy*D was used to verify the identity of the two strains of *Microcystis*. Ability to produce MC is based on the presence or absence of the synthetase genes (Ouellette & Wilhelm, 2003). Conventional PCR was conducted to verify the presence of *Microcystis* and the MC synthetase genes *mcy*B and D using specific PCR primer sets targeting 16S rDNA and conditions described in Ouellette & Wilhelm (2003). The *Microcystis* strains were processed for genomic DNA extraction using a modified phenol–chloroform procedure (Sambrook & Russell, 2001).

Qualitative confirmation of Microcystis ingestion

To verify ingestion, the presence of *Microcystis* cells in copepod guts was determined in a separate feeding experiment. Copepods were starved for 5 h to evacuate gut contents before the experiment, which took place under the same conditions as the survival tests. Copepod density was kept at 10 adults in a 1-L beaker with clean media, and then fed the same treatment diets (MC+ and MC−) described earlier. Following a 30-min feeding period, animals were gently filtered, rinsed three times to remove any attached *Microcystis* cells and narcotised immediately with carbonated water. For each treatment diet, 10 copepods were dissected to expose the gut under a dissecting microscope. Guts were observed for phycocyanin fluorescence (a pigment specific to cyanobacteria), as a qualitative indication of *Microcystis* cells in copepod guts, using an epifluorescent microscope.

Statistical analysis

The effect of diet type on the survival of each copepod species was evaluated by a parametric survival analysis which modelled survival with a Weibull distribution curve (JMP version 7.0; SAS, Cary, NC, U.S.A). Individual probability of mortality or survival (expected proportion of the population dead or surviving) for each treatment and species was calculated by a test of deviance based on the mean number of surviving copepods on the last day of the experiment. Then, the effect of diet type (e.g. *Microcystis* versus no *Microcystis*) and copepod species on survival was calculated to compare differences in the effects of the treatments and controls. Finally, the *Microcystis* only treatments were further broken down to strain (MC+ or MC−) and ratio of dietary *Microcystis* (% *Microcystis*) to analyse differences in the effect of these parameters on copepod survival. Time to 50% mortality was analysed by linear regression of individual replicate survival responses and calculating when 50% mortality occurred on average per treatment. All differences noted are significant at $P = 0.05$ unless otherwise mentioned.

Results

Survival test

Both dietary composition and quantity had very pronounced effects on copepod survival. The presence and proportion of *Microcystis* drastically reduced survival in all treatments for either copepod species, regardless of *Microcystis* strain and MC content (Fig. 1). In contrast, survival with the control diets was significantly higher than corresponding *Microcystis* treatments (Fig. 1; $P < 0.001$).

Reducing the concentration of IA in the controls without *Microcystis* reduced survival. At the end of the experiment, 100% IA had the highest survival (85% ± 3.4), while survival in the 50% IA treatment was lower (75% ± 2.3 for *E. affinis*, 60% ± 8.2 for *P. forbesi*) (Fig. 1). Starved copepods (i.e. those fed 0% IA and 0% *Microcystis*) survived for several days, reaching 100% mortality after 7–9 days for both species, though *P. forbesi* had slightly longer average persistence than *E. affinis*.

Compared to *E. affinis*, *P. forbesi* was more sensitive to differences between the *Microcystis* strains (MC+ versus MC−) and had a higher survival rate on a MC+ diet (Fig. 1). Differences in survival of *P. forbesi* between the two different *Microcystis* strains took several days to emerge. Both strains (MC+ or MC−) caused comparable mortality until day 6, and thereafter *P. forbesi* mortality on the MC− diet was greater than that on the MC+ diet (Fig. 1). Towards the end of the experiment, *P. forbesi* mortality began to decline in most of the MC+ diets, but not in the MC− diets (Fig. 1). For treatments with <50% *Microcystis*, the MC− diet killed more *P. forbesi* than the MC+ diet, while these diets caused similar mortality for *E. affinis* (Fig. 1).

Survival analysis

The increased proportion of *Microcystis* in the diet caused higher mortality and increased the probability of mortality (Fig. 2). The effect test showed that diet type had a significant effect on survival and that differences in survival were not because of copepod species (Table 1a). When comparing results among the treatments (Table 1b), differences in survival were caused by the *Microcystis* strains (MC) and the ratio of *Microcystis* in food (% *Microcystis*). There was a significant difference in how each species responded to the MC+ versus the MC− strains, as well as the ratio of *Microcystis* in food, as shown by the interaction terms (species × MC) and (species × % *Microcystis*), respectively, in Table 1b.

Survival/mortality probability tests confirmed that copepods responded differently, both to the individual strains (MC+ versus MC−) and to the ratio of *Microcystis* in their food. *Pseudodiaptomus forbesi* was more likely to survive on the MC+ diet compared to an MC− diet when the diet contained <50% *Microcystis* (Fig. 2). In contrast, differences in the *Microcystis* strain had no significant effect on *E. affinis* survival at the end of the experiment. Additionally, *P. forbesi* was more sensitive to changes in the *Microcystis* content of its diet, as increased *Microcystis* resulted in higher mortality. In contrast, increasing *Microcystis* did not significantly change the probability of mortality for *E. affinis* (Fig. 2). Rather, the presence of *Microcystis* was more important in reducing *E. affinis* survival probability by the end of the experiment.

Mean survival time

The time to 50% mortality (TL 50) was inversely proportional to the % *Microcystis* in diets, but varied significantly with copepod species and *Microcystis*

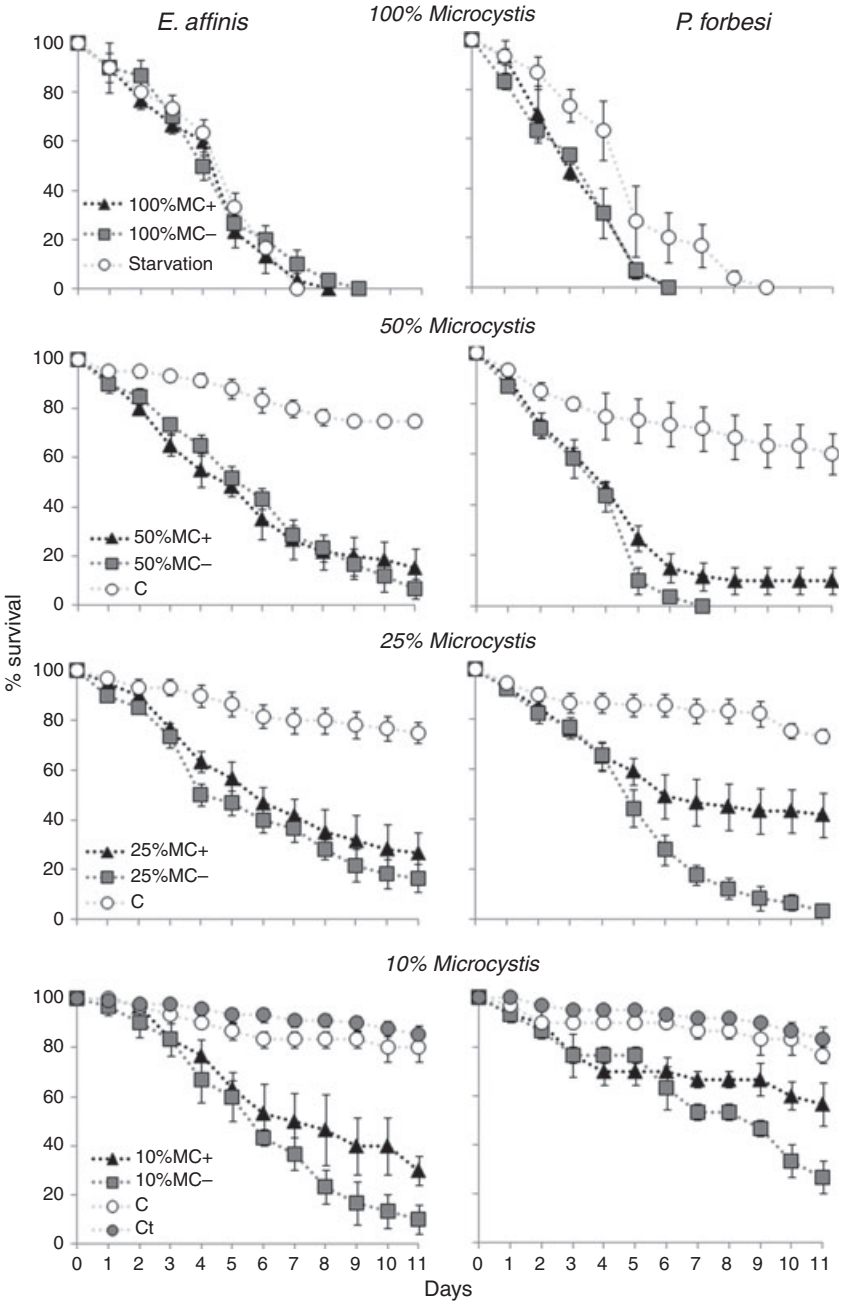

Fig. 1 Survival of *Eurytemora affinis* and *Pseudodiaptomus forbesi* on diets containing a mixture of instant algae (IA) and *Microcystis* (MC+ or MC–), at various proportions of the total food, compared to survival on corresponding control diets containing only IA at the same IA concentration as in the corresponding mixture of IA and *Microcystis*. C_T shows survival on the total amount of control diet (100% IA). Each line represents the mean of a minimum of three replicates (±SE).

strain (Fig. 3). Among the two copepod species, TL-50 was similar when the ratio of dietary *Microcystis* was high (50 and 100%), but diverged at lower ratios of dietary *Microcystis* (10 and 25%) (Fig. 3). Two patterns emerged at these lower *Microcystis* treatments. First, on average, *P. forbesi* lived longer than *E. affinis* when *Microcystis* was in the diet. Second, the variation in *Microcystis* strains caused a significant difference in *P. forbesi* survival time compared to *E. affinis* (Fig. 3). The IA control diets (50, 75, 90 and 100%) all had TL-50 >15 days. Starved copepods had a mean survival time of 3.99 ± 0.04 (*E. affinis*) and 4.23 ± 0.6 (*P. forbesi*) days.

Verification of Microcystis strains, dissolved MC and ingestion

Cross-contamination between the MC+ and MC– strains of *Microcystis* was not observed in the

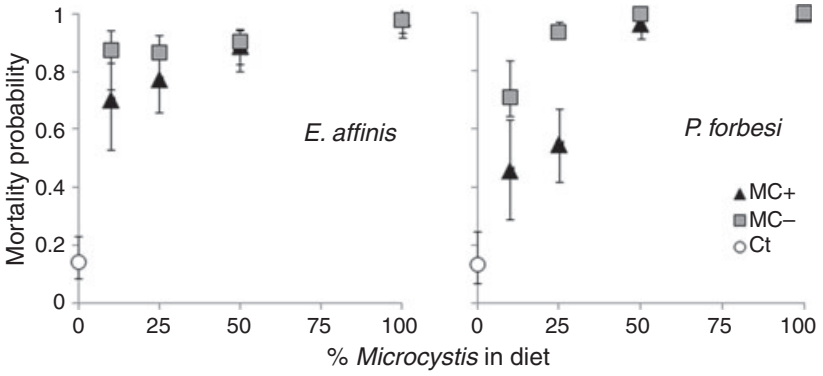

Fig. 2 Probability of copepod mortality in relation to the proportion of *Microcystis* (MC+ or MC−) in the diet. Values are based on survival estimates of the parametric model (test of deviance), for the final day of feeding experiment (day 11). For comparison, C_T refers to mortality on the total amount of control diet with no *Microcystis* (100% instant algae). Bars indicate 95% CI.

Table 1 Summary results for the parametric survival fit (test of deviance) and differences in the effect of (a) species and diet (between all diets, controls and *Microcystis*) on copepod survival probability; results show the factorial interaction of species (*Pseudodiaptomus forbesi* and *Eurytemora affinis*) with each diet treatment and (b) the effect of species, MC presence and % *Microcystis* on copepod survival probability (excludes control diets); results show the factorial interaction of species (*P. forbesi* and *E. affinis*) with MC presence (MC+ or MC− strain) and % of *Microcystis* in the diet (10, 25, 50 and 100%)

Source	No. of parameters	d.f.	χ^2	Prob > χ^2
(a)				
Species	1	1	0.928	0.335
Diet	12	12	730.193	<0.001
Species × diet	12	12	42.383	<0.001
(b)				
Species	1	1	0.833	0.361
MC	1	1	22.372	<0.001
% *Microcystis*	3	3	141.288	<0.001
Species × MC	1	1	8.917	0.003
Species × % *Microcystis*	3	3	30.938	<0.001
MC × % *Microcystis*	3	3	13.236	0.004

MC, microcystin.

treatment diets during the trials as verified by periodic measurements of MC-LR concentration with an ELISA. Furthermore, PCR confirmed the presence of *Microcystis*-specific 16S rDNA genes in both *Microcystis* strain but, as expected, the MC synthetase genes *mcy*B and *mcy*D were detected only in the MC+ strain. Mean cell-bound MC-LR concentration in the 100% MC+ treatment was 1.62 μg L^{-1} (±0.18, $n = 7$) and 348 μg L^{-1} (±49, $n = 8$) in the MC+ *Microcystis* culture. This corresponds to an estimated 4.87 μg mgC^{-1} (±0.98, $n = 7$) of MC-LR per *Microcystis* biomass. The mean cell density in the 100% *Microcystis* treatment was 93 000 cells mL^{-1} (±1458, $n = 14$). Dissolved MC-LR was not detected in the treatment beakers during the experiment. Copepods ingested *Microcystis* in all treatments as observed qualitatively from the fluorescence of phycocyanin.

Discussion

Our results provide strong evidence that the mortality of both *P. forbesi* and *E. affinis* was because of dietary *Microcystis*. As predicted, an increase in the ratio of *Microcystis* in the diet reduced copepod survival within days, and even the smallest addition to the diet (10%) significantly reduced copepod survival. High survival on the control diet suggests that mortality was driven by toxicity from *Microcystis* and that background mortality was low. Similar toxic effects of dietary *Microcystis* on zooplankton have often been observed (DeMott *et al.*, 1991; Kirk & Gilbert, 1992; Wilson *et al.*, 2006). What is unique to this study is the contrast between the two copepod species in their response to the dietary ratio and strains of *Microcystis* and, more surprisingly, the greater tolerance of *P. forbesi* for the MC+ strain relative to the MC− strain.

The hypothesis that MC+ cells would cause higher mortality was not supported, as the MC− strain caused similar (*E. affinis*) or higher (*P. forbesi*) mortality. *Microcystis* produces several non-MC metabolites that are toxic when ingested and probably resulted in the harmful effects found here. Examples include lipopolysaccharides, which reduce the efficiency of detoxification, microviridin, which reduces protease activity, and various other unidentified toxins (Kurmayer & Juttner, 1999; Rohrlack *et al.*, 2004; Wiegand & Pflugmacher, 2005). As shown with other zooplankton, our results highlight the potential toxicity of non-MC metabolites to copepods (Wilson *et al.*, 2006).

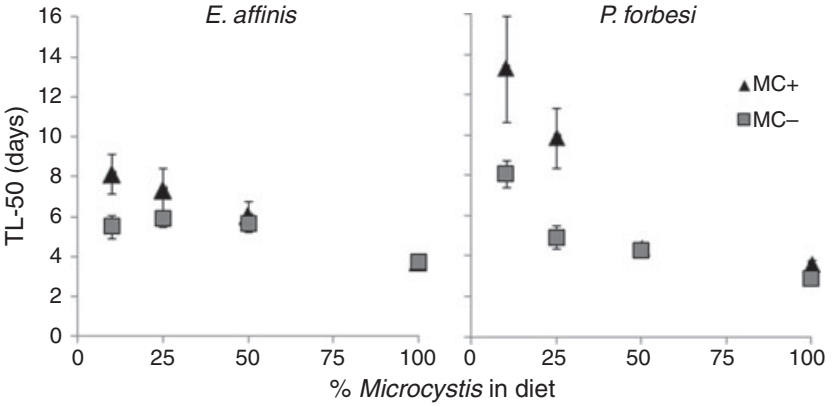

Fig. 3 Mean time to mortality (TL-50) of each treatment and copepod species as a function of the ratio of *Microcystis* (MC+ or MC−) in diet (±SE).

Declining mortality of *P. forbesi* following sustained exposure to the MC+ *Microcystis* suggests that prolonged exposure may prompt an adaptation to this strain, either via physiological tolerance or a change in feeding behaviour. Species-specific tolerance has ecological significance, since it may explain how some zooplankton can dominate during blooms of toxic algae (Kirk & Gilbert, 1992; Work & Havens, 2003; Karjalainen *et al.*, 2007).

Previous exposure to *Microcystis* over evolutionary timescales may improve physiological tolerance (i.e. detoxification) in zooplankton (DeMott *et al.*, 1991), which may be naturally higher for some copepod species (Kumar, 2003). While increased tolerance of *Microcystis* in daphniids can also be triggered by short-term exposure, this ability varies among species and the relationship between evolutionary exposure (genotypic effects) and improved detoxification remains unknown (Gustafsson & Hansson, 2004; Sarnelle & Wilson, 2005). However, detoxification is a process that follows ingestion and probably not the mechanism by which *P. forbesi* was better able to tolerate MC+ *Microcystis*. For MC detoxification to occur, *P. forbesi* in the MC+ treatments would first have to ingest the *Microcystis* cells, thereby ingesting both MC and the non-MC metabolites. In such a case, we would expect to see higher survival for the copepods in the MC− treatments (relative to MC+), since MC detoxification causes added stress to zooplankton (Pflugmacher *et al.*, 1998).

Nevertheless, higher mortality in the MC− treatments confirms that the ingestion of non-MC metabolites is indeed lethal, excluding physiological tolerance of MC as the main cause for coexistence with MC+ *Microcystis*. A more likely mechanism is selective feeding, which is a common adaptation in copepods exposed to toxic cyanobacteria for extended periods (DeMott & Moxter, 1991; Kleppel, 1993; Koski, Engstrom & Viitasalo, 1999). Incidental mortality later in the experiment may be explained by accidental ingestion, as copepods can passively ingest *Microcystis* cells despite selective feeding (Panosso *et al.*, 2003). High mortality at the onset of exposure to the MC+ diet suggests an acclimation phase when *P. forbesi* optimises MC avoidance and subsequently improves its ability to survive in the presence of *Microcystis* by feeding selectively on the more palatable food particles.

Assuming that *P. forbesi* can reject harmful particles such as MC+ cells, why does it not also reject the fatal MC− cells with comparable success? Zooplankton have developed different means of detecting and avoiding cyanobacterial toxins, which are considered as evolved defences against zooplankton grazing (DeMott & Moxter, 1991; DeMott *et al.*, 1991). Although MCs have been shown to deter feeding in daphniids, some copepods rely on more general and unidentified lipophylic toxins to detect and avoid ingesting harmful cyanobacteria (Kurmayer & Juttner, 1999; Rohrlack *et al.*, 1999). Comparing different calanoid copepods exposed to the cyanobacteria *Nodularia*, the hepatotoxin nodularin was the grazing deterrent for *E. affinis*, which selectively fed on nodularin-free cyanobacteria (Engstrom *et al.*, 2000). In contrast, *Acartia* grazing was deterred by a more general cyanobacterial cue other than nodularin, as it selected against *Nodularia* regardless of its nodularin content. This is consistent with our results and shows that deterrence of copepod grazing on cyanobacteria is commonly mediated by species-specific chemosensory

cues. There is no real evidence in the current study showing that differences in the survival of *E. affinis* and *P. forbesi* exposed to each *Microcystis* strain is caused by differences in feeding selectivity. Nevertheless, based on the role of species-specific cues for copepod feeding selectivity, we speculate that MC may be the dominant feeding deterrent for *P. forbesi*, resulting in an ability only to avoid MC+ cells.

Higher survival of *P. forbesi* in the MC+ treatments is surprising and contrary to the presumption of most zooplankton–*Microcystis* interactions. Typically, the MC content of food either reduces or has no significant effect on survival (Rohrlack *et al.*, 1999; Lurling, 2003; Wilson *et al.*, 2006). Following this general prediction, and in contrast to *P. forbesi*, MC content of *Microcystis* had no significant effect on *E. affinis*, as both MC+ and MC− strains caused similar mortality. Despite such species-specific differences, our results indicate that the non-MC compounds in *Microcystis* cells can be the main factor causing negative effects on some copepods.

Eurytemora affinis and *P. forbesi* also responded differently to the proportion of *Microcystis* in their food, regardless of strain. In general, *P. forbesi* did better for a given *Microcystis* diet, and its survival improved with an increased proportion of good food. Calanoid copepods can show reduced ingestion for toxic cyanobacteria when alternative food is available, which probably explains the negative relationship between the ratio of dietary *Microcystis* and *P. forbesi* survival (DeMott & Moxter, 1991; Burns & Hegarty, 1994; Koski *et al.*, 1999). In contrast, *E. affinis* survival varied much less with the ratio of dietary *Microcystis*. This may indicate inefficient feeding selection by this species, which was also shown in the Baltic Sea (Engstrom *et al.*, 2000). *Pseudodiaptomus forbesi* may be better adapted to survive in the presence of *Microcystis* via feeding selection, and further investigation would help verify the mechanism.

In addition to toxicity, *Microcystis* is also nutritionally inadequate for zooplankton (DeMott *et al.*, 1991; Christoffersen, 1996). Relatively high survival in the controls indicates that sufficient nutrition was provided by the IA part of the *Microcystis* – IA mixed diet. Hence, even if *Microcystis* nutritional deficiency was a significant issue, IA should have sustained survival at least as well as the controls. Since survival with *Microcystis* is significantly lower than the controls without *Microcystis*, the toxic effects of *Microcystis* dominate any potential consequence of nutritional inadequacy. Toxic cyanobacteria can also harm zooplankton by inducing feeding inhibition (DeMott *et al.*, 1991; Rohrlack *et al.*, 1999; Lurling, 2003; Wiegand & Pflugmacher, 2005). However, this is unlikely for calanoid copepods that avoid lower quality food particles without reducing their ingestion rate on high-quality food (DeMott & Moxter, 1991; Kleppel, 1993; Koski *et al.*, 1999; Tackx *et al.*, 2003). Surviving copepods in *Microcystis* treatments were actively swimming, had full guts and continued to produce faecal pellets, indicating that copepods continued ingestion during the experiment. Both strains of *Microcystis* and the IA controls were uni- and bi-cellular, ruling out any potential effects from colonial morphology, which can also hinder ingestion (Fulton & Paerl, 1987; Ghadouani, Pinel-Alloul & Prepas, 2003).

Empirical evidence from the field for the toxic effects of cyanobacteria on zooplankton is less common and typically muted in comparison to laboratory interactions, where variables can be strictly controlled (Ferrao-Filho & Azevedo, 2003; Work & Havens, 2003). Ecosystem impacts of cyanobacteria at larger scales are more subtle because of stochastic variability, species-specific adaptations, colonial morphology, multiple trophic links and unpredictable food web dynamics (Kozlowski-Suzuki *et al.*, 2003; Rohrlack *et al.*, 2004; Wilson *et al.*, 2006; Beninca *et al.*, 2008). However, the impacts of cyanobacteria at the ecosystem scale have been demonstrated, and include the disruption of the benthic food web via bioaccumulation of toxins, shifts in zooplankton and phytoplankton species composition, and possible indirect effects shifting the pelagic community towards the microbial loop (Fulton & Paerl, 1987; Christoffersen, 1996; Karjalainen *et al.*, 2007).

We found strong evidence that dietary *Microcystis* reduces copepod survival even at low dietary ratios, suggesting possible direct impacts on copepods in the San Francisco Estuary where *Microcystis* seasonally dominates phytoplankton (Lehman *et al.*, 2005). During blooms in the estuary, *Microcystis* dominates the phytoplankton community and its density fluctuates, between about 0×10^3 and 2×10^3 cell mL^{-1} in the euphotic zone, depending on the location and time, though densities up to 2.2×10^6 mL^{-1} have been recorded (Lehman *et al.*, 2008). The average range monitored in the estuary corresponds with the

treatment diets in this study, at least in terms of cell density, as the highest concentration of *Microcystis* fed to copepods was 9.3×10^3 mL^{-1}. During blooms, persistent concentrations of *Microcystis* between 10^3 and 10^6 cell mL^{-1} are often observed in eutrophic waters globally, making the results of this study relevant to the zooplankton community of the San Francisco Estuary as well as other regions (Chorus & Bartram, 1999; Rinta-Kanto *et al.*, 2005; Costa *et al.*, 2006).

Nevertheless, as mentioned previously, *Microcystis* impacts measured in the laboratory may not always represent natural processes in the field. Depending on the intensity of the bloom and the availability of alternative food, including microbial sources such as ciliates, selectively feeding copepods may or may not persist in the presence of *Microcystis* (DeMott & Moxter, 1991; Work & Havens, 2003). Zooplankton in the San Francisco Estuary can be limited by low phytoplankton abundances (Jassby, Cloern & Cole, 2002; Muller-Solger *et al.*, 2002), and such food limitation can intensify the negative impacts of cyanobacteria on copepods by reducing the effectiveness of selective feeding (DeMott & Moxter, 1991; Engstrom *et al.*, 2000). Conversely, natural blooms of *Microcystis*, including in the estuary, are typically dominated by colonial forms (Lehman *et al.*, 2005; Wilson *et al.*, 2006), and copepods are probably more effective at selective feeding in the presence of colonies, when the particle size is larger (Tackx *et al.*, 2003). However, copepods in the San Francisco Estuary accumulate MC during *Microcystis* blooms, suggesting that ingestion in the field occurs, despite the dominance of colonies (Lehman *et al.*, 2008). For these reasons, although we found negative impacts of *Microcystis* on copepods, prediction of the ecological impacts must also be informed by coupled laboratory and *in-situ* field observations of ingestion and food limitation and evaluation of the ability of copepod species to detect and avoid harmful cells.

Acknowledgments

We greatly appreciate Ida Flores for assistance with culturing copepods and survival experiments, Dr Tomofuri Kurobe for help with development of the PCR, Dr Monika Winder for assistance with epifluorescence microscopy, Dr Emilio Laca for help with statistical analysis and the suggestions of the two anonymous reviewers, which improved the manuscript considerably. Funding of this study is supported by Dr Swee Teh's Aquatic Toxicology Program fund and partially by the California Interagency Ecological Program Pelagic Organisms Decline (IEP-POD), CALFED (project# PO685515) and California Department of Water Resources (Contract Nos. 4600007499 and 4600008137).

References

Beninca E., Huisman J., Heerkloss R., Johnk K.D., Branco P., Van Nes E.H., Scheffer M. & Ellner S.P. (2008) Chaos in a long-term experiment with a plankton community. *Nature*, **452**, 822–826.

Burns C.W. & Hegarty B. (1994) Diet selection by copepods in the presence of cyanobacteria. *Journal of Plankton Research*, **16**, 1671–1690.

Chorus I. & Bartram J. (1999) *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring, and Management*. E & FN Spon, London, U.K.

Christoffersen K. (1996) Ecological implications of cyanobacterial toxins in aquatic food webs. *Phycologia*, **35**, 42–50.

Costa I.A.S., Azavedo S.M.F.O., Senna P.A.C., Bernardo R.R., Costa S.M. & Chellappa N.T. (2006) Occurrence of toxin producing cyanobacteria in a Brazilian semiarid reservoir. *Brazilian Journal of Biology*, **66**, 211–219.

DeMott W.R. & Moxter F. (1991) Foraging on cyanobacteria by copepods: responses to chemical defenses and resource abundance? *Ecology*, **75**, 1820–1834.

DeMott W.R., Zhang Q.X. & Carmichael W. (1991) Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. *Limnology and Oceanography*, **36**, 1346–1357.

Engstrom J., Koski M., Viitasolo M., Reinikainen M., Repka S. & Sivonen K. (2000) Feeding interactions of the copepods *Eurytemora affinis* and *Acartia bifilosa* with the cyanobacteria Nodularia. *Journal of Plankton Research*, **22**, 1403–1409.

Ferrao-Filho A.S. & Azevedo S.M. (2003) Effects of unicellular and colonial forms of toxic *Microcystis aeruginosa* from laboratory cultures and natural populations on tropical cladocerans. *Aquatic Ecology*, **37**, 23–35.

Fulton R.S. & Paerl H.W. (1987) Toxic and inhibitory effects of the blue-green alga *Microcystis aeruginosa* on herbivorous zooplankton. *Journal of Plankton Research*, **9**, 837–855.

Ghadouani A., Pinel-Alloul B. & Prepas E.E. (2003) Effects of experimentally induced cyanobacterial

blooms on crustacean zooplankton communities. *Freshwater Biology*, **48**, 363–381.

Gustafsson S. & Hansson L.-A. (2004) Development of tolerance against toxic cyanobacteria in *Daphnia*. *Aquatic Ecology*, **38**, 37–44.

Horning W.B. & Weber C.I. (1985) *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*. pp. 58–75. EPA/600/4/85/014, USEPA, Cincinnati, OH, U.S.A.

Jassby A.D., Cloern J.E. & Cole B.E. (2002) Annual primary production: patterns and mechanisms of change in a nutrient rich tidal ecosystem. *Limnology and Oceanography*, **47**, 698–712.

Karjalainen M., Engstrom-Orst J., Korpinen S., Peltonen H., Paakonen J.-P., Ronkkonen S., Suikkanen S. & Viitasalo M. (2007) Ecosystem consequences of cyanobacteria in the Northern Baltic Sea. *Ambio*, **36**, 195–202.

Kirk K.L. & Gilbert J.J. (1992) Variation in herbivore response to chemical defenses: zooplankton foraging on toxic cyanobacteria. *Ecology*, **73**, 2208–2217.

Kleppel G.S. (1993) On the diets of calanoid copepods. *Marine Ecology Progress Series*, **99**, 183–195.

Koski M., Engstrom J. & Viitasalo M. (1999) Reproduction and survival of the calanoid copepod *Eurytemora affinis* fed with toxic and non-toxic cyanobacteria. *Marine Ecology Progress Series*, **186**, 187–197.

Kozlowski-Suzuki B., Karjalainen M., Lehtiniemi M., Engström-Öst J., Koski M. & Carlsson P. (2003) Feeding, reproduction and toxin accumulation by the copepods *Acartia bifilosa* and *Eurytemora affinis* in the presence of the toxic cyanobacterium *Nodularia spumigena*. *Marine Ecology Progress Series*, **249**, 237–249.

Kumar R. (2003) Effect of different food types on developmental rates and demographic parameters of *Phyllodiaptomus blanci* (Copepoda:Calanoida). *Archiv für Hydrobiologie*, **157**, 351–377.

Kurmayer R. & Juttner F. (1999) Strategies for the co-existence of zooplankton with the toxic cyanobacterium *Planktothrix rubescens* in Lake Zurich. *Journal of Plankton Research*, **21**, 659–683.

Lehman P.W., Boyer G., Hall C., Waller S. & Gehrts K. (2005) Distribution and toxicity of a new colonial *Microcystis aeruginosa* in the San Francisco Bay Estuary, California. *Hydrobiologia*, **541**, 87–99.

Lehman P.W., Boyer G., Satchwell M. & Waller S. (2008) The influence of environmental conditions on the seasonal variation of *Microcystis* cell density and microcystins concentration in San Francisco Estuary. *Hydrobiologia*, **600**, 187–204.

Lurling M. (2003) *Daphnia* growth on microcystin-producing and microcystin-free *Microcystis* aeruginosa in different mixtures with the green alga *Scenedesmus obliqus*. *Limnology and Oceanography*, **48**, 2214–2220.

Muller-Solger A., Jassby A.D. & Muller-Navarra D. (2002) Nutritional quality of food resources for zooplankton (*Daphnia*) in a tidal freshwater system (Sacramento-San Joaquin River Delta). *Limnology and Oceanography*, **47**, 1468–1476.

Ouellette J.A. & Wilhelm S. (2003) Toxic cyanobacteria: the evolving molecular toolbox. *Frontiers in Ecology and the Environment*, **1**, 359–366.

Paerl H.W. (1988) Nuisance phytoplankton blooms in coastal, estuarine, and inland waters. *Limnology and Oceanography*, **33**, 823–847.

Panosso R., Carlsson P., Kozlowsky-Suzuki B., Azevedo S.M.F.O. & Graneli E. (2003) Effect of grazing by a neotropical copepod *Notodiaptomus*, on a natural cyanobacterial assemblage of toxic and non-toxic cyanobacterial strains. *Journal of Plankton Research*, **25**, 1169–1175.

Pflugmacher S., Wiegand C., Oberemm A., Beattie K.A., Krause E., Codd G. & Steinberg C.E.W. (1998) Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: The first step of detoxification. *Biochimica et Biophysica Acta*, **1425**, 527–533.

Reynolds C.S. & Jaworski G.H.M. (1978) Enumeration of natural *Microcystis* populations. *British Phycological Journal*, **13**, 269–277.

Rinta-Kanto J.M., Ouellette A.J.A., Boyer G.L., Twiss M.R., Bridgeman T.B. & Wilhelm S.W. (2005) Quantification of toxic *Microcystis* spp. during the 2003 and 2004 blooms in Western Lake Erie using quantitative real-time PCR. *Environmental Science and Technology*, **39**, 4198–4205.

Rohrlack T., Dittman E., Borner T. & Christoffersen K. (1999) Effects of cell-bound microcystins on survival and feeding of *Daphnia* spp. *Applied and Environmental Microbiology*, **67**, 3523–3529.

Rohrlack T., Christoffersen K., Kaerbernick M. & Neilan B.A. (2004) Cyanobacterial protease inhibitor Microviridin J causes a lethal molting disruption in *Daphnia pulicaria*. *Applied and Environmental Microbiology*, **70**, 5047–5050.

Sambrook J. & Russell D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Sarnelle O. & Wilson A.E. (2005) Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. *Limnology and Oceanography*, **50**, 284–289.

Sommer T., Armor C., Baxter R. *et al.* (2007) The collapse of pelagic fishes in the upper San Francisco Estuary. *Fisheries*, **32**, 270–277.

Tackx M.L.M., Herman P.J.M., Gasparini S., Irigoien X., Billiones R. & Daro M.H. (2003) Selective feeding of *Eurytemora affinis* (Copepoda, Calanoida) in temperate

estuaries: model and field observations. *Estuarine and Coastal Shelf Science*, **56**, 305–311.

Turriff N., Runge J.A. & Cembella A.D. (1995) Toxin accumulation and feeding behaviour of the planktonic copepod *Calanus finmarchicus* exposed to the red-tide dinoflagellate *Alexandrium excavatum*. *Marine Biology*, **123**, 55–64.

Wiegand C. & Pflugmacher S. (2005) Ecotoxicological effects of selected cyanobacterial metabolites: a short review. *Toxicology and Applied Pharmacology*, **203**, 201–218.

Wilson A.E., Sarnelle O. & Tillmanns A.R. (2006) Effects of cyanobacterial toxicity and morphology on the population growth of freshwater zooplankton: meta-analyses of laboratory experiments. *Limnology and Oceanography*, **51**, 1915–1924.

Work K.A. & Havens K.A. (2003) Zooplankton grazing on bacteria and cyanobacteria in a eutrophic lake. *Journal of Plankton Research*, **25**, 1301–1307.

(Manuscript accepted 11 November 2009)