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Sublethal dietary effects of *Microcystis* on Sacramento splittail, *Pogonichthys macrolepidotus*

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ABSTRACT

The presence of the toxic cyanobacterium *Microcystis* in the upper San Francisco Estuary (SFE) since 1999 is a potential but to date an unquantified threat to the health and survival of aquatic organisms, such as fish and zooplankton. The microcystins (MCs) predominantly in the LR-form (MC-LR) contained in *Microcystis* is hepatotoxic and a potential threat to the fishery. This study was conducted to determine the effects of dietary exposure of the endemic Sacramento splittail, *Pogonichthys macrolepidotus* in SFE to *Microcystis* and its toxin, MC-LR. Juvenile splittail (12.59 ± 0.7 g fish^{-1}) were exposed to five diets for 28 d with MC-LR obtained from: (1) *Microcystis* harvested from the SFE and (2) a synthetic purified form of MC-LR. Three of the test diets contained 3.55 (D5), 9.14 (D10) and 17.13 (D20) mg MC-LR kg^{-1} from *Microcystis*. The other two diets contained either purified MC-LR at 3.89 mg MC-LR kg^{-1} (D5R) or no MC-LR (D0). The RNA/DNA ratio of fish muscle was significantly lower for all treatments fed test diets containing MC-LR compared to the control diet D0, suggesting *Microcystis* adversely affected nutritional status. Protein phosphatase 2A expression in the fish from the D5, D10 and D20 treatments were inversely affected by increasing concentrations of MC-LR. Cytoplasmic inclusion bodies and single cell necrosis were more prevalent and greater in severity in the fish exposed to the diets D10 and D20 compared to fish from the D0 treatment and indicate severe liver toxicity in splittail exposed to MC-LR. The sublethal effects on splittail characterized by this study suggest cyanobacterial blooms have the potential to affect splittail nutritional status and health in SFE.

1. Introduction

Microcystins (MCs) are monocyclic heptapeptide hepatotoxins produced by different species of cyanobacteria such as *Microcystis*, *Anabaena* and *Oscillatoria* (Carmichael, 1995; Sivonen and Jones, 1999). Cyanobacteria form harmful algal blooms in temperate and tropical eutrophic freshwater systems that threaten human and ecosystem health (Backer et al., 2008; Paerl and Huisman, 2009; Sivonen and Jones, 1999; Verspagen et al., 2006). There are currently over 80 variants of MCs, with the LR (MC-LR) form being the most toxic (Zurawell et al., 2005) and exposure to these toxins can promote tumors and result in liver damage in humans and wildlife (Fujiki and Suganuma, 2009). The MC have been detected in drinking water and in aquatic organisms used for human consumption (Kim et al., 2010; Pathak and Singh, 2010; Sivonen and Jones, 1999). Chronic exposure to cyanotoxins can cause widespread and serious health problems in animals and humans such as liver, digestive and skin diseases, neurological impairment, and death (Babica et al., 2006; Leao et al., 2009; Sivonen and Jones, 1999).

Toxicity from MCs results from the inhibition in the activity of serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) by the covalent binding of MC to these enzymes (Fujiki and Suganuma, 2009). Protein phosphatases regulate phosphorylation by protein kinases. Upon inactivation, the enzymes PP1 and PP2A cause hyperphosphorylation of structural proteins and increased gene expression of unregulated protein kinases (Fujiki and Suganuma, 2009). Affected cells exhibit impaired cellular function, tissue hemorrhaging, and necrosis in the livers of mice (Bu et al., 2006) and rainbow trout, *Oncorhynchus mykiss*, and medaka fish, *Oryzias latipes* (Mezhoud et al., 2008; Tencalla et al., 1994), and in the kidneys and gills of carp, *Cyprinus carpio* (Carbis et al., 1996). Microcystins were first identified in the cyanobacteria *Microcystis* (Carmichael, 1995) which may be a potential threat to fishery production in the upper San Francisco Estuary (SFE) (Lehman et al., 2005).

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Table 1
Dietary formulation of test diets fed to splittail for 28 days. Diet D5, D10 and D20 contained *Microcystis* and D5R contained purified MC-LR.

Ingredients	Splittail diet (g kg dry diet^{-1})				
	D0	D5	D10	D20	D5R
Casein (vitamin free)[a]	380	380	380	380	380
Arginine[b]	4	4	4	4	4
Glycine[b]	10.7	10.7	10.7	10.7	10.7
Lycine[b]	2.3	2.3	2.3	2.3	2.3
Dextrin[a]	270	264	258	247	265
Cellulose[a]	103	103	103	103	103
Carboxymethyl cellulose[a]	20	20	20	20	20
BML vitamin mix[b]	40	40	40	40	40
BTm mineral mix[b]	30	30	30	30	30
Corn oil[a]	20	20	20	20	20
Cod liver oil[a]	50	50	50	50	50
Canthaxanthin (10%)[e]	20	20	20	20	20
Sodium Alginate[f]	50	50	50	50	50
Microcystis[c]	0	6	12	23	0
Purified MC-LR[d]	0	0	0	0	5
Analytical concentration of MC-LR (mg/kg)	0	3.55	9.14	17.13	3.89

[a] USB Corporations (Cleveland, OH).
[b] ICN Biomedicals, Inc. (Irvine, CA).
[c] Collected in the San Francisco Estuary on August 2007.
[d] Axxora (San Diego, CA).
[e] DSM Nutritional Products (Parsippay, NJ).
[f] TIC Gums (White Marsh, MD).

The SFE has been experiencing reduced pelagic populations such as striped bass (*Morone saxatilis*), increased listing of threatened or endangered species (e.g., delta smelt, *Hypomesus transpacificus* and green sturgeon, *Acipenser medirostris*) and decreased abundance of indicator species (e.g., Sacramento splittail, *Pogonichthys macrolepidotus*) has occurred in SFE since 2000 (Adams et al., 2007; Baerwald et al., 2007; Feyrer et al., 2006; Moyle et al., 2004; Sommer et al., 2007). Potential causes of these problems have been studied including altered organic carbon accumulation (Canuel et al., 2009), hydrology (Brown et al., 2009) and habitat volume (Kimmerer et al., 2009), and *Microcystis* seasonal variation (Lehman et al., 2008). *Microcystis* blooms are listed among these potential factors because *Microcystis* blooms first recognized in 1999 (Lehman et al., 2005). *Microcystis* is present seasonally in freshwater reaches of the SFE where MC-LR, the most toxic form of microcystin, is also commonly detected (Lehman et al., 2005). The distribution of *Microcystis* blooms extends over the freshwater regions of the SFE from June to November and peaks in September (Lehman et al., 2005). *Microcystis* migrate vertically to the surface during daylight hours and return to the benthic at twilight, exposing benthic and pelagic organisms to the toxins produced by the blooms.

Although the seasonal recurrence of *Microcystis* blooms has been suggested as a threat to pelagic organisms in the SFE by the Interagency Ecological Program of California (IEP) in 2005, exposure to *Microcystis* has not been established as a significant risk factor to important fish species in the SFE (Armor et al., 2005). For fish, the hypothetical routes of exposure to MCs are through: (1) direct consumption of *Microcystis* and other toxin producing cyanobacteria, (2) interaction with the food web by feeding on grazers consuming *Microcystis* and other toxin producing cyanobacteria, or (3) exposure to dissolved MC during the senescence of the bloom. While toxicity is associated with the production of MC toxins, the actual mechanism linking *Microcystis* with the decline of fish populations in the SFE has not been determined (Armor et al., 2005).

The Sacramento splittail, *P. macrolepidotus*, is a native endemic fish in the SFE (Moyle, 2002). As a benthic forager, splittail may be exposed to *Microcystis* near the bottom during blooms and can potentially consume MCs by direct feeding of *Microcystis* in the sediment or contaminated prey. The presence of *Microcystins* in the food web was supported by the presence of microcystins in clam, zooplankton, amphipod and fish tissue collected during field surveys over multiple years (Lehman et al., 2005, 2008, 2010). The impact of the *Microcystis* bloom on the splittail population in the SFE was unknown, however their numbers were fluctuating since the cyanobacteria appeared in the SFE (Feyrer et al., 2006; Moyle et al., 2004). Characterizing the impacts of *Microcystis* on splittail health and survival is important. This native fish can serve as an indicator species for ecosystem quality and was a species of special concern in the SFE by the US Fish and Wildlife Service and the California Department of Fish and Game (Baerwald et al., 2007). It was listed as threatened in 1999 and although it was delisted in 2003 following a brief resurgence in abundance, the long-term persistence of this population remains a key concern (Baerwald et al., 2007).

To date, there has been no dietary exposure study determining the effects of toxic *Microcystis* blooms to the health of fish species in the SFE. The purpose of this study was to determine whether MCs from *Microcystis* are toxic to splittail fed graded concentrations of spiked diets and to characterize the biomarkers of exposure for use in future field analyses. Such information is invaluable to the management of splittail populations, particularly to understand the effects of *Microcystis* blooms on splittail recruitment as well as other fish species in the SFE.

2. Materials and methods

2.1. Diet preparation

Five diets were formulated using graded levels of MC-LR and other purified ingredients (Table 1). The treatment diets containing MC-LR were obtained from two sources: (1) natural form of MC-LR from *Microcystis* harvested from the SFE in August 2007 and (2) commercially purified MC-LR (Axxora, San Diego, CA). The natural *Microcystis* contained 872 mg MC-LR kg^{-1} of dry weight as determined by Liquid Chromatography/Mass spectrometry (LC/MS) Lehman et al. (2005). Sub-samples of *Microcystis* were also analyzed by conventional polymerase chain reaction (PCR) that used primers designed for the conserved *Microcystis*-specific 16S ribosomal DNA (rDNA) region (Ouellette et al., 2006). These tests confirmed the presence of MC producing *Microcystis* spp. in the samples that were processed for the graded MC-LR spiked diets

(Baxa et al., 2010). Three diets containing increasing concentrations of MC-LR from *Microcystis* were designated as D5, D10 and D20. Two control diets were also tested; a positive control diet with the purified MC-LR (D5R) and the control diet (D0) without *Microcystis* or synthetic MC-LR (Table 1). Following collection from the field, colonial *Microcystis* spp. were rinsed several times in distilled water, freeze dried, ground into a powder and stored at −20 °C. The diets were prepared by mixing the dry ingredients with oil and water using a Hobart mixer and extruded into pellets with a Cuisinart stand mixer (Conair, East Windsor, NJ). The pellets were freeze dried, crumbled and sieved into various particle sizes suitable for juvenile splittail and then stored at −20 °C until use.

2.2. Fish and experimental conditions

Splittail juveniles were reared from natural spawning of captive broodstocks in June 2006 at the Center for Aquatic Biology and Aquaculture (CABA), University of California, Davis (UCD). The initial average body weight was 12.6 ± 0.7 g fish^{-1}. The fish were cultured in a recirculating aquaculture system (RAS) with 15 tanks (20 L tank^{-1}). A total of 150 fish were used with 10 fish per treatment tank and three replicates per treatment. The water in the RAS was maintained at 22 ± 0.5 °C and was equipped with charcoal and cartridge filters, moving bed biofilter and UV sterilizer. The RAS had a flow rate of 4 L min^{-1} and maintained at 16L:8D photoperiod. The fish were acclimated to culture conditions and fed the control diet (2.1 Diet Preparation) for 28 d before the dietary exposure trials. The test diets were randomly assigned to three replicate tanks. The fish were fed 2% body weight day^{-1} for each treatment. The amount of diet was split equally and fed to the fish twice daily (9:00 a.m. and 4:00 p.m.). The inlet water was stopped during feeding for 0.5 h after which feces and uneaten food were removed by siphoning. External lesions, feeding behavior and mortality were recorded twice daily. Dissolved waste in the water was removed by chemical, biological and UV filtration. At the end of each 0.5 h feeding, water from the system was purged for 1 h by turning the inlets back on and allowing the outlet water to be removed from the RAS to remove any dissolved MC-LR. The volume of the RAS was maintained by replacing the flushed water with reconstituted water. Concentrations of dissolved MC-LR in the water were tested before and after purging once a week by ELISA kit (Envirologix, USA). The feeding trial lasted for 57 d. General care, maintenance, and handling of the juvenile fish followed procedures approved by the Campus Animal Use and Care Administrative Advisory Committee at UCD.

2.3. Sample collection for gross morphology, biochemistry, and histopathology

After feeding for 57 d, all fish were euthanized with an overdose of 3-aminobenzoic acid ethyl ester (MS-222, Sigma, St. Louis, MO). The euthanized fish were blotted dry with a paper towel, observed and measured for weight, total length, clinical signs (lesions, hemorrhaging and deformities) and liver weight. The liver, gill, kidney and gut tissue were removed and stored in 10% buffered formalin. The liver from each fish was split into two portions. One portion was added to a combined sample for all fish in the treatment and frozen in liquid nitrogen for analysis of caspase, an indicator of apoptosis and protein phosphatase 2A (PP2A), an indicator of exposure to MCs. The second portion of the liver was preserved individually for each fish in 10% neutral buffered formalin and used for histopathology. White muscle samples were taken from the dorsal section of each fish and frozen in liquid nitrogen for determination of the RNA/DNA ratio, a general biomarker of growth.

2.3.1. Biochemical analysis

Protein supernatant was extracted from homogenized liver samples by using ice-cold T-PER tissue protein extraction reagent as described by Deng et al. (2009). The primary and secondary antibodies for PP2A were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The PP2A in the supernatant were detected using western blot technique (Deng et al., 2009). The 25 μg protein was loaded onto 10% Tris–HCl precasted gels and separated by one-dimensional SDS-PAGE gel. Methods for performing western blot and enhanced chemiluminescence (ECL) detection were detailed by Hemre et al. (2004). A GS-710 imaging densitometer (Bio-Rad, Hercules, CA) was used to quantify the protein bands. The PP2A standard (Assay Designs Inc., Ann Arbor, MI; Biotechnology Inc. Santa Cruz, CA) and molecular weight markers (Amersham Biosciences Corp, Piscataway, PA) were loaded into the SDS-PAGE gel to confirm the molecular weight of the bands. Levels of PP2A were expressed as relative band density against the standard.

Caspase protein was extracted from homogenized liver samples by using ice-cold T-PER tissue protein extraction reagent as described by Deng et al. (2010). Caspase activity was determined by Apo-One Homogenous caspase Assay kit (Promega Corporation, Madison, WI). Fluorescence activity with an excitation wavelength of 485 nm and emission wavelength of 530 nm was read with a microplate reader. The caspase protein was expressed as fluorescence (mg protein)$^{-1}$.

Muscle nucleic acids were measured by an ethidium bromide fluorometric technique (Caldarone et al., 2001). Sample protein was dissociated from nucleic acid and the fluorophore ethidium bromide was used to measure total nucleic acids. RNAse was added to differentiate RNA concentration and calculation of DNA concentration.

2.3.2. Determination of MC concentration

The concentration of MC-LR was determined for all the diets by LC/MS. Microcystis tissue samples were stored in a −20 °C freezer and transported in dry ice to the laboratory overnight for analysis of total microcystin concentration as detailed by Lehman et al. (2005). An ELISA assay for water analysis (Envirologix, USA) with a detection range of 0.5–3.0 μg L^{-1} was used to detect dissolved MC-LR in water samples and was used to determine the effectiveness of the RAS filtration and flushing procedures to remove MC-LR from fish tanks.

2.3.3. Histopathology

Histopathological analysis was conducted on liver, gill, kidney and gut of each fish following the methods of Teh et al. (2004). After 48 h in 10% neutral buffered formalin, tissues were dehydrated in a graded ethanol series and embedded in paraffin. For each tissue block, serial sections (2–3 μm thick) were cut and stained with hematoxylin and eosin. Tissues were screened for a variety of histopathological features and lesions. The tissues were analyzed for lesions of glycogen depletion (GD), lipidosis (LIP), macrophage aggregate (MA), infiltration of inflammatory cells (INF), cytoplasmic inclusion bodies (CI) and single cell necrosis (SCN), then scored on an ordinal ranking system of 0 = none/minimal, 1 = mild, 2 = moderate, and 3 = severe using a BH-2 Olympus microscope. Glycogen depletion was 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 was characterized by excess lipid that appeared as clear, round, and well demarcated cytoplasmic vacuoles. Macrophage aggregate was described as a cluster of macrophages packed with coarsely granular yellow-brown pigment. Lymphocytic inflammation was identified by focal to multifocal aggregates of lymphocytes infiltrated the connective

tissue around bile ducts or blood vessels or parenchyma. Cytoplasmic inclusion bodies were characterized by the presence of proteins appearing as refractile, eosinophilic (pink coloration), round, and well-demarcated cytoplasmic droplets. Single cell necrosis was identified by the presence of cells having eosinophilic (i.e., pink coloration) cytoplasm with nuclear pyknosis and karyorrhexis.

2.4. Biological and statistical analyses

Condition factor (CF) was used to determine relative health of the splittail between treatments. The CF was calculated by using the individual wet weight of the fish in grams (W_B) and the cube of the individual length of the fish in centimeters (L) from head to the end of the tail and then multiplied by 100,

$$CF = 100 \times W_B L^{-3}. \quad (1)$$

Hepatosomatic index (HSI) was used to determine relative effect of MC-LR toxicity on the liver by the relative change in the liver and body weight ratios. The HSI was calculated using the individual wet weight measurements of the liver (W_L) in g for each fish divided by the weight of the same fish in grams (W_B) then multiplied by 100,

$$HSI = 100 \times W_L W_B^{-1}. \quad (2)$$

All data were analyzed using JMP ver. 8.0 (SAS Institute Inc. Cary, NC). Analysis was conducted using one-way analysis of variance (ANOVA) to determine the dietary effects of the treatments on average body weight, total length, CF, HSI, RNA/DNA, mean lesion scores, Caspase activity and PP2A expression. Significant differences ($p < 0.05$) were compared using Tukey's multiple mean comparison tests. The assumptions of the ANOVA were tested using Shapiro–Wilks and Levene's test. Differences in toxicity between the pure MC-LR (D5R) and the MC-LR from *Microcystis* tissue (D5) were tested using Student's *t*-test. Data were presented as mean ± SD.

3. Results

3.1. Concentrations of MC-LR in the diet and ambient water

The dietary MC concentration in each diet was confirmed by analysis: 0 (D0), 3.89 (D5R), 3.55 (D5), 9.14 (D10) and 17.13 (D20) mg MC-LR kg^{-1} dry diet (Table 1). No MC-LR was detected in the RAS water from treatments fed diet D0, D5, D5R and D10 during the 0.5 h feeding period. Microcystin-LR was only detected within the 0.5–3 μg L^{-1} range in the ambient water of the treatment D20 after 0.5 h of feeding. After flushing the RAS for 1 h, the MC-LR concentrations in the ambient water were below the minimum detectable level of 0.5 μg L^{-1} for all treatments.

3.2. Growth parameters, HSI, % survival

There was no significant ($p > 0.05$) dietary effect on the growth parameters examined including average body weight, total length, HSI and % survival (Table 2). Mortalities were observed in all diets but were not significant. The first mortalities were observed for fish in D20 after 24 d and D10 after 27 d. The percent survival for D10 and D20 were 90.0 and 96.7% respectively, after 28 d. There was no significant difference in % survival, average body weight, length and CF between D5 and D5R by Student's *t*-test. The HSI of fish fed the D5R diet were significantly lower than in fish fed the D5 diet by Student's *t*-test.

3.3. Biochemistry

After 28 days of feeding, the ratio of RNA/DNA was significantly ($p < 0.05$) lower in the muscle of fish fed the diets containing MC-LR compared to fish fed the control diets (Table 3). Caspase was not significantly different in MC-LR diet treatments (Table 3). Protein Phosphatase 2A expression was significantly depressed in the fish of the D20 treatment (Table 3). In addition, when examining only fish fed *Microcystis*, PP2A expression and MC-LR concentration were inversely significant (Fig. 1). RNA/DNA ratios and caspase activity were not significantly different in treatments D5 and D5R.

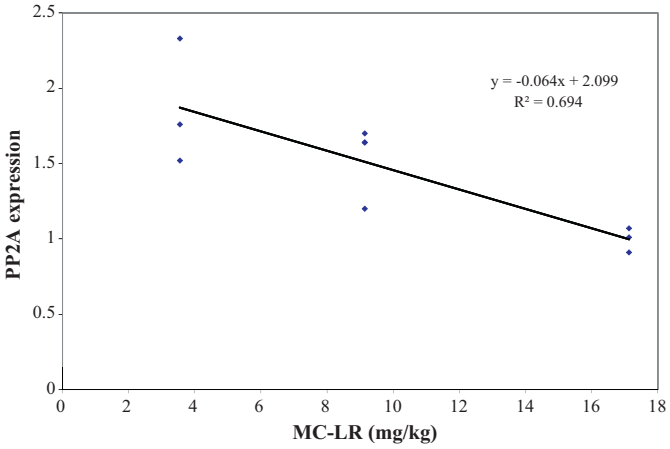

Fig. 1. Significant[a] inverse relationship of expression of protein phosphatase 2A in liver of splittail after 28 days of dietary exposure to Microcystis with graded concentrations of MC-LR. [a] p-Value < 0.0001.

3.4. Histopathology

Histopathological examination of liver, gill, kidney and gut showed that damage was restricted to the liver. Cytoplasmic inclusion bodies or single cell necrosis were not detected in the control but were observed in D5, D5R, D10 and D20 (Figs. 2 and 3). Moderate to severe lesions for CI and SCN were observed in treatments D5, D5R, D10 and D20. The severity of CI was also significantly greater for D10 and D20 compared to D0 (Table 4). There were increases in SCN mean lesion scores in fish exposed to D10 and in CI mean lesion scores in fish exposed to D10 and D20 (Table 4). Liver sections did not show significant changes in lipidosis, macrophage aggregate, and the infiltration of inflammatory cells for all treatments (Table 4).

4. Discussion

The SFE has been experiencing systemic negative trends in fish abundance including the population of the indicator species Sacramento splittail (Baerwald et al., 2007; Feyrer et al., 2006; Moyle et al., 2004). The appearance of *Microcystis* blooms since 1999 and the presence of the associated MCs in the prey of splittail may be a factor affecting the abundance of the fish. The purpose of this study was to examine the effects of dietary exposure to *Microcystis* because the two species co-occur in the same habitat (Baerwald et al., 2007; Lehman et al., 2005), thus splittail are continuously exposed to MCs in their diet. Concentrations encountered by fish in the field are often similar to those tested for this study. Microcystins were measured in the prey of splittail such as worms and amphipods at concentrations as high as 12.0 ± 0.00 and 2.62 ± 1.88 μg g^{-1} dry weight, respectively, throughout SFE (Lehman et al., 2008). Most of the diets in this study are within this range of exposure therefore the results of this study can potentially occur in the SFE. At the end of the 28 d dietary study indices of liver lesions, PP2A expression and RNA/DNA ratios were found to be the most sensitive to exposure to MC-LR from *Microcystis*. This study demonstrated the potential toxicity of *Microcystis* and

Table 2
Growth parameters, hepatosomatic index (HSI) and % survival of splittail after 28 days of dietary exposure to MC-LR. Values are presented as mean ± SD. Diets contained 3.89 (D5R), 3.55 (D5), 9.14 (D10), and 17.13 (D20) mg/kg of MC-LR.

Diet	Average body weight (g)	Total length (cm)	Condition factor	HSI %	% Survival
D0	10.66 ± 0.65	12.51 ± 0.40	0.52 ± 0.02	0.79 ± 0.14	96.7 ± 5.8
D5R	10.58 ± 0.57	12.45 ± 0.05	0.51 ± 0.02	0.70 ± 0.03	93.3 ± 5.8
D5	11.79 ± 2.34	12.97 ± 0.69	0.51 ± 0.03	0.75 ± 0.03	96.7 ± 5.8
D10	10.24 ± 1.54	12.33 ± 0.63	0.50 ± 0.03	0.80 ± 0.05	90.0 ± 10.0
D20	10.86 ± 0.53	12.69 ± 0.15	0.51 ± 0.03	0.72 ± 0.10	96.7 ± 5.8

Table 3
Caspase activity, RNA/DNA and PP2A expression for splittail after 28 days of dietary exposure to MC-LR. Values are given as mean ± SD. Diets contained 3.89 (D5R), 3.55 (D5), 9.14 (D10), and 17.13 (D20) mg/kg of MC-LR.

Diet	Liver		Muscle
	Caspase (Fluorescence mg of protein^{-1})	PP2A (% relative band density)	RNA/DNA
D0	48.68 ± 2.14	1.27 ± 0.21	1.16 ± 0.19
D5R	56.04 ± 15.88	1.44 ± 0.22	0.66 ± 0.10[a]
D5	52.00 ± 12.87	1.87 ± 0.42	0.72 ± 0.09[a]
D10	66.20 ± 18.57	1.51 ± 0.27	0.62 ± 0.09[a]
D20	60.70 ± 15.87	1.00 ± 0.08[a]	0.63 ± 0.08[a]

[a] Significant difference between treatments and D0 are indicated ($p < 0.05$).

the accompanying MCs at concentrations relevant to the field, to juvenile Sacramento splittail through dietary exposure.

Morphometric indices were not sensitive to exposure to MC-LR in either the commercial (D5R) form or from natural sources (D5, D10 and D20). Although previous experiments on brown trout, *Salmo trutta* (Bury et al., 1998), zebrafish, *Danio rerio* (Cazenave et al., 2006; Oberemm et al., 1987), and Medaka, *O. latipes* (Deng et al., 2010) have found negative effects of MC-LR exposure to morphometric indices, increasing concentrations of MC-LR had no apparent affect splittail length, weight, CF, and HSI. As a cyprinid, splittail may be more resistant to the negative impacts of MC-LR. For example, common carp were found to only exhibit negative effects at much higher concentrations than were in this experiment (Carbis et al., 1996; Mares et al., 2009).

RNA/DNA ratios were found to be more sensitive than the morphometric indices. RNA:DNA ratios respond quickly to short term impacts on growth such as the 28 d dietary exposure of this study and is an indicator of nutritional status (Buckley et al., 1999). The low RNA/DNA ratios suggested the nutritional status of splittail was poor in diets with MC-LR. Reduced ratios of RNA/DNA were also reported following poor feeding conditions in olive flounder (*Paralichthys olivaceous*) (Gwak and Tanaka, 2001), herring (*Clupea harengus*) (Clemmesen, 1987), and turbot (*Scophthalmus maximus*) (Clemmesen, 1987) as well as in medaka exposed to *Microcystis* (Deng et al., 2010). Poor feeding or increased body burdens with the MC toxin may also exacerbate the poor nutritional status thereby shifting the energy from growth to immunology in response to toxic exposure. Detoxification of MCs increased energetic costs of brown trout (Bury et al., 1998) and zebrafish (Cazenave et al., 2006; Oberemm et al., 1997) exposed to MCs. The energetic costs of repairing liver damage in brown trout was significantly increased when exposed to MCs from lysed *Microcystis* cells (Bury et al., 1998). Zebrafish exposed to MCs experienced reduced growth rates (Oberemm et al., 1987) and impaired embryonic development (Cazenave et al., 2006) under increasing concentrations of the hepatotoxin.

Inhibition of PP2A expression is a clear indication of MC-LR toxicity (Carmichael, 1995; Fujiki and Suganuma, 2009). Microcystins inhibits PP2A resulting in hyperphosphorylation of structural proteins and increased gene expression of unregulated protein kinases (Fujiki and Suganuma, 2009). Affected cells exhibit impaired cellular function, hemorrhaging, and necrosis in the livers of mice (Bu et al., 2006) and rainbow trout, *O. mykiss*, and medaka fish, *O. latipes* (Mezhoud et al., 2008; Tencalla et al., 1994), and in the kidneys and gills of carp, *C. carpio* (Carbis et al., 1996). In this study PP2A expression was affected by MC-LR exposure but only at the highest concentration of MC-LR (Table 3). However PP2A expression was inversely related to increasing MC-LR concentrations (Fig. 1), suggesting that even at low concentrations there may be an effect. We postulate that *Microcystis* strains in the SFE contain known (e.g., MC-LR and MC-LA toxins) (Lehman et al., 2008) and potentially unknown components (e.g., secondary metabolites) that have negative or positive effects on PP2A expression. When fish were fed the low MC diet (D5), the positive nutritional effect may have muted the negative MC-LR toxic effect resulting in no significant difference in PP2A expression for splittail feed diet D5 than D0. When compared to the diet containing no *Microcystis* (D5R) the PP2A expression (1.44 ± 0.22) appeared to be marginally ($p = 0.09$) higher and HSI (0.7 ± 0.03) was significantly ($p < 0.05$) lower in the fish from the D5 treatment (1.87 ± 0.42 and 0.75 ± 0.03,

Table 4
Mean histological lesion scores in the liver of splittail after 28 days of dietary exposure to MC-LR. Values are given as mean ± SD. Diets contained 3.89 (D5R), 3.55 (D5), 9.14 (D10), and 17.13 (D20) mg/kg of MC-LR. Lesions are glycogen depletion (GD), lipidosis (LIP), macrophage aggregate (MA), infiltration of inflammatory cells (INF), cytoplasmic inclusion bodies (CI) and single cell necrosis (SCN).

Diet	Mean lesion scores					
	GD	LIP	MA	INF	CI	SCN
D0	1.27 ± 0.42	1.60 ± 0.20	0.20 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
D5R	1.07 ± 0.50	1.40 ± 0.20	0.40 ± 0.00	0.27 ± 0.12	0.40 ± 0.20	0.73 ± 0.70
D5	1.33 ± 0.23	1.53 ± 0.23	0.13 ± 0.23	0.13 ± 0.23	0.33 ± 0.31	0.40 ± 0.20
D10	1.67 ± 0.61	1.27 ± 0.46	0.07 ± 0.12	0.20 ± 0.35	1.20 ± 0.20[a]	0.67 ± 0.31
D20	2.07 ± 0.42	1.40 ± 0.20	0.27 ± 0.12	0.33 ± 0.42	0.87 ± 0.50[a]	0.33 ± 0.12

[a] Significant difference between treatments and D0 are indicated ($p < 0.05$).

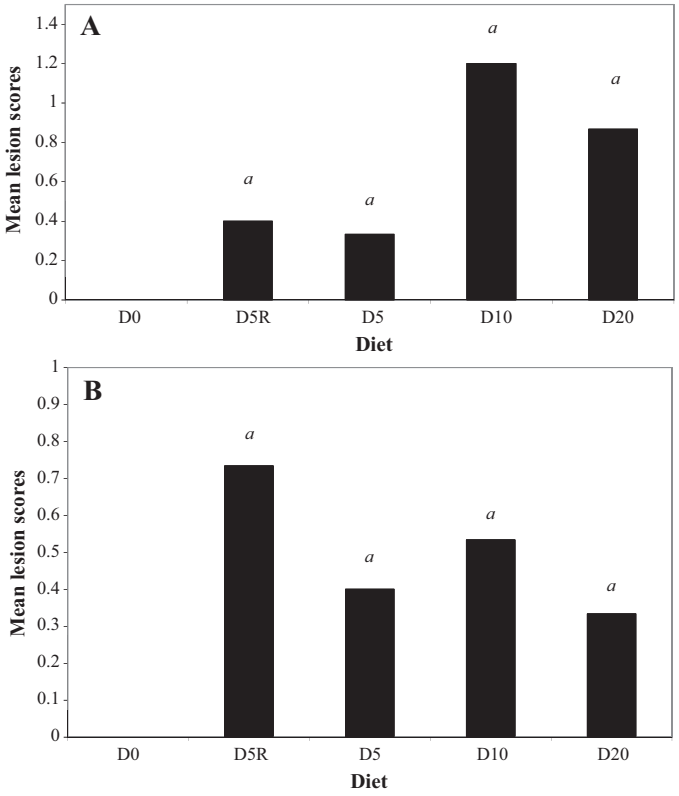

Fig. 2. Mean histological lesion scores with increased prevalence but not significance. (A) Cytoplasmic inclusion bodies (CI) in the liver of splittail after 28 days of dietary exposure to MC-LR. (B) Single cell necrosis (SCN) in the liver of splittail after 28 days of dietary exposure to MC-LR. [a]Increased prevalence of lesions compared to D0.

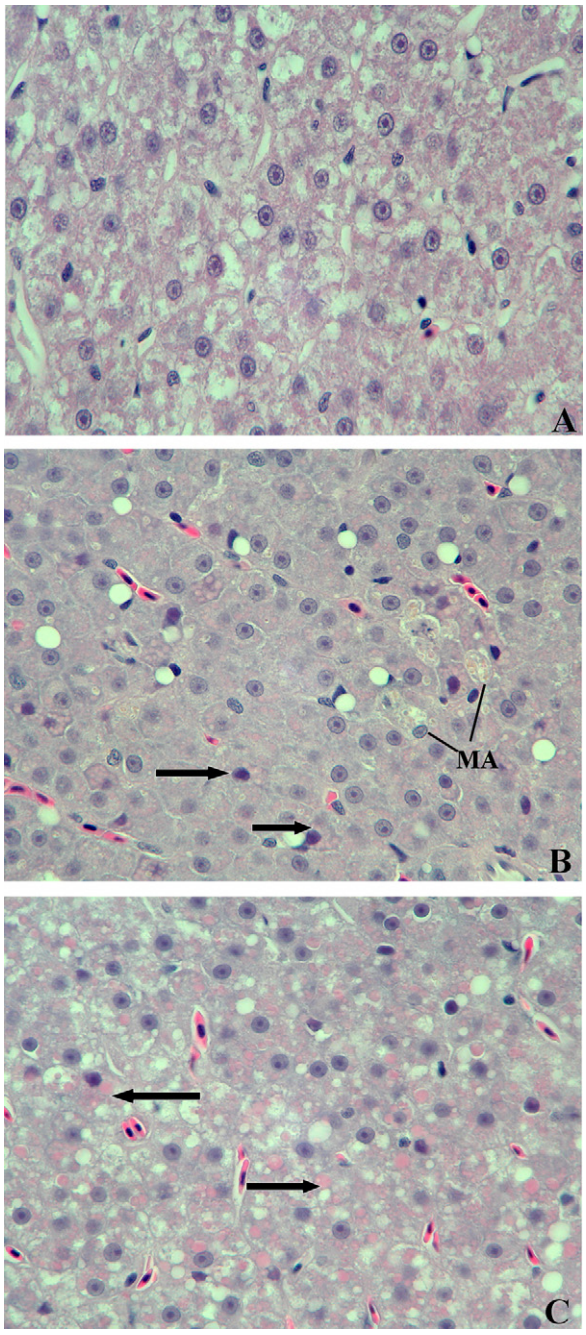

Fig. 3. Liver sections of juvenile splittail fed with different concentrations of MC-LR. (A) Normal morphology at 0 ppm MC-LR (D0). (B) Moderate single cell necrosis (arrows) at 5 ppm of purified MC-LR (D5R). (C) Severe cytoplasmic inclusion bodies (arrows) at 20 ppm MC-LR (D20). MA = macrophage aggregates.

respectively), suggesting there may have been a beneficial effect from low concentrations of *Microcystis* compared to exposure to the pure form of the toxin. At the highest concentration (D20), MC-LR toxicity predominated over the nutritional effects and enhanced PP2A inhibition. The cyanobacteria at low concentrations may have beneficial nutrients that may have mitigated the effects of the MCs. Similarly, the calanoid copepod *Acartia tonsa* exhibited increased mortalities and reduced egg production when fed *M. aeruginosa* but small additions of cyanobacteria actually promoted egg production (Schmidt and Jónasdóttir, 1997). Further investigation on the nutritional value of *Microcystis* to splittail in small quantities is needed to elucidate this observation.

Fish diets with *Microcystis* containing MC-LR concentrations greater than 3.55 mg (kg^{-1}) dry weight significantly impaired the health of splittail based on histopathology of the liver (Table 4). The formation of lesions was a more sensitive biomarker of toxicity than morphometric indices. Liver lesions demonstrated impacts from exposure to MC-LR, particularly for CI and SCN lesions were more prevalent in all treatments containing MC-LR. Cytoplasmic inclusion bodies can occur when proteins are assembled but they cannot be exported due to the impairment of the hepatocyte or misfolding of the protein resulting in accumulation of these proteins within the cell (Strnad et al., 2008). Protein phosphatase inhibition can result in the impairment of normal function of the hepatocyte due to the loss of the protein kinase/protein phosphatase regulation (Carmichael, 1995). The energy required to produce the proteins and failure to export them may predispose the development of single cell necrosis or apoptosis (Fladmark et al., 1999). Similarly, liver lesions observed in this study were indicative of MC exposure and were similar to those reported in the liver of brown trout (Bury et al., 1997) and common carp (Carbis et al., 1996). The prevalence for SCN was greater for D10 then the other treatments, including D20. The greater prevalence of SCN in D10 over D20 may be due to the decreased feeding rate observed where most of the diet was not consumed by the splittail of D20 as compared to D10 fish. Splittail in the D10 treatment may have been exposed to greater quantities over the course of the experiment than D20 by feeding more readily upon the diet. The higher concentration of MC-LR in D20 diet may have impaired normal feeding behavior of splittail. In a study with silver carp (*Hypophthalmichthys molitrix*) and tilapia (*Oreochromis niloticus*), the fish selected against ingestion of toxic strains of *M. aeruginosa* (Beveridge et al., 1993).

Although the formation of SCN was common, the biomarker of apoptosis (caspase activity) was not altered. Splittail may have a

different mechanism for apoptosis when exposed to MC-LR than caspase induced apoptosis or SCN was a result of increased oxidative stress from reactive oxygen species. Although the activation of caspase induced apoptosis by MC-LR exposure is known (Fladmark et al., 1999), rat hepatocytes exposed to MC-LR did not exhibit this mechanism however, expression of calpain, which is another cysteine protease involved in cell death, did induce apoptosis (Ding et al., 2002). Microcystin is detoxified by glutathione *S*-transferase (GST), an import enzyme for mitigating damage from oxidative stress (Pflugmacher et al., 1998). Exposure to significant levels of MCs can reduced the available GST (Amado and Monserrat, 2010; Pflugmacher et al., 1998) resulting in increased oxidative toxicity ultimately resulting in increased necrosis (Amado and Monserrat, 2010; Prieto et al., 2006).

Establishing the toxicity of MCs produced from *Microcystis* in the SFE as demonstrated by the biomarkers of RNA/DNA, PP2A expression and liver lesions of CI and SCN is an important step needed for future studies on determining the impacts of *Microcystis* in the SFE. This study indicated *Microcystis* is toxic to Sacramento splittail, the toxicity can be linked to the toxin microcystin and the mechanisms of toxicity leading to PP2 inhibition and necrosis may be explained by alternative metabolic pathways such as the nutritional effects of *Microcystis* and calpain activity. In addition, the frequency and severity of histopathological biomarkers measured in this study suggest that longer exposure of splittail to MCs in the wild may result in significant health effects that may ultimately result in death.

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