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Sublethal dietary effects of microcystin producing *Microcystis* on threadfin shad, *Dorosoma petenense*

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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 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) produced by Microcystis is hepatotoxic and a potential threat to the fishery. Concurrently, in the SFE significant declines in pelagic fish, known as the Pelagic Organism Decline (POD), has been recognized by state and federal agencies since 2000. In 2005, the presence of the toxic algal bloom, Microcystis has been hypothesized as a link to the POD by the Interagency Ecology Program Management Team. This study aims to characterize the toxic effects of Microcystis in one of the POD species, threadfin shad (*Dorosoma petenense*) by exposure to diets containing Microcystis harvested from the SFE. The diets contained Microcystis with 4.4 (D5) and 10.0 (D10) μg g^{-1} MC-LR that was fed to threadfin shad for 57 days. The treatments were compared to the control diet, 0 μg g^{-1} MC-LR (D0). Results showed that ingested Microcystis was localized in the gut by *in situ* hybridization and MCs were localized in the tissues of the gut, kidney and liver. Condition factor (CF) and liver and gonadal lesions were sensitive to MC exposure. There was a significant inverse relationship between CF and MC-LR with exposed fish exhibiting severe cachexia. Liver lesions of sinusoidal congestion and glycogen depletion significantly increased with increasing MC-LR concentrations, indicating hemorrhaging in the liver and poor nutritional status, respectively. In females, there was a significant increase in severe ovarian necrosis with increasing MC-LR concentration, indicating loss of reproductive potential. The results indicate that MC-LR from Microcystis significantly impairs the health and reproductive potential of threadfin shad has a potential negative effect on populations in the SFE.

1. Introduction

In 2005, a consortium of federal and California state agencies known as the Interagency Ecological Program (IEP) recognized an alarming trend in the upper San Francisco Estuary (SFE). The four most abundant pelagic fish, delta smelt (*Hypomesus transpacificus*), longfin smelt (*Spirinchus thaleichthys*), striped bass (*Morone saxatilis*) and threadfin shad (*Dorosoma petenense*) were in significant decline since 2000 (Sommer et al., 2007). The IEP labeled the trend as the Pelagic Organism Decline (POD) (Armor et al., 2005; Sommer et al., 2007). To address the potential causes of the POD, the IEP developed a workplan to determine the contributing factors affecting the decline (Armor et al., 2005). The key factors identified were bottom-up (food quality/quantity), top-down (water diversion and predation), reproductive success, and the environment (water quality, disease, toxic agents) (Armor

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et al., 2005). Research conducted to determine the potential causes of the systemic fish declines have addressed several factors including changes in organic carbon accumulation (Canuel et al., 2009), altered hydrology (Brown et al., 2009), changes to habitat volume (Kimmerer et al., 2009), and *Microcystis* seasonal variation (Lehman et al., 2008). *Microcystis* blooms were listed among these potential factors, because they were first observed in the estuary only a year before the POD occurred.

Microcystis blooms have been linked to damage to the wildlife and ecosystems (Carmichael, 1995; Leao et al., 2009; Paerl and Huisman, 2009; Pathak and Singh, 2010) including the SFE (Lehman et al., 2005, 2010). *Microcystis* is a cyanobacterium that produces hepatotoxins called microcystins (MCs). The cyanobacterium can form dense blooms that can dominate the phytoplankton community in highly altered and eutrophic freshwater systems. These cyanobacterial blooms are considered harmful and can threaten human and ecosystem health (Backer et al., 2008; Paerl and Huisman, 2009; Sivonen and Jones, 1999; Verspagen et al., 2006). Chronic exposure to MCs causes widespread and serious health problems in animals and humans such as hepatic, gastric and epidermic diseases, neurological impairment, and death (Babica et al., 2006; Leao et al., 2009; Sivonen and Jones, 1999). *Microcystis* is present seasonally in freshwater reaches of the SFE from the end of June to the beginning of November where the LR (MC-LR) form, the most toxic variant of microcystin, is common (Lehman et al., 2005; Zurawell et al., 2005).

There are over 80 variants of MCs, and exposure can promote tumors and result in liver damage in humans and wildlife (Carmichael, 1995; Fujiki and Suganuma, 2009). Toxicity from MCs results from the inhibition in the activity of serine/threonine protein phosphatase 1 (PP1) and 2A (PP2A) by 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 function, loss of structural integrity including cell to cell binding that result in hemorrhaging and necrosis and subsequently affect growth, stress protein activity, and lesion formation (Fujiki and Suganuma, 2009). MCs have been found to cause impaired cellular function, hemorrhaging, and necrosis in the livers of mice (Bu et al., 2006), rainbow trout, *Oncorhynchus mykiss* (Tencalla et al., 1994), and medaka, *Oryzias latipes* (Deng et al., 2010; Mezhoud et al., 2008). Necrosis from exposure to MCs has also been detected in the kidneys and gills of carp, *Cyprinus carpio* (Carbis et al., 1996). Microcystins have already been detected in the SFE in clams, zooplankton, amphipods, and fish tissue collected during field surveys over multiple years (Lehman et al., 2005, 2008, 2010). Thus, *Microcystis* and associated MCs may be a potential threat to fishery production in the upper SFE (Lehman et al., 2005, 2010).

Threadfin shad (TFS), a clupeidae, is one of the four species included in the POD in the SFE and is therefore a focus of study in determining the causes of the decline (Armor et al., 2005). The TFS is a small freshwater pelagic species from open waters of reservoirs, lakes, and rivers in the freshwaters of North and Central America that drain into the Gulf of Mexico (Feyrer et al., 2009; Moyle, 2002). As a popular prey species for sport fish, it has been introduced throughout the warm waters of the United States (DeVries and Stein, 1990). It was introduced to reservoirs in California in 1953 and soon invaded the SFE in 1962 (Dill and Cordone, 1997; Feyrer et al., 2009; Kimsey, 1954). Threadfin shad feed on small zooplankton, phytoplankton, and detritus particles via filter feeding therefore it can be exposed to MCs by directly feeding on *Microcystis* or from consuming prey that graze on *Microcystis* (Feyrer et al., 2003, 2009; Ingram and Ziebell, 1983; Turner, 1966). Threadfin shad can live for two years and attain reproductive maturity after one year (Johnson, 1970). Spawning of TFS occurs between April to August in the SFE (Feyrer, 2004; Grimaldo et al., 2004; Moyle, 2002), which overlaps the seasonal range of *Microcystis* blooms between June and November (Lehman et al., 2005). Previous studies on disease (Baxter et al., 2008) and abiotic factors (Feyrer et al., 2009) have not been able to explain the loss of TFS abundance in the SFE since 2000. In a comprehensive review of the biology and ecology of TFS in the SFE, Feyrer et al. (2009) suggest that *Microcystis* may be one of several underlying factors affecting TFS abundance. Since larval and juvenile TFS co-occur with *Microcystis* blooms, it is important to examine potential linkages between *Microcystis*/MCs and TFS health and survival in the SFE.

To date, there has been no dietary exposure study determining the potential adverse effects of toxic *Microcystis* blooms to the general health of POD species in the SFE. The purpose of this study is to determine whether MCs from *Microcystis* are toxic to TFS and to characterize the biomarkers of exposure. Some of the techniques utilized in this study will be optimized for use in future studies on archived samples from routine monitoring programs performed by the California Department of Fish and Game (CDFG). Samples from CDFG are preserved in 10% formalin therefore some of the analyses optimized in this dietary study reflect the constraint of being applied in future studies to formalin fixed samples from CDFG. As MCs have been detected in the pelagic food web of the SFE (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. This study will provide valuable information needed for management of TFS populations and to delineate *Microcystis* blooms as a potential factor affecting TFS abundance in the SFE. It can also provide directions for future research on the impacts of *Microcystis* on pelagic fish.

2. Materials and methods

2.1. Diet preparation

Three diets were formulated with graded concentrations of *Microcystis* and other purified ingredients (Table 1). The treatment diets contained *Microcystis* harvested from the SFE in August 2007. Following collection from the field, colonial *Microcystis* were rinsed several times in distilled water, freeze dried, ground into a powder and stored at −20 °C. The *Microcystis* was verified independently and

Table 1
Dietary formulation of test diets fed to threadfin shad for 57 days. Diet D5 and D10 contained *Microcystis* showing corresponding microcystin concentrations.

Ingredients	Splittail Diet (g/kg dry diet)		
	D0	D5	D10
Casein (vitamin free)[a]	380	380	380
Arginine[b]	4	4	4
Glycine[b]	10.7	10.7	10.7
Lycine[b]	2.4	2.4	2.4
Dextrin[a]	270	264	258
Cellulose[a]	103	103	103
Carboxymethyl cellulose[a]	20	20	20
BML Vitamin mix[b]	40	40	40
BTm mineral mix[b]	30	30	30
Corn oil[a]	20	20	20
Cod liver oil[a]	50	50	50
Canthaxanthin (10%)[d]	20	20	20
Sodium Alginate[e]	50	50	50
Microcystis[c]	0	5.73	11.5
Analytical concentration of microcystin (mg/kg)	0	4.4	10.0

[a] USB Corporations (Cleveland, OH).
[b] ICN Biomedicals, Inc. (Irvine, CA).
[c] Collected in the San Francisco Estuary on August 2007.
[d] DSM Nutritional Products (Parsippay, NJ).
[e] TIC Gums (White Marsh, MD). Microcystin concentrations were analyzed by LC/MS.

contained 872 mg MC kg^{-1} of dry weight as determined by Liquid Chromatography/Mass spectrometry (LC/MS) as described by 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 MC spiked diets (Baxa et al., 2010). The two diets containing concentrations of microcystins from *Microcystis* were designated as D5 (5 mg mg MC kg^{-1}) and D10 (10 mg MC kg^{-1}) diets. Previous attempts to make this diet have recovered only 50% of the total MC therefore the concentrations were confirmed by LC/MS to determine the actual concentrations. The concentrations were selected based on field data showing concentrations as high as 10.81 $\pm$ 0.23 μg L^{-1} in the SFE (Lehman et al., 2008) therefore the concentrations were well within ecologically relevant concentrations. A diet prepared without *Microcystis* (D0) served as the control treatment and was used to acclimate the TFS for two weeks before the diet study (Table 1). 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 TFS and then stored at −20 °C until use. The concentrations of MCs for the diets were analyzed by LC/MS by Dr. Birgit Puschner, (UC Davis) and reported in Table 1.

2.2. Fish and experimental conditions

Juvenile TFS were collected from the Tracy Fish Collection Facility at the Central Valley Water Project. The TFS were held in filtered and sterilized flow through tanks for 1 month prior to transport. No *Microcystis* contamination was detected. The TFS suffered less than 4% mortality following transport to the University California, Davis (UCD) using in-house protocols. The fish were acclimated for 2 weeks in three recirculating aquaculture system (RAS) with 4 fiberglass tanks in each system (70 L $tank^{-1}$) and 2 weeks on the control diet (D0). A total of 90 fish were used with 10 fish per treatment tank and three replicates per treatment. The initial average body weight was 2.82 $\pm$ 0.1 g $fish^{-1}$. The water in the RAS was maintained at 25 $\pm$ 0.5 °C and was equipped with charcoal and cartridge filters, fluidized sand 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 (Section 2.1) for 14 d before the dietary exposure trials. The test diets were randomly assigned to the three RAS systems. The fish were fed 3% body weight day^{-1} for each treatment. The amount of diet was split equally and fed to the fish twice daily (0900 and 1600 h). 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 0.5 h by turning the inlets back on and allowing the outlet water to be removed from the RAS to remove any dissolved MCs. The volume of the RAS was maintained by replacing the flushed water with reconstituted water. Concentrations of dissolved MCs 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

After feeding for 57 d, all fish were euthanized with an overdose of tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO). The euthanized fish were blotted dry with a paper towel, observed and measured for weight, total length, and clinical signs (lesions, hemorrhaging and deformities). The whole fish was fixed and stored in 10% buffered formalin in accordance with CDFG protocols. Fixed samples were analyzed by histopathology, *in situ* hybridization, and immunohistochemistry.

2.3.1. Determination of MC concentration

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

procedures by CDFG samples were preserved in 10% formalin therefore the LC/MS could not be utilized.

2.3.2. 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), sinusoidal congestion (SC), eosinophilic droplets (EDP), and single cell necrosis (SCN). In addition gonads were examined for lesions, gonocyte necrosis (GN) for males and oocyte necrosis (ON) for females. The lesions were 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). Sinusoidal congestion was identified as blood cell profusion in the interstitial spaces of the liver. Eosinophilic droplets were characterized by the presence of proteins appearing as eosinophilic (pink coloration), round, and well-demarcated cytoplasmic droplets. Single cell necrosis in the liver, gonadal necrosis in males, and oocyte necrosis in females was identified by the presence of cells having eosinophilic (i.e., pink coloration) cytoplasm with nuclear pyknosis and karyorrhexis.

2.3.3. In situ *hybridization*

In situ hybridization (ISH) was performed on gut samples preserved in 10% formalin (Section 2.3). The ISH analysis can be applied in a more efficient manner than conventional taxonomy which requires exhaustive analysis and confirmations. The probe for the analysis was labeled with digoxigenin (DIG) using a PCR DIG probe synthesis kit (Roche Applied Science, Indianapolis, IN). Primers for *Microcystis* spp. were designed from the 16S rDNA sequences of *Microcystis* present in the San Francisco Estuary. The plasmid containing the partial *Microcystis* DNA polymerase was used as a template (ca. 50 pg) in the PCR for the probe generation using MIC16S F (AAA GCG TGC TAC TGG GCT GTA) and MIC16S R (CCC TTT CGC TCC CCT AGC T) primers (Baxa et al., 2010). The PCR for probe preparation and conditions were as previously described in Baxa et al. (2010). DIG labeling of the probes was conducted according to the manufacturer's instructions. Following PCR, subsamples of the labeled probe were run on a gel to confirm successful tagging of the DIG. Prior to use in the *in situ* hybridization assay, the probes were denatured for 5 min at 95 °C, immediately placed on ice, and stored at −20 °C until used.

Whole fish sections were used for non-radioactive *in situ* hybridization. Samples were processed to paraffin wax blocks, cut to 5 μm sections and placed on Superfrost plus slides (Fisher). Except for modifications on the probe labeling as described above, hybridization temperature (52 °C), and signal development at 4 °C overnight with the substrate solution, all steps in the ISH assay were followed according to a previous protocol established for fish pathogens (Antonio et al., 1998).

2.3.4. Immunohistochemistry

Fixed TFS samples (Section 2.3) were embedded in paraffin, cut to 5 μm sections, placed on Superfrost plus slides (Fisher), and assessed by immunohistochemistry (IHC) for protein phosphatase 2A (PP2A) activity, the presence of MCs, and CYP1A activity. Primary antibodies for PP2A and CYP1A were used on the liver and kidney, respectively and were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The primary antibody for MCs was used on the gut, liver and kidney and was obtained from Axxora Biochemicals (San Diego, CA). The MC-LR antibodies cross reacts with all the MCs detected in the diets and were reported as microcystin equivalents (MC_{eq}). A 1:200 dilution for all antibodies was utilized. Staining of the hybridized antibodies was developed utilizing the immunoperoxidase reaction of the goat ABC staining system kit (Santa Cruz Biotechnology, Inc. Santa Cruz, CA). Controls included a serial section of one of the slides stained positive for PP2A, MCs, or CYP1A. The control slide was not treated with the primary antibody but only with the secondary antibody and the goat ABC Staining system.

2.4. Biological and statistical analyses

Fulton's condition factor (K) was used to determine the relative health of the TFS between treatments. The K 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,

$$K = 100 \times W_B\, L^{-3} \tag{1}$$

Specific growth rate was used to determine relative growth rates of TFS between treatments. The specific growth rate (μ) was calculated by taking the natural log of the final weight (W_f) over the initial weight (W_i) divided by change in time from the beginning of the experiment (T_i) to the end (T_f) and then multiplied by 100 (Ricker, 1958),

$$\mu = 100 \times Ln(W_f/W_i) \times (T_f - T_i)^{-1} \tag{2}$$

All data except the histopathology results were analyzed using JMP ver. 8.0 (SAS Institute Inc. Cary, NC.). One-way analysis of variance (ANOVA) was used to determine the extent of differences among dietary treatments in average body weight, total length, μ, K, % survival. 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. Data were presented as mean ± standard deviation. Histopathology is a qualitative analysis therefore it was not analyzed by the ANOVA.

3. Results

3.1. Concentrations of MC in the diet and ambient water

The dietary MC concentration in each diet was confirmed by LC/MS analysis: 0 (D0), 4.4 (D5), and 10.0

(D10) mg total MCs kg^{-1} dry diet (Table 1). No MC was detected by ELISA in the RAS water from treatments fed diet D0, D5, and D10 during the 0.5 h feeding period. After flushing the RAS for 0.5 h, the MCs concentrations in the ambient water remained below the minimum detectable level of 0.5 μg L^{-1} for all treatments.

3.2. Growth parameters, % survival

Growth parameters as determined by total length, body weight, specific growth rate (μ), Fulton's condition factor (K), and % survival was compared among treatments and between males and females. Gross examination of the emaciated fish revealed significant loss of muscle mass also known as cachexia (Fig. 1). After 57 d *Microcystis* exposure, 10% of the fish in the D5 and 27% in the D10 treatments exhibited cachexia. There was no significant difference ($p > 0.05$) in total length, average body weight, % survival, and μ between TFS in D0 and TFS exposed to different MC diet concentrations in D5 and D10 (Table 2). There was a significant negative relationship for K, with increasing MC concentrations (Fig. 2) with fish from D10 exhibiting the lowest average K (0.71) and the lowest overall K at 0.33. There was no significant effect of MC on total length, average body weight, and K between males and females. Mortalities were not significant.

3.3. Histopathology

Histopathological examination of liver, gill, kidney, gut and gonads showed that damage was restricted to the liver and in female gonads. TFS exposed to increasing concentrations of MCs showed microscopic changes in the liver such as severe glycogen depletion (GD), eosinophilic droplets (EDP), single cell necrosis (SCN), and sinusoidal congestion (SC) (Table 3, Fig. 3). There was no apparent interaction between sex and treatment on GD, SCN, SC, and EDP. Gonadocyte necrosis was not detected in males (Table 3) but there were more females showing an increase in prevalence and severity of ON (Table 3, Fig. 4 and 5).

3.4. In situ *hybridization*

Using ISH, *Microcystis* was positively localized in the formalin preserved gut of the TFS following exposure to diets spiked with *Microcystis* from the 2007 blooms. The purple precipitates in the intestines and gut contents (Fig. 6) indicate the *Microcystis* DNA from the diets. Corresponding sections stained with unlabeled probes did not show these signals validating the analysis.

3.5. Immunohistochemistry

The IHC analyses localized MCs in exposed TFS and showed enhanced CYP1A activity but there was no difference in PP2A activity. CYP1A activity was more sensitive with enhanced staining detected in the kidneys of exposed fish compared to controls (Fig. 7) suggesting an increase in stress in the exposed TFS. The IHC analysis for MC_{eq} did not localize the hepatotoxin in control fish, confirming that these fish were not exposed to MCs. In exposed fish (D5 and D10), MC_{eq} was localized in the gut, liver and kidney (Fig. 8), confirming the fact that these fish were exposed to MCs. The validation of the analysis by using corresponding sections stained without the primary antibody for all the IHC performed in this study confirmed the results by exhibiting no stain.

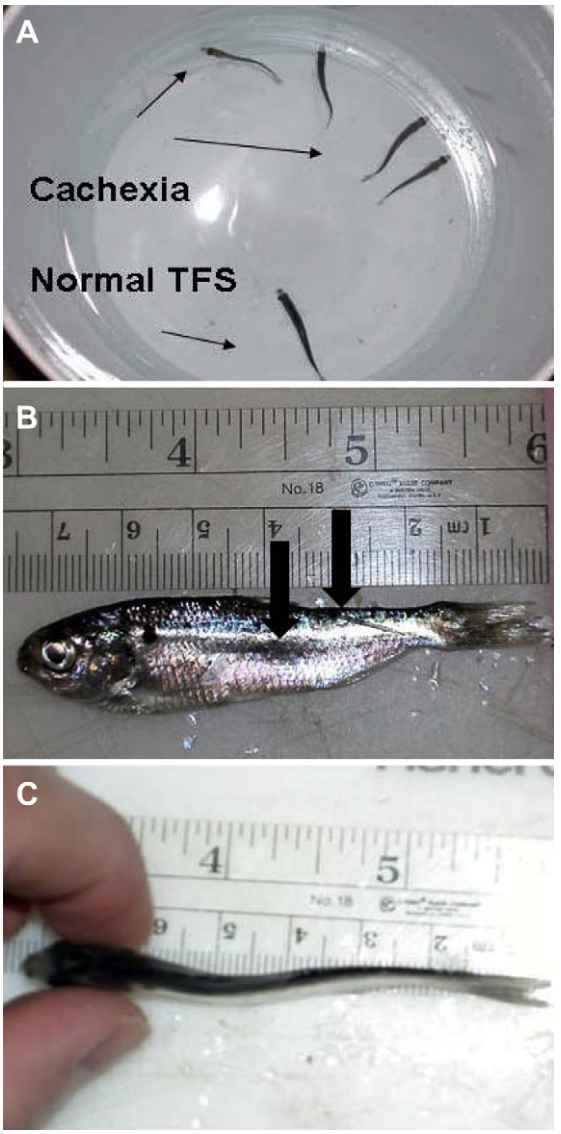

Fig. 1. A) Threadfin shad (TFS) exhibiting cachexia ($N = 4$) and 1 asymptomatic TFS fed 10 ppm microcystin diet (D10) after 57 d dietary exposure. B) Lateral view of the abnormal TFS indicating severe emaciation characteristic of cachexia (severe ill health and malnutrition). Arrows point to degenerating muscle tissues. C) Dorsal view of the same fish.

4. Discussion

Microcystin producing *Microcystis* blooms have occurred in the SFE since 1999 from the end of June to the beginning of November and their potential role in the declined abundance of pelagic fish have alarmed state and

Table 2
Average growth parameters and % survival of threadfin shad after 57 days of dietary exposure to microcystin. Values are presented as mean ± SD. Diets contained 4.4 (D5) and 10.0 (D10) mg/kg of microcystin.

Diet	Body weight (g)	Total length (cm)	Fulton's condition factor	Specific growth rate	% survival
D0	2.89 ± 0.11	7.03 ± 0.08	0.82 ± 0.03	0.045 ± 0.068	92.13 ± 6.9
D5	2.67 ± 0.41	7.03 ± 0.21	0.74 ± 0.05	−0.110 ± 0.269	89.63 ± 10.0
D10	2.68 ± 0.18	7.02 ± 0.24	0.71 ± 0.05[a]	−0.093 ± 0.117	100.0 ± 0.0

[a] Significant difference between treatments are indicated ($P < 0.05$).

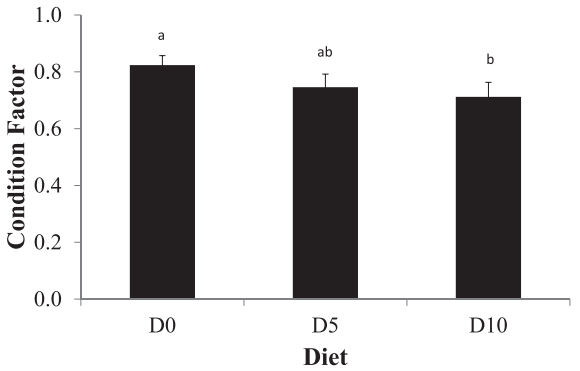

Fig. 2. Fulton's condition factor in threadfin shad fed different concentrations of microcystin spiked diets after 57 days. Different letters between groups denote significant difference using Tukey pairwise comparison ($P < 0.05$).

federal agencies in California (Baxter et al., 2008). *Microcystis* is a threat to wildlife and ecosystem health (Carmichael, 1995; Leao et al., 2009; Lehman et al., 2010; Paerl and Huisman, 2009; Pathak and Singh, 2010) therefore it is an important species to study thus it was imperative to determine the extent to which *Microcystis* is toxic to species listed in the POD. *Microcystis* blooms are present in the habitat of TFS and associated MCs are present in the prey of TFS (Dill and Cordone, 1997; Feyrer et al., 2009; Lehman et al., 2005; Moyle, 2002), therefore this study aimed to examine the effects of dietary exposure to *Microcystis*. Threadfin shad may feed on *Microcystis* grazers (e.g. zooplankton) or directly ingest *Microcystis* (Feyrer et al., 2003, 2009; Ger et al., 2009a, 2009b; Ingram and Ziebell, 1983). Microcystins were measured in TFS prey such as copepods were as high as 3.39 μg g^{-1} dry weight and in the *Microcystis* as high as 10.81 ± 0.23 μg L^{-1} in the SFE (Baxa et al., 2010; Lehman et al., 2008) therefore the concentrations of 4.4 (D5) and 10 ppm (D10) were ecologically relevant. Microcystins have been found to bioaccumulate in fish (Martin and Vasconcelos, 2009) and has also been observed in organisms in the SFE (Lehman et al., 2010) therefore the body burdens in TFS can be potentially higher than has been previously detected in their prey (Lehman et al., 2008). At the end of the 57 d dietary study, indices of *K* and liver lesions were found to be the most sensitive biomarkers of exposure to MCs from *Microcystis* (Table 2 and 3). In addition, *Microcystis* and MCs were localized among exposed TFS. This study demonstrated the potential toxicity of *Microcystis* and the accompanying MCs and optimized the various analytical techniques to characterize the adverse effects and localize the cyanobacteria and MCs in juvenile TFS through dietary exposures in the laboratory. The analytical techniques optimized in this study can be utilized on formalin fixed tissue that has been archived by routine state fish surveys such as those performed by the California Department of Fish and Game (CDFG).

Exposure to increasing concentrations of MCs had a negative impact on the nutritional status and health of the TFS. Gross examination of TFS at the end of 57 days of exposure revealed that 10% of the fish in the D5 (4.4 ppm MCs) and 27% in the D10 (10 ppm MCs) 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 may be due to the impaired liver (a key organ in digestion and assimilation of nutrients) and/or an increase in energy costs from exposure to M*icrocystis* resulting in increased muscle protein catabolism (Kirilenko and Ermolaev, 1976; Mares et al., 2009; Zhao et al., 2006). Muscle quality and density was severely impacted by exposure to *Microcystis* as observed in common carp and silver carp (*Hypophthalmichthys molitrix*) (Mares et al., 2009), tilapia (*Oreochromis mossambicus*) (Kirilenko and Ermolaev, 1976), and in gibel carp (*Carassius gibelio*) (Zhao et al., 2006). In addition, K was significantly affected among TFS feeding on *Microcystis*-spiked diets suggesting that the health of the exposed TFS was

Table 3
Mean histological lesion scores in the liver of threadfin shad after 57 days of dietary exposure to microcystin. Liver lesions were scored for glycogen depletion (GD), single cell necrosis (SCN), sinusoidal congestion (SC), eosinophilic droplets or proteinaceous material (EDP) including oocyte necrosis (ON) in TFS. Values are presented as mean ± SD. Diets contained 4.4 (D5) and 10.0 (D10), and mg/kg of microcystin.

Diet	Mean lesion scores					
	GD	SCN	SC	EDP	ON	GN
D0	0.87 ± 0.31	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
D5	1.93 ± 0.31	0.40 ± 0.40	0.13 ± 0.12	0.33 ± 0.42	0.07 ± 0.12	0.00 ± 0.00
D10	2.33 ± 0.42	0.53 ± 0.31	0.53 ± 0.31	0.47 ± 0.50	1.00 ± 0.80	0.00 ± 0.00

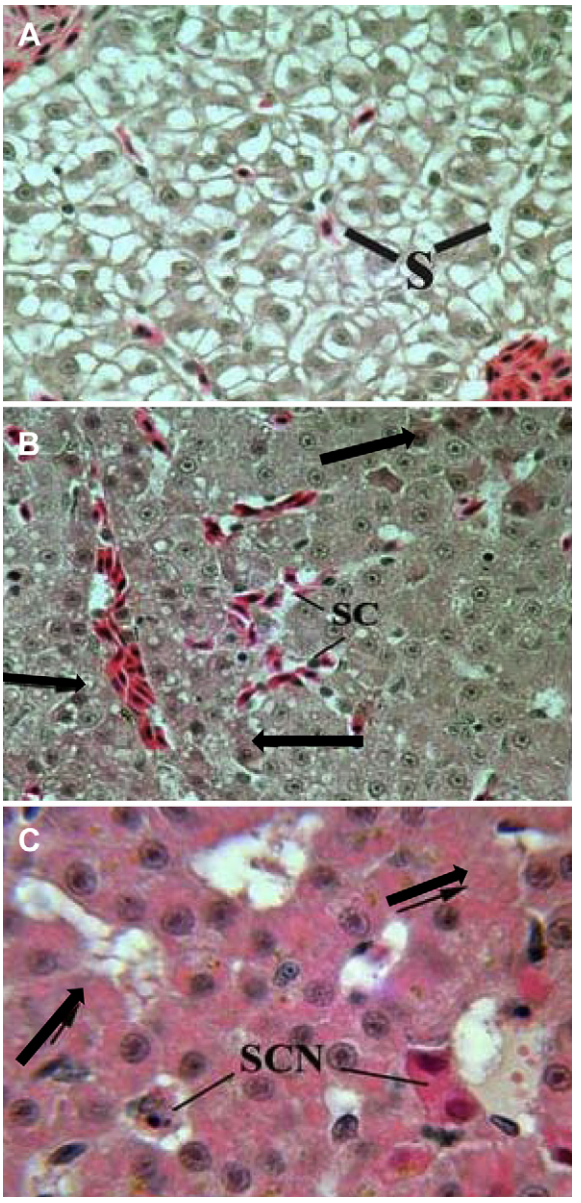

Fig. 3. Liver sections of threadfin shad stained with H & E. A) Normal glycogen-rich liver; Note that the sinusoids (S) are usually one red blood cell thick; C) moderate sinusoidal congestion (SC), hepatocellular degeneration (arrows) and glycogen depletion in liver of TFS fed 10 ppm microcystin diet for 57 days; E) severe eosinophilic protein droplets (arrows) and moderate (single cell necrosis) SCN in liver of TFS exposed to 4.4 ppm microcystin diet for 57 days.

impaired. Exposure to MCs can result in malnutrition and reduced health due to liver damage (Bury et al., 1997; Cazenave et al., 2006; Deng et al., 2010; Leao et al., 2009; Oberemm et al., 1997), utilization of energy for detoxification (Amado and Monserrat, 2010; Pflugmacher et al., 1998; Prieto et al., 2006) and reduced feeding (Beveridge et al., 1993). The results of this study confirm the adverse effects of MC exposure on the health and nutritional status of threadfin shad.

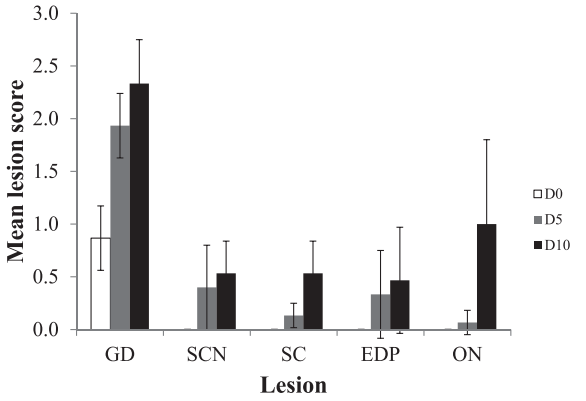

Fig. 4. Mean lesion scores in the liver of threadfin shad exposed to the different *Microcystis*-spiked diets: 4.4 ppm microcystin (D5) or 10 ppm microcystin (D10), and controls (no microcystin or D0) for 57 days of exposure. Liver lesions were scored for glycogen depletion (GD), single cell necrosis (SCN), sinusoidal congestion (SC), eosinophilic droplets or proteinaceous material (EDP) including oocyte necrosis (ON) in TFS. SCN, SC, EDP, and ON were not observed in control fish. There were no testicular lesions observed in the control and exposed fish.

To verify that the toxic effects were due to *Microcystis*, ingestion of the cyanobacterium was confirmed by *in situ* hybridization (ISH) and exposure to MCs was confirmed by immunohistochemistry (IHC). ISH affectively localized the

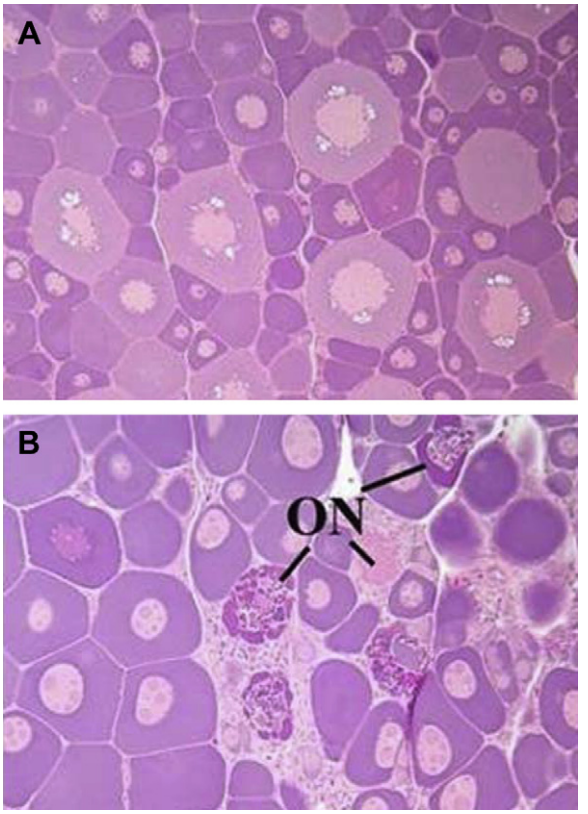

Fig. 5. Threadfin shad ovarian sections stained with H & E. A) Normal ovary; B) Severe stage II oocyte necrosis (ON) in TFS after 57 days of exposure to 10 ppm microcystin.

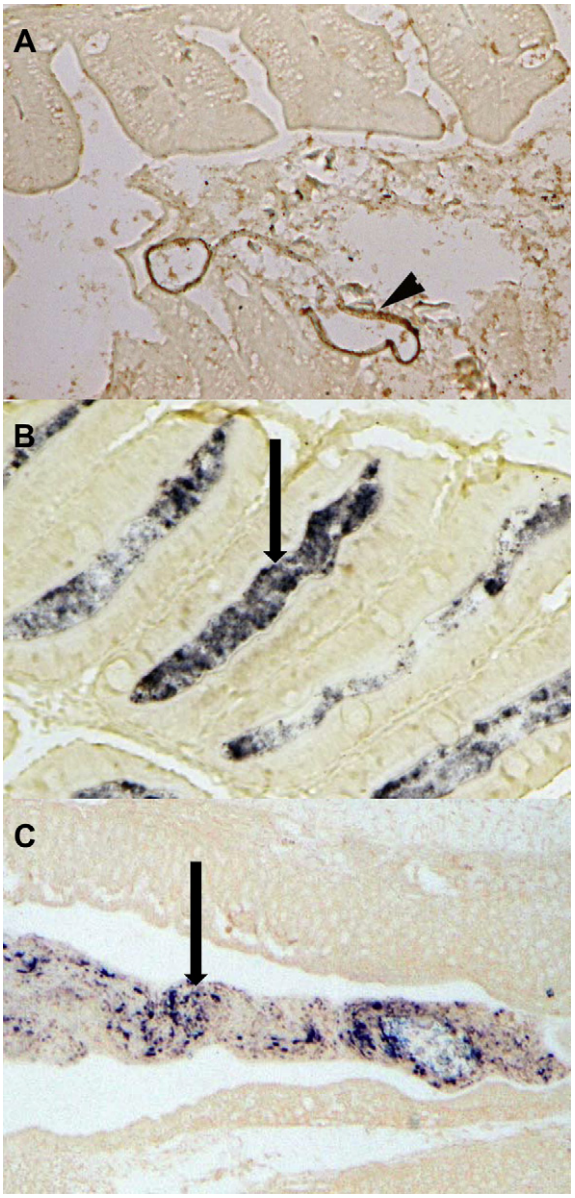

Fig. 6. *In situ* hybridization of threadfin shad guts at the end of the 57 d feeding study. A negative staining of *Microcystis* DNA is shown in fish fed 0 ppm (A). Note *Microcystis* DNA has dark-blue coloration staining (arrows) while unknown food debris has dark brown coloration (arrowhead). *Microcystis* DNA are shown between intestinal wall and lining of fish fed with 4.4 ppm microcystin diet (B), and gut contents (C) of fish fed with 10 ppm microcystin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Microcystis in the guts of TFS receiving D5 and D10 treatments (Fig. 6). Importantly, ISH can be applied to examine the gut contents of archived TFS and other fish from the SFE to confirm historical and future exposures to blooms of *Microcystis*. The results confirm that ISH can be performed on preserved samples with a high degree of accuracy due to the specificity of the analysis. In addition, the ISH analysis can be applied more efficiently than taxonomy. The benefits of IHC analysis were mixed as both control and exposed TFS showed no apparent differences in PP2A activity. The protein phosphatase enzyme is an essential enzyme and it continuously regulates protein kinase activity therefore PP2A would be present in unexposed and exposed threadfin shad. Relative staining intensity was used to determine enzyme inhibition. The reduced staining of the fish liver tissue exposed to MCs was too subtle to determine relative differences in inhibition. Standard IHC may have been too crude to detect a difference and that quantitative immunohistochemistry may have been more sensitive than visual comparisons (Matkowskyj et al., 2003). As such, PP2A immunohistochemistry was determined to be an insensitive biomarker for assessment of MC exposure in TFS. The quantitative method outlined by Matkowskyj et al. (2003) was not pursued in this study as the purpose of this study was to develop high-throughput screening techniques for presence/absence of *Microcystis*/MC exposure for field samples. Like PP2A, CYP1A is an essential component of detoxification therefore it is likely to be detected in both the exposed and unexposed TFS. Although CYP1A in the kidneys of the control TFS was detected the exposed fish exhibited enhanced staining showing darker pigmentation (Fig. 7) suggesting toxic effects from *Microcystis* may be due to additional sources. For example, *Microcystis* have a variety of associated toxins including lipopolysaccharides (Carmichael, 1995; Sivonen and Jones, 1999). The lipopolysaccharides of cyanobacterial cell walls can have significant toxic effects such as inflammatory response and oxidative stress. Although it was important to know that MCs are not the only toxin present, due to the sensitivity of the IHC analysis for CYP1A and the difficulty in determining relative staining in some of the samples conducted, it would be impractical to use this technique on field samples. Although other toxins may be present, the majority of the damage was found in the liver, the target organ of MC toxicity and is the most likely factor causing the impaired health among exposed TFS (Bury et al., 1997; Deng et al., 2010). The IHC analysis localized MC binding sites in the intestine, liver, and kidney of TFS receiving low (D5) and high (D10) concentrations of MC-spiked diets (Fig. 8). The results of the IHC MC stain were easily obtained as the signal was not present in the controls. The IHC results suggest that consuming *Microcystis* can lead to the absorption of MCs. As with the ISH the IHC analysis can be performed on archived samples that cannot be conducted by conventional analysis such as instrumentation. In addition, we wanted a cost effective analysis to determine presence/absence on archived samples from routine fish surveys to determine the extent of the MC exposure. The results of the ISH and IHC confirmed that 1) exposure to *Microcystis* came from the diet, 2) MC is subsequently absorbed through the intestines, 3) the toxin goes to the target organ (liver), and 4) MC was localized in the kidneys indicating detoxification by glutathione, the enzyme responsible for detoxifying MCs (Amado and Monserrat, 2010; Pflugmacher et al., 1998; Prieto et al., 2006). The presence of MC and prevalent lesions in the liver confirm the toxic effects of MC in the liver of TFS upon ingesting the toxin. The hepatic lesions indicate that the TFS were unable to sufficiently detoxify MCs to prevent damage to the liver.

Histopathological examination of the liver revealed significant effects from MC exposure. Microcystin inhibits

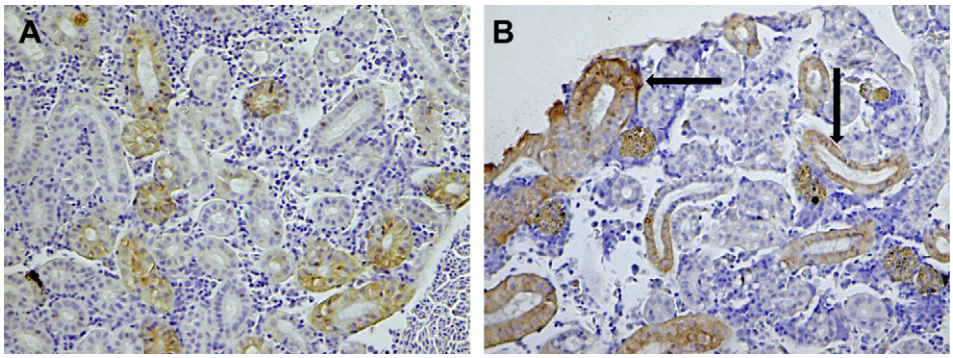

Fig. 7. Cytochrome P450 (CYP1A) immunohistochemistry in kidney of threadfin shad fed with A) 0 ppm, and B) 4.4 ppm microcystin spiked diets. Note that CYP1A activity is more enhanced (arrows) in exposed compared to control fish.

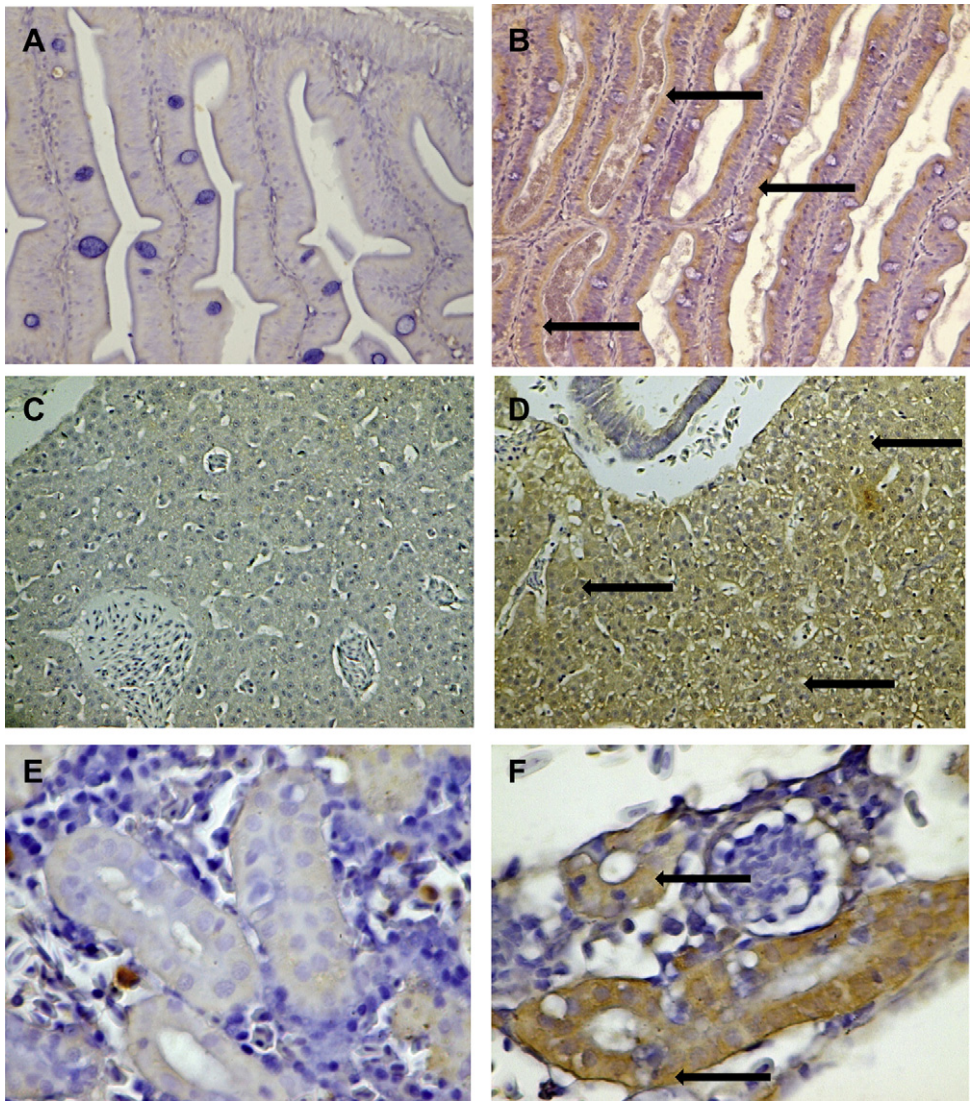

Fig. 8. Immunohistochemistry: Microcystin-LR equivalent localization in threadfin shad receiving no microcystin: A) intestine, C) liver and E) kidney and 4.4 ppm microcystin spiked diet: B) intestine, D) liver, and F) kidney. Arrows indicate positive brownish stains of microcystin.

the activity of PP1 and PP2A and upon inactivation, the enzymes cause hyperphosphorylation of structural proteins resulting in impaired liver function (Fujiki and Suganuma, 2009; Leao et al., 2009). TFS exposed to increasing concentrations of MCs showed microscopic changes in the liver such as severe eosinophilic droplets or proteinaceous materials, glycogen depletion, single cell necrosis, and sinusoidal congestion (Table 3, Fig. 3). 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 (Strnad et al., 2008; Zatloukal et al., 2002). In addition to direct toxic stress from MCs, reduced nutritional status as indicated by cachexia and reduced K, and increased energy needs may have caused significant glycogen depletion (GD) in the liver. Depleted glycogen debilitates energy reserves that may impair activity and increase susceptibility to disease, predation, and starvation (Fladmark et al., 1999). Protein kinases can be significantly unregulated in MC-exposed fish resulting in the loss of structural integrity within and between cells (Carmichael, 1995; Fujiki and Suganuma, 2009; Leao et al., 2009). Inhibition of PP1 and PP2A can result in reduced cell to cell binding, increased single cell necrosis (SCN) and hemorrhaging in the liver (SC) (Bury et al., 1997). The liver lesions SC, SCN and EDP indicate impaired liver function and were observed in greater prevalence among fish exposed to 10 ppm MC (D10) compared to 4.4 ppm MC (D5) (Fig. 4). The severe liver lesions observed in TFS, strongly indicated MC exposure and were demonstrated in other species such as brown trout, *Salmo trutta* (Bury et al., 1997), common carp (Carbis et al., 1996), and medaka (Deng et al., 2010) exposed to MCs.

Although there was similar liver damage characterized between genders, there was a difference detected in the gonads of female TFS. Females showed an increase in the prevalence and severity of oocyte necrosis (ON) (Figs. 4 and 5). Increasing concentrations of MCs resulted in increasing prevalence and severity of ON. Liver and oocyte necrosis was observed in fish exposed to MCs such as in catfish, *Heteropneustes fossilis* (Gupta and Guha, 2006). The catfish exposed to microcystin developed severe liver and gonadal damage. As an essential part of the reproductive development, damage to the liver can impair the function of the ovary. The liver mobilizes nutrients to support the oocyte and regulates the maturation of the ovary. Liver damage and oocyte necrosis reduces the fecundity of the TFS by impairing maturation and reducing the number of viable oocytes in the ovary. The extent and severity of ON may indicate that TFS exposed to MCs can have impaired gonadal development and reduced reproductive success, which may influence population dynamics in the SFE. As a short-lived fish (2 years), reduction of reproductive potential can affect recruitment of TFS, and can ultimately have significant negative population effects (Lewontin, 1965; Winemiller and Rose, 1992). Threadfin shad mature early and have a short life span therefore they exhibit an 'opportunistic' reproductive strategy (Winemiller and Rose, 1992). The opportunistic strategy allows threadfin shad to repopulate habitat after short term or small scale disturbances but are susceptible to large scale and chronic disturbances such as the *Microcystis* blooms in the SFE (Lewontin, 1965; Winemiller and Rose, 1992). Recurring *Microcystis* blooms and toxicity effects on the growth, health and reproductive status of the TFS may be one of the many risk factors associated with the persistent decrease in population abundance in the SFE.

Establishing the toxicity of MCs produced from *Microcystis* in the SFE as demonstrated by the biomarkers of health (*K* and liver lesions) and reproductive status (ON) is an important step needed for future studies on determining the impacts of *Microcystis* in the SFE. The diagnostic tools (ISH and IHC) are fast and cost effective analyses that can be used to screen for MC exposure on fish archived from the routine monitoring fish surveys by CDFG that would otherwise be unavailable for analysis by instrumentation. Development of a quantitative analysis should be optimized to better characterize the exposure. Although a longer duration of exposure would have reflected the impact of seasonal *Microcystis* blooms from the end of June to the beginning of November in the SFE, the results from this study clearly show that microcystins are toxic to TFS even after only 2 months of exposure. The reduced *K*, severe cachexia, and the increased frequency and severity of histopathological biomarkers, taken together, provide compelling evidence of significant health and reproductive effects among TFS populations exposed to recurring *Microcystis* blooms in the SFE and validate further study of the effects of the bloom on TFS and other vulnerable species in the SFE.

Ethical statement

The authors guarantee that the manuscript is an original work and it is not submitted for publication anywhere. This manuscript follows the rules of Ethical Guidelines as stated by Elsevier. The authors have no relationship with any manufacture of any product used in this manuscript. No unlawful statements are contained in this manuscript. All authors have contributed significantly to the research contained in the manuscript and the creation of the manuscript.

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Conflict of interest

None.

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