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Global Gene Expression Profiling in Larval Zebrafish Exposed to Microcystin-LR and *Microcystis* Reveals Endocrine Disrupting Effects of Cyanobacteria

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Supporting Information

ABSTRACT: *Microcystis* blooms occur worldwide and threaten aquatic ecosystems and human health. Sublethal effects on early developmental stages of fish are largely unknown, and research has mainly focused on microcystin toxins (such as MC-LR) rather than *Microcystis* cells. We exposed (96 h) zebrafish larvae to purified MC-LR (0−1000 μg/L) or lyophilized *Microcystis aeruginosa* containing 4.5 μg/L MC-LR and evaluated changes in global gene expression (Affymetrix GeneChip zebrafish genome arrays). Significant changes in gene expression (≥1.7-fold change, $p < 0.0001$) were determined with Rosetta Resolver 7.0, and ontology analysis was conducted with the DAVID bioinformatics tool. The number of differentially expressed genes relative to control increased with MC-LR concentration and included genes related to known mechanisms of action for MC-LR in mammals and older life stages of fish, as well as genes unique to larval zebrafish. Up-regulation of vitellogenin genes (*vtg*) (19.2-fold to >100-fold on arrays; 619.3-fold confirmed by quantitative PCR) was observed in *Microcystis*-exposed larvae but not in larvae exposed to MC-LR. Up-regulation of *vtg* indicates exposure to estrogenic substance(s) and suggests that *Microcystis* may be a natural source of environmental estrogens. Concerns about effects of *Microcystis* blooms may extend beyond those associated with the microcystin toxin.

INTRODUCTION

Toxin-producing harmful algal blooms occur throughout the world and are a major public health and ecological concern. While there are many different types of harmful algal blooms, cyanobacterial blooms are especially important because their occurrence is widespread and toxin concentrations regularly exceed levels considered safe for humans and wildlife.[1] Microcystins (MC) are a diverse group of toxins mainly produced by cyanobacteria of the genus *Microcystis* and are cyclic heptapeptides, with more than 80 variants described.[2,3] Microcystin-LR (MC-LR) is generally recognized as being the most toxic microcystin variant, and concentrations in surface waters often exceed the World Health Organization advisory level of 1 μg/L.[4]

The mechanisms of MC toxicity and detoxification in fish are believed to be similar to those reported in mammals. The liver is the major target organ,[5] and a primary mechanism of MC toxicity is inhibition of protein phosphatases (PPs) 1 and 2A.[6] Inhibition of PPs results in hyperphosphorylation of cytokeratins,[7,8] resulting in cytoskeletal rearrangement and compromised liver function, including necrosis, apoptosis, and intrahepatic hemorrhage.[5] The binding affinity of various MC variants to PPs is believed to govern variant potency.[9] Disruption of Na^+/K^+ ATPase pumps and subsequent dysfunction of ion regulation has been reported to be a consequence of inhibition of PPs by MC and a secondary mechanism of MC-induced toxicity in fish.[10,11] The degree to which MC disrupts ion homeostasis in situ is not well understood and may be an effect of fatty acids associated with *Microcystis* rather than the microcystin toxins themselves.[12,13] Detoxification of MC occurs in the liver via glutathione conjugation catalyzed by glutathione S-transferase,[14] subsequently resulting in biliary excretion.[15]

To date the major focus of research on MC effects in fish early life stages has been directed toward developing an understanding of toxicity during the embryo stage (before hatching), and in most cases where effects on larvae have been assessed, the exposure was terminated before hatching and downstream effects were observed while larvae were grown in clean water.[16] In experiments where larvae were assessed following acute embryonic exposure to microcystin, effects included decreased survival,[17−20]

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severe skeletal malformations,[20] and hepatobiliary hypertrophy.[19] In the few cases where fish were exposed to microcystin during the larval stage, effects were more severe than embryo exposure. Wiegand et al.[21] demonstrated that uptake of MC-LR in larval zebrafish was greater than that of embryos. Loach larvae exposed to MC-LR had significantly lower survival rates than embryos, and malformations, including alterations of hepatocytic organelles, heart muscle, and erythrocytes, were also more severe.[22] This information suggests that larval fish may be especially susceptible to MC-LR exposure, and a better understanding of mechanisms of toxicity during this stage is needed.

Recently, other investigators have applied gene expression analyses in an effort to better characterize biochemical pathways influenced by MC-LR in fish. Microarray investigations with adult zebrafish injected (intraperitoneal) with MC-LR revealed that numerous immune-related genes, in addition to genes involved in tumorigenesis and cell cycling, were differentially regulated in liver tissue.[23] In larval zebrafish, immune-related genes and heat-shock proteins were also differentially expressed in targeted analyses as assessed by quantitative PCR.[24] These studies have just begun detailing sublethal effects of MC-LR in fish.

Exposure of larval fish to *Microcystis* blooms in the environment is a complex issue because larval fish are not exposed to MC-LR alone but rather to *Microcystis* cells and lysates that contain other substances in addition to MC-LR. *Microcystis* can produce numerous peptides classified as aeruginosins,[25] micropeptins,[26] and microviridins[27] that putatively have some type of biological function.[28,29] In addition, *Microcystis* cell walls contain lipopolysaccharides that can be toxic.[30] In several cases where fish were exposed to *Microcystis* and MC-LR during early development, the toxicity of *Microcystis* was greater than that of purified MC-LR.[17,18,31,32] As such, it is important to consider the effects of both *Microcystis* and MC-LR in larval fish.

The objective of this study was to compare the responses of larval zebrafish exposed to the purified MC-LR toxin with those exposed to *Microcystis*. A global gene expression approach was used to distinguish the biochemical pathways affected by MC-LR from those pathways influenced by exposure to *Microcystis*, which contains MC-LR and numerous other bioactive compounds. We predicted that MC-LR and *Microcystis* exposure to larval fish would result in distinctive sets of differentially expressed genes related to toxicity mechanisms and pathways, potentially impacting larval development and survival.

EXPERIMENTAL SECTION

Experimental Fish. Zebrafish (*Danio rerio*) were obtained from the Zebrafish Research Facility in the Center for Environmental Biotechnology at the University of Tennessee. Fish husbandry, spawning, and experimental procedures were conducted with approval from the University of Tennessee Institutional Animal Care and Use Committee (protocol 1690-1007). Water for holding fish and conducting experiments (hereafter referred to as fish water) consisted of Milli-Q water (Millipore, Bedford, MA) with ions added: 19 mg/L $NaHCO_3$, 1 mg/L sea salt (Instant Ocean Synthetic Sea Salt, Mentor, OH), 10 mg/L $CaSO_4$, 10 mg/L $MgSO_4$, and 2 mg/L KCl. Embryos were obtained by spawning adult fish with no history of contaminant exposure. Fertilization of embryos took place at the same time (±15 min), such that larvae used in experiments were of similar age at the time of exposure. All activities (maintenance of adult fish, spawning, and experiments) were conducted in an environmental chamber with a temperature of 27 ± 1 °C and 14:10 h light:dark photoperiod.

Preparation of Exposure Solutions. The *Microcystis* treatment was prepared from lyophilized cells of *Microcystis aeruginosa*. *M. aeruginosa* PCC-7806 was obtained from the Pasteur Culture Collection of Cyanobacteria and cultured in BG-11 medium according to the same methods described for *M. aeruginosa* LE-3 isolates in Rinta-Kanto and Wilhelm.[33] Live cultures of *M. aeruginosa* were centrifuged in 250 mL batches at 3500 rpm for 1 h to concentrate cells into a pellet, and pellets obtained after centrifugation of 6 L of culture were combined. Cells were lyophilized for 48 h in a freeze-dry system (Labconco, Kansas City, MO) and the total dry weight mass of algal cells obtained was 300 mg. For exposure of larval zebrafish, lyophilized *Microcystis* was reconstituted back to its original nominal concentration of 50 mg of lyophilized cells/L.

Solutions for MC-LR treatments were prepared by dissolving 1 mg of purified microcystin-LR (Alexis Biochemicals, San Diego, CA) in 0.5 mL of ethanol and dilution to 100 and 1000 μg/L in fish water. The concentration of ethanol in all treatments was ≤0.05%, and a treatment of 0.05% ethanol was used as a vehicle control. Fish water served as the negative control.

Experimental Design. At 72 h postfertilization, larvae were exposed to lyophilized *Microcystis* and purified MC-LR at concentrations of 100 and 1000 μg/L. Controls consisted of zebrafish system water (negative control) and zebrafish system water containing 0.05% ethanol (vehicle control). Larvae from both control groups as well as 100 μg/L MC-LR, 1000 μg/L MC-LR, and lyophilized *Microcystis* were exposed in groups of 50 with three replicates per treatment (independent biological replicates) and were sacrificed after 96 h for RNA extraction and subsequent microarray analysis (one array was used for each replicate and three independent arrays were used for each treatment). All larvae were exposed in beakers containing 100 mL of solution. Water samples for water quality measurements and microcystin analysis were taken during the experiment, and mortality and behavioral observations were recorded at 24-h intervals.

Water Quality and Chemical Analyses. Water quality parameters measured following 96-h exposure included dissolved oxygen (6.7 mg/L), pH (6.9), total alkalinity (36 mg/L as $CaCO_3$), total hardness (18 mg/L as $CaCO_3$), and ammonia (<0.2 mg/L). Analysis of MC-LR in samples was conducted at the State University of New York College of Environmental Science and Forestry (Syracuse, NY) by protein phosphatase inhibition assay following the methods of Carmichael and An.[34] Lyophilized *M. aeruginosa* pellets were extracted in 50% acidified methanol by use of ultrasound (25 W; three 20 s bursts with 20 s of cooling on ice between bursts), and water samples were analyzed directly without concentration. Measured MC-LR concentrations (mean ± SD) were 140 ± 12 μg/L (in the 100 μg/L MC-LR solution), 1703 ± 71 μg/L (in the 1000 μg/L MC-LR solution), and 4.5 μg of MC-LR equiv/L (in the lyophilized *Microcystis*). LC-MS analysis[35] of the MC-LR standards and PCC7806 *Microcystis* cell material indicated that microcystin-LR was the only toxin variant present in these samples.

Total RNA Extraction. Larvae were centrifuged for 10 min at 13 000 rpm to separate larvae from exposure water, and pellets containing larvae were stored at −80 °C until RNA extraction was performed the following week. Total larval RNA was extracted by use of the RNeasy mini extraction kit for

animal tissues (Qiagen, Valencia, CA) and quantified on a UV-spectrophotometer (Nanodrop, Wilmington, DE) as previously described.[36]

Microarray Methods. Microarray analysis was conducted at the Affymetrix Core Facility located on the University of Tennessee campus, with which our lab has previously conducted microarray experiments with zebrafish.[37] Equal amounts of RNA from controls and treatments were used for cDNA synthesis and subsequent biotin labeling for microarray analysis (Message Amp II biotin enhanced kit, Ambion, Austin, TX). Samples were then applied to GeneChip zebrafish genome arrays (≈15 000 gene transcripts), and hybridization and scanning procedures were conducted according to Affymetrix GeneChip expression analysis technical manual.[38]

Statistical Analysis of Microarray Data. Analysis of array data was conducted with Rosetta Resolver 7.0 gene expression data analysis system (Rosetta Informatics, Seattle, WA) via methods similar to those of Twiner et al.[39] By use of a rank consistency filter, features were subjected to a combination linear and Lowness normalization algorithm. On the basis of the Rosetta error model, a composite array was generated for each treatment and control, in which the data underwent a weighted averaging based on feature quality in the triplicate arrays making up the composite. A list of "signature" gene features was then generated for each time point from the composite array by *p*-value sorting and absolute differential expression (≥1.7-fold, $p < 0.0001$). The software does not assign an absolute value to expression ratios >100-fold or to *p*-values $<10^{-45}$. Signature gene lists for each treatment were further characterized by ontology by use of the Database for Annotation Visualization and Integrated Discovery (DAVID). DAVID calculates a modified Fishers Exact *p*-value to demonstrate GO enrichment, where *p*-values less than 0.05 after Benjamini multiple test correction are considered to be strongly enriched in the annotation category. This global correction minimizes the family-wide false discovery rate to less than 5%.[40,41]

Quantitative Reverse Transcriptase Polymerase Chain Reaction. Aliquots of the same RNA samples used for arrays were analyzed to confirm expression of vitellogenin type 1 (*vtg1A/B*) by quantitative reverse transcriptase PCR (qRT-PCR). The qRT-PCR protocol and primer/probe sets are described in Henry et al.,[36] and zebrafish β-actin gene was used as the internal control. Briefly, amplicons for qPCR standards for zebrafish *vtg1A/B* and β-actin were generated by use of Taq DNA polymerase (Fisher Scientific, Pittsburgh, PA) and T/A cloned into pCR2.1 (Invitrogen, Carlsbad, CA) for propagation and generation of template for qPCR. Plasmid DNA was linearized with *Bam*HI (Promega), enzyme was removed with QIAquick PCR purification kit (Qiagen), and reverse transcription was performed with T7 RiboMAX Express large-scale RNA production system (Promega). Taqman qPCR was performed with an MJ Research PTC-200 (GMI, Inc. Ramsey, MN) and a QuantiTect Probe RT-PCR Kit (Qiagen). Each reaction contained 1 μg of total RNA, 7.5 pM primers, and 5 pM TaqMan probe, and each sample was run in triplicate. Reverse transcription was carried out at 50 °C for 30 min followed by 95 °C for 15 min, then followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min for qPCR. Each plate contained a standard curve of 10-fold dilutions from 10^8 to 10^1 transcript copies and the efficiencies of the PCR reaction were >93% for both *vtg1A/B* and β-actin. Relative quantification of *vtg1A/B* expression (C_T) was obtained by the $\Delta-\Delta C_T$ method.[36]

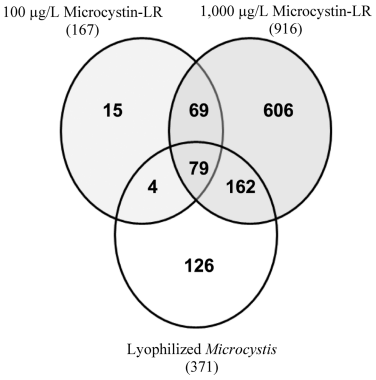

Figure 1. Numbers of genes in each treatment that differ significantly from controls (≥1.7-fold change, $p < 0.0001$). Numbers in overlapping regions represent genes common to multiple treatments, and numbers in nonoverlapping regions represent genes expressed only in that treatment.

RESULTS

Differential Gene Expression. The selected doses of MC-LR and *Microcystis* caused no significant mortality (<2%) and no observable behavioral changes in larval zebrafish during the 96-h exposure. However, changes in gene expression were observed after fish were exposed to MC-LR and *Microcystis*. Out of ~15 000 gene transcripts assessed on the zebrafish arrays, there was greater than 99.9% similarity in gene expression between the two controls (ethanol vs fish water). As such, all treatment comparisons were made relative to the ethanol control. The total number of genes with significant changes in expression (≥1.7-fold change, $p < 0.0001$) relative to the control increased with concentration of MC-LR, with 167 significant genes in fish exposed to 100 μg/L MC-LR and 916 significant genes in the 1000 μg/L MC-LR treatment. Of the 916 differentially regulated genes in the 1000 μg/L MC-LR treatment, 69 were also differentially expressed in the 100 μg/L MC-LR treatment (Figure 1). In larval fish exposed to *Microcystis*, 371 genes were significantly altered compared to control, and 79 of these genes were also differentially regulated in the MC-LR treatments. Of the 371 genes identified in the *Microcystis* treatment, 126 were not differentially expressed in either MC-LR treatment. All data are publicly available at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12214).

Functional Classification of Genes. Differentially expressed genes were functionally categorized by use of DAVID. Genes responding to exposure to MC-LR at 100 and 1000 μg/L (n = 69) (Figure 1) were related to detoxification and metabolism, lipid binding and transport, cell signaling and development, blood clotting and oxygen transport, ion transport, liver function, maintenance of tight junctions, and thermoregulation (see Table S1, Supporting Information). Almost all genes in this group were down-regulated, and *x*-fold change values of fatty acid binding protein 1B (−19.9), type IV antifreeze (−14.6), phospholipase A2 (−11.2), fibrinogen B (−6.1), serpin peptidase inhibitor A7 (−4.8), and selenoprotein p1B (−4.5), showed the greatest decrease in expression in the 1000 μg/L treatment compared to control.

The majority of differentially expressed genes common to all treatments (n = 79) (Figure 1) were cell signaling and developmental genes including calpain genes, CD9 and CD81 antigens, and an apoptosis inducing factor (see Table S2, Supporting

Table 1. Genes Unique to *Microcystis* Treatment[a]

sequence code	accession number	description	gene symbol	*x*-fold change[a]	*p*-value
		Cell Signaling and Development			
Dr.15260.1.S1_at	BI429195	nuclear receptor subfamily 1, group D, member 2B	*nr1d2b*	−3.4	6.03×10^{-7}
Dr.20567.1.S1_at	AW567115	calcium binding protein 39-like	*cab39l*	−2.9	1.48×10^{-6}
Dr.26399.1.A1_at	AL719041	PAR-6 γ protein[b]	*pard6gb*	−2.8	4.43×10^{-6}
Dr.25140.8.A1_at	AL730238	CD81 antigen	*cd81*	−2.8	1.36×10^{-9}
Dr.12138.2.A1_at	AI957874	exostoses 1A	*ext1a*	−2.7	2.00×10^{-5}
Dr.18414.1.S1_at	AW165356	programmed cell death 4A	*pdcd4a*	−2.7	2.00×10^{-5}
Dr.19421.1.A1_at	AL725987	PHD finger protein 8	*phf8*	−2.4	6.54×10^{-6}
Dr.11726.1.S1_at	NM_131877.1	caspase 3	*casp3*	−2.4	3.00×10^{-5}
Dr.7424.2.S1_at	BI839632	COP9 constitutive photomorphogenic homologue subunit 5	*cops5*	−2.3	4.44×10^{-6}
Dr.3282.1.S1_at	NM_131691.1	endothelial differentiation sphingolipid G-protein-coupled receptor 1	*edg1*	−2.3	8.63×10^{-6}
Dr.25322.1.S1_at	AL726472	lin-7 homologue C	*lin7c*	−2.2	2.71×10^{-8}
Dr.13009.2.S1_at	BE556991	sprouty homologue 2	*spry2*	−2.2	4.48×10^{-6}
Dr.1710.2.S1_at	AI794095	eukaryotic translation initiation factor 4e family 3	*eif4e3*	−2.1	7.67×10^{-8}
Dr.7804.1.S1_at	AB069858.1	antizyme inhibitor 1	*azin1*	−2.1	3.00×10^{-5}
Dr.6932.3.S1_at	AL730217	high-mobility group box 3A	*hmgb3a*	−2.0	2.07×10^{-11}
Dr.15361.1.S1_at	AL926178	ras-related protein rab-22A[c]	*rab22a*	−2.0	2.60×10^{-6}
Dr.11764.1.S1_at	NM_131774.1	annexin A13	*anxa13*	−2.0	1.00×10^{-5}
Dr.7100.1.A1_at	BQ091992	immediate early response 2[b]	*ier2*	−1.9	9.07×10^{-10}
Dr.11698.1.S1_at	AL725759	transcription factor AP-2 γ	*tfap2c*	−1.9	1.51×10^{-9}
Dr.16550.1.A1_at	BI709565	TM2 domain-containing 2	*tm2d2*	−1.9	1.82×10^{-9}
Dr.24242.2.S1_at	BQ131454	mitochondrial processing peptidase α[c]	*pmpca*	−1.9	3.11×10^{-8}
Dr.578.2.S1_a_at	U96848.1	thyrotroph embryonic factor	*tef*	−1.9	7.04×10^{-8}
Dr.5572.1.S1_at	NM_131101.1	homeo box B5A	*hoxb5a*	−1.8	6.73×10^{-10}
Dr.3238.1.A1_at	AI793363	Kruppel-like factor 11A	*klf11a*	−1.8	9.45×10^{-9}
Dr.7225.1.S1_at	BC045952.1	MOB1, Mps one binder kinase activator-like 1A (yeast)	*mobkl1a*	−1.7	4.88×10^{-6}
Dr.23293.1.A1_at	BE016153	tubulin polymerization-promoting protein family member 3	*tppp3*	−1.7	4.00×10^{-5}
Dr.26344.2.S1_a_at	AL717083	cell division cycle 42	*cdc42*	−1.7	6.00×10^{-5}
Dr.3472.1.A1_at	AI545021	spectrin, β, nonerythrocytic 1	*sptbn1*	1.7	7.95×10^{-13}
Dr.15630.1.S1_at	CD594794	similar to tubulin folding cofactor E-like	*tbcel*	1.7	2.77×10^{-6}
DrAffx.1.10.S1_at	AY151045.1	cysteine-rich transmembrane BMP regulator 1	*crim1*	1.8	1.82×10^{-7}
Dr.3421.1.A1_at	AW342746	ribosome binding protein 1 homologue (dog)	*rrbp1*	1.8	6.00×10^{-5}
Dr.12403.1.S1_at	NM_131633.1	roundabout homologue 2	*robo2*	1.9	2.46×10^{-7}
Dr.6210.1.S1_at	BM184670	cartilage acidic protein 1[b]	*crtac1*	2.0	1.91×10^{-11}
Dr.15261.1.A1_at	BI710394	cytoplasmic polyadenylation element binding protein 4	*cpeb4*	2.0	6.00×10^{-5}
Dr.52.1.A1_at	AA495026	phosphatidylinositol binding clathrin assembly protein, like	*picalml*	2.2	3.51×10^{-6}
		Neurological Function			
Dr.17557.1.S1_at	AL730871	neurocalcin δ[d]	*ncald*	−2.7	1.53×10^{-14}
Dr.24196.1.S1_at	NM_131452.1	embryonic lethal abnormal vision-like 1	*elavl1*	−2.1	4.11×10^{-10}
Dr.4230.1.S1_a_at	NM_130909.1	HuG	*hug*	−2.0	5.79×10^{-17}
Dr.1968.1.S1_at	BC049308.1	fusion involved in malignant liposarcoma	*fus*	−1.8	6.11×10^{-9}
Dr.25322.2.S1_at	BI888421	lin-7 homologue C	*lin7c*	−1.7	1.94×10^{-13}
DrAffx.1.10.S1_at	AY151045.1	cysteine-rich transmembrane BMP regulator 1	*crim1*	1.8	1.82×10^{-7}
Dr.12617.1.A1_at	NM_131806.1	ephrin B3	*efnb3*	1.8	1.00×10^{-5}
Dr.12403.1.S1_at	NM_131633.1	roundabout homologue 2	*robo2*	1.9	2.46×10^{-7}
Dr.23350.1.S1_at	AF425739.1	parvalbumin 8	*pvalb8*	4.7	2.00×10^{-5}

Table 1. Continued

sequence code	accession number	description	gene symbol	*x*-fold change[a]	*p*-value
		Visual Perception			
AFFX-Dr-NM_131175−1_s_at	NM_131175-1	opsin 1, long-wavelength-sensitive 1	*opn1lw1*	−3.0	4.92×10^{-13}
Dr.26436.1.S1_at	AF210644.1	retinal degradation slow 4	*rds4*	−2.6	1.83×10^{-11}
Dr.12204.1.S1_at	BI706778	retinal degradation slow 4	*rds4*	−2.3	3.00×10^{-5}
Dr.8102.1.S1_at	NM_131253.1	opsin, medium-wavelength-sensitive 1	*opn1mw1*	−2.0	1.38×10^{-8}
Dr.9899.1.S1_at	NM_131868.1	guanine nucleotide binding protein, α transducing activity 1	*gnat1*	−1.9	1.08×10^{-9}
Dr.10433.1.S1_at	NM_131791.1	cryptochrome 2A	*cry2a*	−1.9	5.00×10^{-5}
Dr.8194.1.S1_at	BI879950	opsin, short-wavelength-sensitive 1	*opn1sw1*	−1.8	1.20×10^{-8}
Dr.8097.1.S1_at	NM_131192.1	opsin, short-wavelength-sensitive 2	*opn1sw2*	−1.7	1.65×10^{-7}
		Endocrine Activity			
Dr.10788.1.S1_at	NM_131804.1	nothepsin	*nots*	4.0	1.02×10^{-18}
Dr.10461.1.S1_at	NM_131642.1	cytochrome P450, family 19, subfamily A, polypeptide 1B	*cyp19a1b*	4.0	6.68×10^{-8}
Dr.25009.6.A1_at	BI878405	**vitellogenin**[d]	*vtg*	>100	$<1.00 \times 10^{-45}$
Dr.25009.1.S1_a_at	NM_170767.1	**vitellogenin 1**	*vtg1*	>100	$<1.00 \times 10^{-45}$
Dr.25009.6.A1_a_at	BI878405	**vitellogenin 1**	*vtg1*	>100	$<1.00 \times 10^{-45}$
Dr.25009.4.A1_at	BG303658	**vitellogenin 2**	*vtg2*	>100	$<1.00 \times 10^{-45}$
Dr.2978.1.S1_at	AI477604	**vitellogenin 3**	*vtg3*	19.2	$<1.00 \times 10^{-45}$
		Ion Regulation			
Dr.3.1.S1_at	AF469651.1	ATPase, Na^+/K^+ transporting, β 3A	*atp1b3a*	−2.2	4.44×10^{-11}
Dr.26437.1.S1_at	NM_131669.1	ATPase, Na^+/K^+ transporting, β 2A	*atp1b2a*	−2.0	5.40×10^{-7}
Dr.3613.1.S1_at	BC048037.1	ceruloplasmin	*cp*	2.4	1.73×10^{-9}
		Apoptosis			
Dr.18414.1.S1_at	AW165356	programmed cell death 4A	*pdcd4a*	−2.7	2.00×10^{-5}
Dr.11726.1.S1_at	NM_131877.1	caspase 3	*casp3*	−2.4	3.00×10^{-5}
		Glucose Transport			
Dr.17415.1.S1_at	BC050518.1	insulin receptor substrate 1B[d]	*irs1b*	−2.4	1.86×10^{-7}
		Amino Acid Transport			
Dr.7516.1.A1_at	BM095174	solute carrier family 38, member 4	*slc38a4*	2.1	3.45×10^{-12}
		Muscle Contraction			
Dr.23350.1.S1_at	AF425739.1	parvalbumin 8	*pvalb8*	4.7	2.00×10^{-5}
		DNA Synthesis/Degradation			
Dr.1691.1.S1_at	NM_131450.1	ribonucleotide reductase protein R2 class I	*rrm2*	−2.3	1.98×10^{-6}
Dr.1668.1.S1_at	BC046003.1	hypoxanthine phosphoribosyltransferase 1	*hprt1*	−1.8	2.90×10^{-6}
		Intestinal Function			
Dr.18599.1.S1_at	BQ479899	fatty acid binding protein 6	*fabp6*	1.9	3.81×10^{-6}
		Metabolism			
Dr.14021.3.A1_at	BM095392	3-hydroxyisobutyrate dehydrogenase B	*hibadhb*	−2.0	2.55×10^{-16}
		Protein Targeting			
Dr.3075.1.S1_at	BC049337.1	translocase of outer mitochondrial membrane 34	*tomm34*	−1.8	1.00×10^{-5}

[a] ≥1.7-fold change, $p < 0.0001$ [b] Weak similarity to gene indicated. [c] Moderate similarity to gene indicated. [d] Similar to gene indicated.

Information). Changes in expression of these genes may indicate altered cell cycle progression, carcinogenesis, cell death, or disruption of cellular differentiation during development. Genes showing the greatest degree of fold change were nephrosin (−4.4-fold change, $p = 2.0 \times 10^{-5}$) and mucin 2 (−4.7-fold change, $p = 2.0 \times 10^{-5}$). Immune-related genes were also affected, as well as those associated with cytoskeletal disruption, ion regulation, oxidative stress, and maintenance of tight junctions. Because these genes were affected by exposure to purified MC-LR and by the cyanobacterium responsible for production of this toxin, this group of genes may represent a toxin response that more closely mimics a natural exposure situation than

genes differentially expressed upon exposure to purified MC-LR alone.

Also of interest were genes that responded when larval zebrafish were exposed to *Microcystis* but not the purified MC-LR treatments. The majority of genes expressed only in the *Microcystis* treatment ($n = 126$) (Figure 1) were genes involved in cell signaling and development (28%), neurological function (7%), visual perception (6%), and endocrine activity (6%) (Table 1). Other genes included those affecting ion regulation, apoptosis, glucose and amino acid transport, muscle contraction, DNA synthesis and degradation, intestinal function, metabolism, and protein targeting. The majority of genes were down-regulated, but some genes related to cell signaling development, neurological function, and visual perception were up-regulated.

Vitellogenin Expression and Validation by qRT-PCR. Affymetrix probe sets designed for zebrafish vitellogenin genes indicated high up-regulation (19-fold to >100-fold change) of these genes in larval zebrafish exposed to *Microcystis* (Table 1). In larval zebrafish exposed to MC-LR, there was no significant effect on expression of any of the vitellogenin genes relative to the control. Up-regulation of vitellogenin (accession number NM_170767.1) was confirmed by qRT-PCR of *vtg1A/B* [mean *x*-fold-change $\pm$ SD = 619.3 $\pm$ 130.2 ($n = 9$)].

DISCUSSION

Gene expression results confirmed known mechanisms of action for MC-LR and also identified effects not previously associated with this toxin. When larval fish were exposed to both MC-LR and *Microcystis* treatments, the top functional categories affected (cell signaling and development, cytoskeleton, immune function, ion regulation, oxidative stress, tight junction, liver effects) were associated with effects shown previously for purified MC-LR exposure in fish and mammals[5,10,11,16,20,42−45] (see Table S2, Supporting Information). Because these genes were also affected in the *Microcystis* treatment in the present study, this is consistent with the presence of MC-LR toxin in the *Microcystis* treatment (i.e., MC-LR was generated by the *Microcystis* cells).

We did not test the effects of 4.5 μg/L purified MC-LR and therefore are not able to distinguish the effects on gene expression within the *Microcystis* treatment that were the result of the toxin at the concentration (4.5 μg/L MC-LR) associated with *Microcystis* cultures used in this study. It is likely that some of the differentially expressed genes within the *Microcystis* treatment were affected by the 4.5 μg/L MC-LR. When differentially expressed genes from the *Microcystis* treatment were compared to purified MC-LR treatment (100 μg/L) only 22% (83 genes) of all genes (371 genes) altered by *Microcystis* were consistent with exposure to MC-LR. In this study however, it is the numerous genes that were affected by *Microcystis*, but not MC-LR, that are of most interest. Our objective was to see if there were effects from the *Microcystis* cells that were not due to the presence of the toxin alone, and our observations strongly suggest that there are biological effects beyond just the toxin MC-LR.

MC-LR is known to affect immune response; however, there were few similarities between our work and previous gene expression studies that mainly focused on the response of immune-related genes in zebrafish exposed to purified MC-LR. Of the 11 genes tested by qPCR and found to be up-regulated in zebrafish by Li et al.,[24] none were differentially expressed in our 100 μg/L MC-LR or *Microcystis* treatments, but three heat-shock protein genes were significantly down-regulated (−2.2-fold to −1.9-fold change) at 1000 μg/L MC-LR. When compared to the results of a microarray study by Wei et al.,[23] in which adult zebrafish were exposed to MC-LR by intraperitoneal injection, five genes reported by Wei et al. to be up-regulated were significantly down-regulated (−4.2-fold to −1.8-fold change) in the 1000 μg/L MC-LR treatment, and three of these same five genes were down-regulated (−2.2-fold to −1.9-fold change) in the *Microcystis* treatment. Variation between our work and these studies may be attributed to differences in life stage of the fish, MC-LR concentration, and/or exposure route.

Also when genes differentially expressed in both 100 and 1000 μg/L MC-LR (but not the *Microcystis* treatment) were examined, different effects emerge other than those typically associated with MC-LR. There were additional cell signaling effects not affected by *Microcystis* (five genes) (see Table S1, Supporting Information); however the majority of genes affected by both concentrations of MC-LR tested were related to detoxification/metabolism and lipid binding/transport. Detoxification/metabolism genes included glutathione S-transferase, an enzyme involved in detoxification of MC-LR,[14] as well as cytochrome P450 enzymes, which are typically not associated with microcystin exposure. Lipid transport metabolism genes mainly included apolipoproteins, which are known to influence nutrition and immune response in fish.[46] In addition, strong down-regulation of type IV antifreeze protein (−14.6-fold) may indicate that microcystin-LR may inhibit thermoregulation in fish, which is an effect that has not previously been associated with this compound. It is also interesting that significant expression of PP genes occurred only in exposures of 1000 μg/L and not 100 μg/L, and these genes were also not differentially expressed in larval zebrafish exposed to *Microcystis*. This implies either that larval fish were able to compensate when exposed to low concentrations of MC-LR or that PP enzymes may not be as responsive at this stage in development and a higher dose may be required for inhibition to occur. The absence of this major biomarker of MC-LR toxicity in larval fish exposed to *Microcystis* is also interesting, because it implies that MC-LR may not be the most important compound affecting fish when they are exposed to the cyanobacteria.

Most of the gene function categories affected exclusively by *Microcystis* exposure were different from those associated with MC-LR. Several genes related to neurological development and/or function were differentially expressed, including ephrin B3 (1.8-fold change), a gene in which overexpression has been associated with notochord defects in zebrafish,[47] and neurocalcin δ (−2.7-fold change), a neuronal calcium binding protein involved in visual transduction in the retina.[48] Alteration in the regulation of these genes suggests that *Microcystis* may interfere with proper nervous system development in larval fish. Similarly, several genes involved in visual perception, including opsin genes, were down-regulated, suggesting that development of eye structures may not proceed correctly— an effect that may affect fish at later life stages and interfere with the ability to regulate circadian rhythm, locate food, and avoid predation.

The most highly up-regulated genes were those coding for vitellogenin, and this change in gene expression was observed only after zebrafish were exposed to the *Microcystis* treatment. Vitellogenins are a group of lipoproteins produced in the liver in response to estrogen and are transported through the blood and deposited in the developing oocytes of female fish.[49] Expression of *vtg* in male and immature fish has become a biomarker of exposure to environmental estrogens,[50] and the present study is the first to report

estrogenic effects associated with *Microcystis* exposure in any organism. Recently, a low-level estrogenic response was observed in a human breast carcinoma cell line when cells were exposed to purified MC-LR.[51] Our results, conversely, did not show vitellogenin induction in fish exposed to purified MC-LR, which indicates that the estrogen receptor-mediated induction of *vtg* in zebrafish was not activated by this toxin. Phytoestrogens are compounds identified in plants that can induce vitellogenin;[52,53] and it is possible that the substances(s) produced by *Microcystis* that caused induction of *vtg* in this study are similar to phytoestrogens.

The possibility that *Microcystis* blooms may release estrogenic substances (aka "phycoestrogens") that can interfere with reproduction is of considerable environmental interest. Because compounds that induce vitellogenin in fish are generally able to do so in other species,[50] endocrine disruption from *Microcystis* could extend throughout aquatic ecosystems and also impact the terrestrial environment, including birds and mammals. Human exposure to *Microcystis* has long been a concern due to the microcystin toxin, and most monitoring programs are designed to evaluate presence of this toxin; however, the potential for endocrine disruption to occur that is unrelated to the presence of the toxin suggests that monitoring programs may need to be re-evaluated. Projected global increase in the frequency of *Microcystis* blooms[54] and the potential for estrogenic effects adds to the environmental and public health concerns related to bloom events.

■ ASSOCIATED CONTENT

Supporting Information. Two tables listing genes common to 100 and 1000 μg/L MC-LR treatments and genes common to all treatments. This information is available free of charge via the Internet at http://pubs.acs.org.

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Online Supporting Information

Global gene expression profiling in larval zebrafish exposed to microcystin-LR and *Microcystis* reveals endocrine disrupting effects of cyanobacteria

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Table of Contents

Table S1. Genes common to 100 and 1,000 µg/L MC-LR treatments relative to control[a]

Sequence code	Accession number	Description	Gene symbol	Fold change MC-LR vs. control[a] 100 µg/L	1,000 µg/L
Detoxification/ metabolism					
Dr.11596.1.S1_at	AI545537	tryptophan 2,3-dioxygenase	tdo2a	-3.4	-3.4
Dr.3498.1.S1_at	BC045343.1	methionine adenosyltransferase I, alpha	mat1a	-2.4	-3.6
Dr.18453.1.S1_at	BC044525.1	uridine phosphorylase 2	upp2	-2.4	-2.6
Dr.9478.1.S1_at	AB078927.1	cytochrome P450 1A	cyp1a	-2.4	-3.0
Dr.14058.1.A1_at	CD015351	glutathione s-transferase, theta 1A[c]	gstt1a	-2.3	-2.4
Dr.11729.1.S1_at	NM_152954.1	cytochrome P450 2J28	cyp2j28	-2.2	-2.2
Dr.2132.1.A1_at	BQ262149	hydroxyacid oxidase 1	hao1	-2.1	-3.4
Dr.4189.1.S1_at	BI891596	nad(P)H dehydrogenase quinone 1	nqo1	-1.9	-2.6
Dr.7977.1.S1_at	AW232474	glutathione peroxidase 1A	gpx1a	-1.9	-2.6
Dr.25191.1.S1_at	BC046894.1	isocitrate dehyrodgenase 1	idh1	-1.8	-2.5
Dr.7520.1.A1_at	AW019023	aconitase 1	aco1	-1.8	-2.3
Dr.1041.1.S1_at	BC050158.1	alpha-L-fucosidase 1	fuca1	-1.7	-2.6
Lipid binding/transport					
Dr.24953.1.S1_at	CD014348	apolipoprotein C-II	apoc2	-4.2	-7.8

Sequence code	Accession number	Description	Gene symbol	Fold change MC-LR vs. control[a] 100 µg/L	1,000 µg/L
Lipid binding/transport (continued)					
Dr.24261.1.S1_at	BM182911	fatty acid-binding protein 1	fabp1b	-3.9	-19.9
Dr.13681.1.S1_at	BI867505	apolipoprotein M	apom	-3.6	-3.6
Dr.1323.1.S1_at	NM_131128.1	apolipoprotein A-I	apoa1	-3.2	-6.0
Dr.4002.1.A1_at	BG884597	apolipoprotein B	apob	-3.1	-9.6
Dr.5488.1.S1_at	AI477980	apolipoprotein A-IV	apoa4	-2.5	-4.7
Dr.5674.2.S1_at	BM186239	apolipoprotein C-I precursor[c]	apoc1l	-1.8	-2.7
Dr.59.1.S1_at	AY178793.1	annexin A1A	anxa1a	-1.8	-2.1
Cell signaling and development					
Dr.20054.1.S1_at	NM_131335.1	gastrulation specific protein	g12	-2.6	-3.3
Dr.25140.7.A1_a_at	BQ262802	epithelial cell adhesion molecule	epcam	-1.9	-2.5
Dr.9122.1.S1_at	BM102177	CC chemokine SCYA103[c]	LOC795788	-1.8	-2.4
Dr.7503.1.A1_a_at	AW421072	pituitary tumor-transforming 1 interacting protein[b]	---	-1.8	-2.3
Dr.8149.1.A1_at	NM_131458.1	insulin-like growth factor binding protein 2B	igfbp2b	-1.8	-1.8
Dr.12557.1.A1_at	AW077290	calmodulin-binding transcription activator 1[c]	LOC797322	1.7	1.9
Dr.11457.1.S1_at	BC046887.1	zinc finger and BTB domain containing 16	zbtb16	1.8	2.1

Sequence code	Accession number	Description	Gene symbol	Fold change MC-LR vs. control[a] 100 µg/L	1,000 µg/L
Blood clotting, oxygen transport					
Dr.5462.1.S1_at	BI878927	fibrinogen B	fgb	-2.9	-6.1
Dr.1450.1.S1_s_at	BI896310	hemoglobin alpha embryonic-3	hbae3	-1.9	-2.9
Dr.845.1.A1_at	BG729013	fibrinogen alpha chain	fga	-2.1	-2.7
Liver function					
Dr.8516.1.S1_at	NM_178298.2	selenoprotein P, plasma, 1B	sepp1b	-2.3	-4.5
Tight junction					
Dr.994.1.S1_at	NM_131763.1	claudin B	cldnb	-1.9	-3.0
Thermoregulation					
Dr.696.1.S1_at	AI496864	type IV antifreeze protein	zgc:161979	-4.8	-14.6
Cytoskeletal function					
Dr.9252.1.A1_at	BE605502	sciellin	scel	-1.8	-2.0
Inflammation					
Dr.15332.1.S1_at	AL917567	phospholipase A2, group IB	pla2g1b	-11.1	-11.2
Muscle contraction					
Dr.20153.1.S1_a_at	AF210639.1	myosin light polypeptide 9 like	myl9l	-1.9	-2.2

Sequence code	Accession number	Description	Gene symbol	Fold change MC-LR vs. control[a] 100 µg/L	1,000 µg/L
Integral to membrane					
Dr.23357.1.A1_at	BE201798	transmembrane protein 90A[c]	LOC569467	-2.2	-4.1
RNA processing					
Dr.24764.1.S1_at	AL727764	cleavage and polyadenylation specific factor 3[d]	hbae1	-1.7	-2.1
Actin binding					
Dr.20115.1.S1_at	BC049463.1	cofilin 1 (non-muscle)	cfl1	-1.8	-2.1
Dr.17687.1.A1_at	BQ078227	IQ motif containing GTPase activating protein 1	iqgap1	-1.8	-2.4
Translation					
Dr.20386.3.S1_at	BM141602	eukaryotic translation initiation factor 1A, X-linked, B	zgc:110087	-1.7	-1.8

[a] ≥ 1.7-fold change, *p<0.0001*

[b]weak similarity to gene indicated

[c]similar to gene indicated

[d]strongly similar to gene indicated

Table S2. Genes common to all treatments relative to control.

Sequence code	Accession number	Description	Gene symbol	Fold Change vs. Control[a] *Microcystis* MC-LR (µg/L) 100	1,000
Cell signaling and development					
Dr.17470.1.S1_at	AF498291.1	nephrosin	npsn	-4.6	-4.4	-7.4
Dr.914.1.A1_a_at	BE556864	WH2 domain-containing protein 1[d]		-1.8	-2.6	-5.0
Dr.18186.1.S1_at	BQ093694	S100 calcium-binding protein A1[d]	s100a1	-2.1	-2.5	-3.7
Dr.4236.1.S1_at	BQ092511	calpain 9	capn9	-2.2	-2.4	-3.6
Dr.13076.1.S1_at	BC053138.1	pleckstrin homology containing, family F, member 1	plekhf1	-3.0	-2.2	-5.3
Dr.17116.1.S1_at	AF282675.1	calpain 1	capn1	-2.5	-2.2	-3.0
Dr.11420.1.S1_at	BC050238.1	BAI1-associated protein 2-like 1A	baiap2l1a	-1.9	-2.0	-2.6
Dr.2251.1.A1_at	AI793815	golgi integral membrane protein 4A	golim4a	-2.5	-2.0	-3.7
Dr.1945.1.A1_at	CD015541	calpain 2, large subunit like	capn2l	-2.3	-2.0	-3.7
Dr.1116.1.S1_at	BQ092087	S100 calcium binding protein V2	s100v2	-1.8	-1.9	-2.1
Dr.4409.1.S1_at	BC049036.1	CD9 antigen like	cd9l	-1.9	-1.8	-3.1
Dr.11692.1.S1_at	BG727434	vasodilator-stimulated phosphoprotein[b]	vasp	-2.0	-1.8	-2.4
Dr.23066.1.S1_at	AW019779	apoptosis-inducing factor, mitochondrion-associated 2	aifm2	-1.8	-1.7	-2.6
Dr.10664.1.S1_at	NM_131518.1	cd81 antigen	cd81	-2.2	-1.7	-2.3
Dr.13857.1.A1_at	BM185945	similar to cyclin 1	ccn1	1.9	1.7	2.1

Sequence code	Accession number	Description	Gene symbol	Fold Change vs. Control[a] *Microcystis*	MC-LR (µg/L) 100	1,000
Cell signaling and development (continued)						
DrAffx.2.49.A1_at	AW116899	bromodomain containing 4	brd4	2.3	1.9	2.2
Dr.25935.1.A1_at	CD605501	zinc finger, CCHC domain containing 12[c]	zcchc12	1.9	2.0	2.1
Cytoskeleton						
Dr.7105.1.S1_at	BC053229.1	actin related protein 2/3 complex, subunit 1B	arpc1b	-2.5	-2.3	-4.9
Dr.13076.1.S1_at	BC053138.1	pleckstrin homology containing, family F, member 1	plekhf1	-3.0	-2.2	-5.3
Dr.9531.1.A1_at	BQ074417	myosin, heavy chain 9, non-muscle, like-2	myh9l2	-2.0	-2.0	-3.4
Dr.3432.1.S1_at	BC049461.1	capping protein (actin filament), gelsolin-like	capg	-2.2	-1.8	-4.3
Dr.14768.1.A1_at	BI983132	flavoprotein oxidoreductase mical3	mical3	2.8	2.4	2.7
Immune function, haematopoiesis						
Dr.25714.1.A1_at	AW232464	cathepsin S, B.2	ctssb.2	-1.9	-1.9	-4.5
Dr.4409.1.S1_at	BC049036.1	CD9 antigen like	cd9l	-1.9	-1.8	-3.1
Dr.11692.1.S1_at	BG727434	vasodilator-stimulated phosphoprotein[b]	vasp	-2.0	-1.8	-2.4
Dr.10664.1.S1_at	NM_131518.1	cd81 antigen	cd81	-2.2	-1.7	-2.3
Ion regulation, membrane stability						
Dr.922.1.S1_at	BC044188.1	aquaporin 3	aqp3	-2.0	-1.8	-2.5
Dr.1735.1.A1_at	AI721648	chloride intracellular channel 1	clic1	-2.4	-1.8	-3.2
Dr.10467.1.S1_at	NM_131628.1	sodium channel, voltage-gated, type VIII, alpha A	scn8aa	1.7	1.7	2.0

Sequence code	Accession number	Description	Gene symbol	Fold Change vs. Control[a] *Microcystis* MC-LR (µg/L) 100	1,000
Oxidative stress					
DrAffx.1.74.S1_at	AY216583.1	selenoprotein W2B	sepw2b	-3.0	-2.5 -5.8
Dr.7379.1.A1_at	AW232459	selenoprotein W2B	sepw2b	-1.8	-1.8 -2.6
Dr.17468.1.A1_at	BM956969	glutathione reductase[d]	gsr	-1.7	-1.7 -3.1
Tight junction					
Dr.7692.1.A1_at	BC049304.1	occludin	ocln	-2.2	-1.9 -3.3
Dr. 20610.1.S1_at	NM_131637.1	claudin 7	cldn7	-2.0	-1.9 -2.9
Liver effects					
Dr.8947.2.S1_at	CD594735	Kunitz-type serine protease inhibitor 2[d]	spint2	-3.1	-2.9 -5.6
Dr.2408.2.S1_at	BM571242	matrix metalloproteinase 2[c]	mmp2	-2.4	-1.9 -2.6
Endopeptidase inhibitor					
Dr.8947.2.S1_at	CD594735	Kunitz-type protease inhibitor 2[d]	spint2	-3.1	-2.9 -5.6
Dr.3073.1.A1_at	AI585030	serpin peptidase inhibitor clade A, member 7	serpina7	-2.0	-2.5 -6.5
Detoxification					
Dr.16014.1.S1_at	BM024109	glutathione transferase omega 1[b]	gsto1	-2.6	-2.4 -4.9
Thyroid hormone availability					
Dr.3073.1.A1_at	AI585030	serpin peptidase inhibitor, clade A, member 7	serpina7	-2.0	-2.5 -6.5

Sequence code	Accession number	Description	Gene symbol	Fold Change vs. Control[a] *Microcystis*	MC-LR (µg/L) 100	1,000
Steroid hormone synthesis						
Dr. 10671.1.S1_at	NM_131663.1	steroidogenic acute regulatory protein	star	-3.8	-3.3	-6.9
Endosome formation						
Dr.16802.1.S1_at	BC049333.1	vesicle-associated membrane protein 8	vamp8	-2.2	-1.8	-3.1
Intestinal effects						
Dr.14396.2.A1_at	BI673162	mucin 2[d]	muc2	-4.9	-4.7	-6.7
Bacterial pathogenesis						
Dr.1991.1.A1_at	BM529391	globoside alpha-1,3-N-acetylgalactosaminyltransferase 1, like 4	gbgt1l4	-2.2	-1.7	-2.5

[a] ≥ 1.7-fold change, *p<0.0001*

[b]weak similarity to gene indicated;

[c]moderate similarity to gene indicated;

[d]similar to gene indicated