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Cyanotoxins within and Outside of *Microcystis aeruginosa* Cause Adverse Effects in Rainbow Trout (*Oncorhynchus mykiss*)

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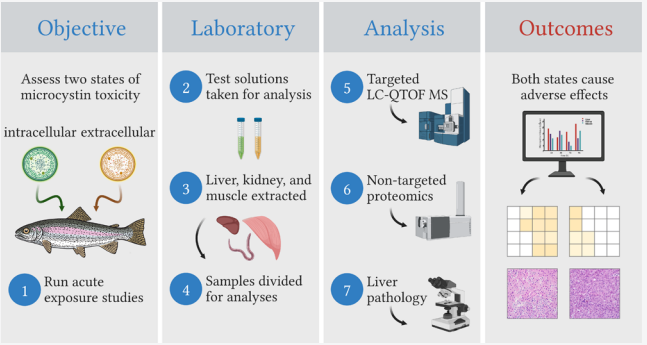
ABSTRACT: The global expansion of toxic *Microcystis* blooms, and production of cyanotoxins including microcystins, are an increasing risk to freshwater fish. Differentiating intracellular and extracellular microcystin toxicity pathways (i.e., within and outside of cyanobacterial cells) in fish is necessary to assess the severity of risks to populations that encounter harmful algal blooms in pre-to-postsenescent stages. To address this, adult and juvenile Rainbow Trout (*Oncorhynchus mykiss*) were, respectively, exposed for 96 h to intracellular and extracellular microcystins (0, 20, and 100 $\mu g\ L^{-1}$) produced by *Microcystis aeruginosa*. Fish were dissected at 24 h intervals for histopathology, targeted microcystin quantification, and nontargeted proteomics. Rainbow Trout accumulated intracellular and extracellular microcystins in all tissues within 24 h, with greater accumulation in the extracellular state. Proteomics revealed intracellular and extracellular microcystins caused sublethal toxicity by significantly dysregulating proteins linked to the cytoskeletal structure, stress responses, and DNA repair in all tissues. Pyruvate metabolism in livers, anion binding in kidneys, and myopathy in muscles were also significantly impacted. Histopathology corroborated these findings with evidence of necrosis, apoptosis, and hemorrhage at similar severity in both microcystin treatments. We demonstrate that sublethal concentrations of intracellular and extracellular microcystins cause adverse effects in Rainbow Trout after short-term exposure.



KEYWORDS: *fish, toxicology, algal bloom, microcystin, cyanobacteria, proteomics, histopathology*

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1. INTRODUCTION

Harmful algal blooms dominated by *Microcystis* are a global concern as their frequency and severity continue to impact the ecological and socioeconomic value of freshwater ecosystems.[1−4] These blooms typically produce cyanotoxins including microcystins, a family of over 250 congeners[5] that can cause lethality and health impairment in humans[6] and wildlife.[7−11]

Microcystin toxicity in humans and wildlife often begins in the liver by active uptake into cells via membrane transporters called organic acid transporter polypeptides (OATPs).[12] OATPs are members of the solute carrier family (SLC) that act as cell gatekeepers by mediating the transport of endogenous and exogenous compounds[13] in tissues, with high expression in the liver.[14,15] With assistance from OATPs, microcystins bind with a high affinity to protein phosphatases (PP1 and PP2A) that are linked to regulatory pathways responsible for cell replication, cytoskeletal structure, stress responses, and DNA repair.[15,16] In response, a series of processes are activated to protect cells from disease and death: glutathione S-transferases (GSTs) conjugate microcystins for the purpose of detoxification;[17] B-cell lymphomas regulate mitochondrial apoptosis;[18] and mitogen-activated protein kinases (MAPKs), which are mediated by PP2A, direct cellular responses to growth and differentiation[19] and regulate cellular proliferation and cancer.[14] Depending on the severity and length of exposure, microcystin toxicity can further promote tumor formation, hemorrhage, and organ failure.[12,20]

Aquatic organisms, including fish, encounter microcystins more frequently than terrestrial organisms through contact with contaminated water or by accumulation in aquatic food webs.[20] Field and laboratory studies have shown that fish and mammals respond similar to microcystins by accumulating in the liver, kidney, and edible muscle tissues.[7,8,21−26] These

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studies uncovered mechanisms of microcystin toxicity through intraperitoneal injection, oral gavage, and waterborne exposure to lysed cells of toxic cyanobacteria or immersion in food and water containing purified microcystins—approaches that utilized microcystins in their extracellular state. However, it is contested that exposure to extracellular microcystins is not the only relevant route of exposure to fish in the field.[8] Microcystins also naturally co-occur with other bioactive metabolites (e.g., aerucyclamides and cyanopeptolins) within *Microcystis* cells[27] and coexposure to these metabolites is critical to evaluating the ecotoxicology of blooms. These major issues concerning microcystin toxicity pathways in fish must be addressed to understand, and ameliorate, the negative effects from *Microcystis* blooms when toxins are intact within or senescing from cells.

Rainbow Trout (*Oncorhynchus mykiss*), the most widely farmed trout species in the world,[28] can encounter *Microcystis* blooms[29] and is an important model organism in toxicological research. Microcystin exposure studies on Rainbow Trout revealed high acute doses (550−6660 μg microcystin-LR L^{-1}) could, in severe cases, cause mortality within 96 h following gavage of lysed cells[7] along with inhibition of protein phosphatases in the liver.[23,25] These studies provide empirical evidence that the observable cellular degradation of hepatocytes was primary manifestations of hepatotoxicity. However, lethal doses overestimate microcystin concentrations common in the field,[8] warranting studies with realistic exposures at sublethal levels (20−100 $\mu g\ L^{-1}$).[12] Indeed, while extracellular microcystin concentrations in water can reach 100 $\mu g\ L^{-1}$,[30] this is rare and recent evidence indicates that no or little $\mu g\ L^{-1}$ extracellular microcystins are detected in freshwater ecosystems even after lysis of harmful algal blooms.[12] The emergence of high-throughput molecular approaches, such as nontargeted proteomics, also enables the understanding of broader ranges of molecular functions and can identify biomarkers related to mechanisms of toxicity.[31] Rainbow Trout is suitable for this exploration because its genome is known and annotated.[32]

We sought to answer two questions about microcystins to better understand their toxicology in fish that encounter harmful algal blooms in pre-to-postsenescent stages. First, will the kinetics between intracellular and extracellular microcystins (i.e., within and outside of *Microcystis* cells) differ in fish? Second, will the kinetics between intracellular and extracellular microcystins result in similar sublethal molecular level effects in tissues?

To address these questions, we: (1) measured and compared the temporal dynamics of microcystins in tissues using a targeted approach; and (2) measured proteome responses in fish to differentiate microcystin toxicity pathways using a nontargeted approach. We additionally performed liver histopathology to determine whether tissue-level effects could occur and corroborate our findings. We utilized adult and juvenile Rainbow Trout to test these inquiries. Experiments followed an acute exposure time relative to the lifetime of Rainbow Trout, which is frequently used in ecotoxicological tests and microcystin studies using Rainbow Trout.[7,22−25,33,34] We hypothesized the kinetics of intracellular and extracellular microcystins will differ in fish tissues, but sublethal molecular level effects will manifest at similar rates of severity.

2. MATERIALS AND METHODS

2.1. Culture and Test Solution Preparation. *Microcystis aeruginosa* strain CPCC 300 was obtained from the Canadian Phycological Culture Center (the University of Waterloo, Waterloo, Canada). This strain produces microcystin-LR (CAS: 101043-37-2, $C_{49}H_{74}N_{10}O_{12}$) and its desmethylated form [D-Asp^3]-microcystin-LR (CAS: 120011-66-7, $C_{48}H_{72}N_{10}O_{12}$), which occur widely[1,12] and are among the most toxic congeners of this family.[35] Details regarding culturing this strain[36] and the procedure for preparing microcystins in their intracellular and extracellular states for the experiments are provided in the Supporting Information (SI 1).

2.2. Test Species. Adult and juvenile Rainbow Trouts were selected for the experiments. Adult fish (300−350 g) were cultured at the Aquatic Toxicology Unit (ATU) of the Ontario Ministry of the Environment, Conservation, and Parks (MECP; Etobicoke, ON, Canada). Juvenile fish (50−70 g) were cultured at the Lyndon Fish Hatchery (Petersburg, ON, Canada) and transported to the ATU. Fish were maintained at 15 °C under a photoperiod of 16:8 h (light/dark) for 2 weeks to allow acclimatization prior to each test. No mortalities were observed during the period of acclimatization. Fish were fed daily with standard commercial pelleted food at a feeding rate of 1−5% of the wet body weight. Fish were not fed 24 h prior to test launch or during the experiments.

2.3. Microcystin Experiments. Adult and juvenile fish were, respectively, tested using static nonrenewal 96 h experiments in the ATU following the MECP Laboratory Services Branch Animal Care Committee Animal Utilization Proposal (Laboratory Lab License #0053). Experiments included one control (i.e., water-only) and two treatments (i.e., intracellular and extracellular microcystins). For the first experiment on adults, intracellular and extracellular treatments were prepared at 24 μg total microcystins L^{-1}, based on the World Health Organization's (WHO) guideline value for recreational waters[15] and concentrations commonly measured in the environment.[37] The results of this experiment indicated no detection of microcystins in the muscle of adult Rainbow Trout. To determine whether microcystins could accumulate in the muscle of Rainbow Trout within environmentally relevant levels,[12] we performed a second experiment on juveniles by exposing them to a higher concentration (100 μg total microcystins L^{-1}).

The control and treatments had four experimental units (i.e., pails were 50 L for adults and 20 L for juveniles, respectively, with a loading density ≤2.5 g fish L^{-1}). Experimental units had four Rainbow Trout (i.e., 16 fish per treatment, 48 fish per test). Fish were randomly assigned to experimental units. Intracellular and extracellular microcystin controls (i.e., solution-only with no fish) were additionally added to monitor degradation of the toxin. Treatments were carried out under constant aeration throughout the experiments. Fish were collected at 0, 24, 48, 72, and 96 h. To maintain the same loading density throughout the experiment, at each time point one experimental unit of four fish was randomly selected from treatments and fish were sacrificed by a forceful blow to the head. Livers, kidneys, and muscles were dissected, and growth parameters [i.e., body length, body weight, liver weight, kidney weight, and liver and kidney somatic indices (LSI and KSI)] were measured prior to being quick-frozen at −80 °C (Table S2).

2.4. Biochemical Analysis. *2.4.1. Water Analysis.* Water samples (5 mL) were collected at 0, 24, 48, 72, and 96 h to quantify the cell concentration, fluorescence, and standard water parameters (Table S3) and quick-frozen at −80 °C

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Environ. Sci. Technol. 2021, 55, 10422−10431

Table 1. Measured Concentrations for Microcystin-LR and [D-Asp^3]-Microcystin-LR in Water (μg L^{-1}) and Corresponding Total Microcystins in the Liver, Kidney, and Muscle (ng g^{-1} w.w.) of Rainbow Trout

species	time (h)[a]	mortality (%)	liver somatic index (%) ± SD (n = 4)	kidney somatic index (%) ± SD (n = 4)	microcystin-LR in water (μg L^{-1}) ± SD	[D-Asp^3]-microcystin-LR in water (μg L^{-1}) ± SD	total microcystins in liver (ng g^{-1} w.w.) ± SD (n = 4)[c]	total microcystins in kidney (ng g^{-1} w.w.) ± SD (n = 4)[c]	total microcystins in muscle (ng g^{-1} w.w.) ± SD (n = 4)[c]
Rainbow Trout (adult)	CTRL 0	0	1.08 ± 0.03	0.59 ± 0.04	0	0	<MDL	<MDL	<MDL
	CTRL 24	0	1.06 ± 0.05	0.50 ± 0.05	0	0	<MDL	<MDL	<MDL
	CTRL 48	0	1.11 ± 0.09	0.53 ± 0.11	0	0	<MDL	<MDL	<MDL
	CTRL 72	0	0.98 ± 0.12	0.59 ± 0.12	0	0	<MDL	<MDL	<MDL
	CTRL 96	0	1.07 ± 0.18	0.57 ± 0.10	0	0	<MDL	<MDL	<MDL
	IN-MC 0	0	1.03 ± 0.04	0.60 ± 0.04	17.6 ± 0.6	6.9 ± 0.2	<MDL	<MDL	<MDL
	IN-MC 24	0	0.99 ± 0.06	0.58 ± 0.07	18.8 ± 1.3	7.0 ± 0.4	<MDL	3.4 ± 1.7	<MDL
	IN-MC 48	0	0.90 ± 0.13	0.72 ± 0.07	19.9 ± 0.7	6.5 ± 0.7	<MDL	2.1 ± 1.0	<MDL
	IN-MC 72	0	0.94 ± 0.07	0.61 ± 0.07	17.4 ± 0.5	6.2 ± 0.3	<MDL	6.2 ± 1.5	<MDL
	IN-MC 96	0	0.94 ± 0.21	0.77 ± 0.25	16.9 ± 0.0	6.4 ± 0.0	<MDL	4.8 ± 3.2	<MDL
	EX-MC 0	0	0.98 ± 0.12[b]	0.59 ± 0.03	10.2 ± 1.9	4.0 ± 0.7	<MDL	<MDL	<MDL
	EX-MC 24	0	1.00 ± 0.19[b]	0.57 ± 0.07	0.1 ± 0.0	0.1 ± 0.1	14.5 ± 9.2	8.5 ± 7.2	<MDL
	EX-MC 48	0	0.89 ± 0.13[b]	0.55 ± 0.14	0.1 ± 0.0	0.1 ± 0.1	7.3 ± 3.6	13.2 ± 14.8	<MDL
	EX-MC 72	0	0.76 ± 0.04[b]	0.55 ± 0.11	0.1 ± 0.0	0.0 ± 0.0	11.6 ± 3.6	25.7 ± 21.2	<MDL
	EX-MC 96	0	0.82 ± 0.06[b]	0.59 ± 0.13	0.1 ± 0.0	0.1 ± 0.0	5.5 ± 2.1	31.7 ± 28.8	<MDL
Rainbow Trout (juvenile)	CTRL 0	0	0.94 ± 0.15	0.62 ± 0.05	0	0	<MDL	<MDL	<MDL
	CTRL 24	0	0.96 ± 0.11	0.60 ± 0.10	0	0	<MDL	<MDL	<MDL
	CTRL 48	0	0.83 ± 0.10	0.55 ± 0.06	0	0	<MDL	<MDL	<MDL
	CTRL 72	0	1.11 ± 0.26	0.70 ± 0.18	0	0	<MDL	<MDL	<MDL
	CTRL 96	0	1.03 ± 0.12	0.58 ± 0.08	0	0	<MDL	<MDL	<MDL
	IN-MC 0	0	0.98 ± 0.13	0.62 ± 0.02	73.9 ± 1.1	25.8 ± 1.4	<MDL	<MDL	<MDL
	IN-MC 24	0	0.93 ± 0.21	0.68 ± 0.17	72.9 ± 4.9	24.6 ± 1.8	6.5 ± 1.0	32.0 ± 33.5	3.2 ± 0.6
	IN-MC 48	0	1.00 ± 0.13	0.61 ± 0.05	66.5 ± 2.2	24.0 ± 0.7	5.7 ± 1.5	2.9 ± 2.1	5.0 ± 5.4
	IN-MC 72	0	1.01 ± 0.15	0.81 ± 0.09	64.6 ± 5.6	24.1 ± 2.3	5.6 ± 2.1	12.9 ± 10.0	3.5 ± 2.5
	IN-MC 96	0	1.20 ± 0.15	0.76 ± 0.22	70.2 ± 0.0	25.2 ± 0.0	7.0 ± 1.3	14.6 ± 5.6	3.0 ± 3.0
	EX-MC 0	0	1.03 ± 0.12	0.60 ± 0.02	69.6 ± 4.0	31.4 ± 5.0	<MDL	<MDL	<MDL
	EX-MC 24	0	1.04 ± 0.10	0.52 ± 0.21	63.4 ± 0.5	20.7 ± 0.9	51.0 ± 28.7	73.6 ± 50.5	6.1 ± 6.9
	EX-MC 48	0	1.04 ± 0.18	0.59 ± 0.09	60.8 ± 4.4	20.8 ± 3.1	120.2 ± 71.6	209.9 ± 42.3	3.5 ± 2.5
	EX-MC 72	0	0.91 ± 0.14	0.72 ± 0.12	48.4 ± 0.2	12.9 ± 0.5	173.1 ± 97.8	119.9 ± 56.6	5.8 ± 3.7
	EX-MC 96	0	1.02 ± 0.11	0.70 ± 0.06	36.8 ± 0.0	9.9 ± 0.0	56.0 ± 19.1	101.0 ± 47.0	8.3 ± 6.9

[a]IN-MC denotes intracellular microcystins; EX-MC denotes extracellular microcystins. [b]Statistically significant from the control ($p < 0.05$) as determined by the Kruskal–Wallis test. [c]The method detection limit (MDL) for microcystins in tissues is 2.18 ng g^{-1} w.w.

before analysis for microcystin congeners at the MECP laboratories (Etobicoke, ON, Canada) using a published method[38] described in the Supporting Information (SI 2).

2.4.2. Tissue Analysis. Samples were analyzed at the MECP laboratories for total microcystins using a modified Lemieux Oxidation method via liquid chromatography coupled to time-of-flight mass spectrometry (LC-QTOF MS)[39] described in the Supporting Information (SI 3). Tissue concentrations are presented on a wet weight basis (w.w.) throughout.

2.5. Proteomics Analysis. Adult tissues were collected at 0, 24, 48, 72, and 96 h for proteomics analysis. Complete details of the proteomics analysis, including tissue preparation,[40] database searches, and analysis of identified proteins, are described in the Supporting Information (SI 4). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[41] partner repository with the dataset identifier PXD026187.

2.6. Histopathology. Adult tissues were collected for histopathology. Juvenile tissues were not collected due to limited quantity as priority was given to measuring microcystins in tissues. Livers were fixed in 10% buffered formalin and processed at the Animal Health Laboratory, the University of Guelph (Guelph, ON, Canada). Tissue sections were 4 μm thick, and stained with hematoxylin and eosin. Photographs were taken on an Olympus BX53 microscope equipped with an Olympus DP72 camera (Olympus Canada Inc., Richmond Hill, ON, Canada).

Tissues were scanned and assessed for dissociated and/or basophilic condensed hepatocytes, necrosis and/or apoptosis, hemorrhage, mitotic figures, and inflammation. Representative affected areas were scaled 1–4, representing normal to most severe, respectively, for each of the above. Five representative areas per liver tissue were scaled. Twenty percent of the tissues were assessed a second time and scores were accepted if within ±1.

2.7. Statistical Analysis. Statistical analyses were conducted on: (1) water concentrations of total microcystins; (2) correlations between total microcystins in water (MC_{water}) and total microcystins in liver (MC_{liver}), kidney (MC_{kidney}), muscle (MC_{muscle}), LSI and KSI; (3) liver histopathology; and (4) label-free proteomics data. Statistical significance was set at $p < 0.05$ and false discovery rate (FDR) correction was applied where appropriate. Detailed descriptions of each analysis are provided in the Supporting Information (SI 5).

3. RESULTS

3.1. Microcystins in Water. For both experiments, MC_{water} was 75 ± 3% microcystin-LR and 25 ± 3% [D-Asp^3]-microcystin-LR (Table 1). For adults, the starting MC_{water} values were 24.4 and 14.2 μg L^{-1} for the intracellular

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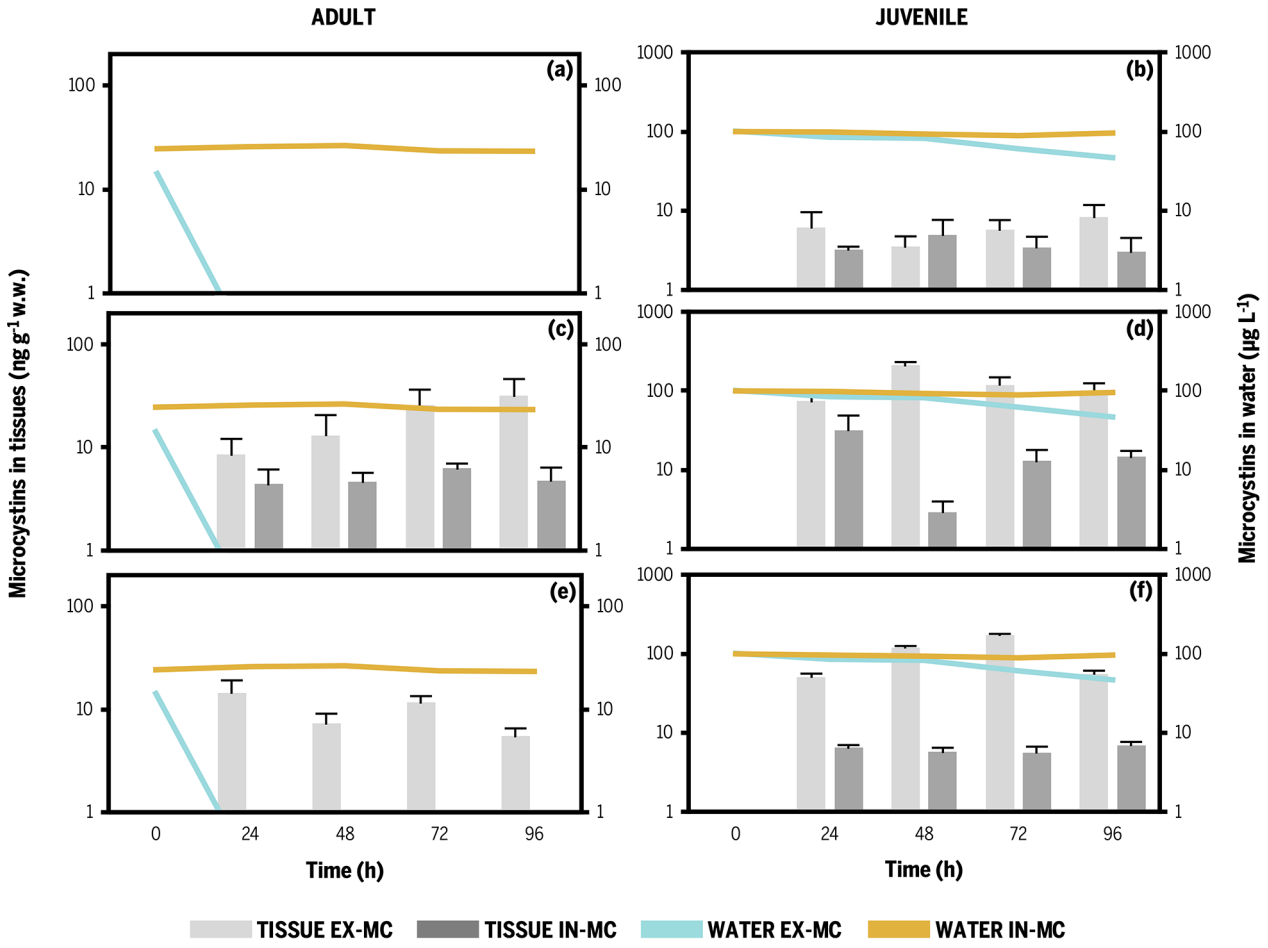

Figure 1. Microcystin concentrations in adult and juvenile Rainbow Trout muscles (a,b), kidneys (c,d), and livers (e,f) from exposure to intracellular and extracellular treatments.

and extracellular treatments, respectively. By 24 h, the mean extracellular MC_{water} decreased to 0.2 $\mu g\ L^{-1}$ (−98.6%), while there was little change for intracellular MC_{water}. For juveniles, the starting MC_{water} values were 99.7 and 101.0 $\mu g\ L^{-1}$ for the intracellular and extracellular treatments, respectively. By 96 h, the mean extracellular MC_{water} decreased to 46.8 $\mu g\ L^{-1}$, but there was a little change in the mean intracellular MC_{water}. Similar observations were noted for the microcystin controls (MC_{water} decreased by 21.67% for extracellular; little change for the intracellular) (Figure S1).

3.2. Microcystins in Tissues. *3.2.1. Liver.* For adults, no intracellular MC_{liver} were detected above the MDL (Figure 1e, Table 1). Extracellular MC_{liver} increased to 14.5 ± 9.2 ng g^{-1} w.w. by 24 h, and gradually declined to 5.5 ± 2.1 ng g^{-1} w.w. by 96 h. Time did not affect MC_{liver} and the LSI in different treatments (F = 2.346, p = 0.051 and F = 0.870, p = 0.526, respectively) (Table S5). For juveniles, intracellular MC_{liver} were detected between 24 h (6.5 ± 1.0 ng g^{-1} w.w.) and 96 h (7.0 ± 1.3 ng g^{-1} w.w.) (Figure 1f, Table 1). Extracellular MC_{liver} were also detected between 24 h (51.0 ± 28.7 ng g^{-1} w.w.) and 96 h (56.0 ± 19.1 ng g^{-1} w.w.) at higher levels. Time and treatment as independent variables significantly increased MC_{liver} (F = 3.323, p = 0.030 and F = 37.997, p < 0.001, respectively), and their joint effect significantly increased MC_{liver}, as shown by the interaction term (F = 3.425, p = 0.009) (Table S5). In contrast, time did not affect LSI across treatments (F = 1.823, p = 0.122) (Table S5). Both life stages readily accumulated significantly higher levels of extracellular MC_{liver} than intracellular MC_{liver}.

3.2.2. Kidney. For adults, intracellular MC_{kidney} were detected between 24 h (3.4 ± 1.7 ng g^{-1} w.w.) and 96 h (4.8 ± 3.2 ng g^{-1} w.w.) (Figure 1c, Table 1). Extracellular MC_{kidney} increased between 24 h (8.5 ± 7.2 ng g^{-1} w.w.) and 96 h (31.7 ± 28.8 ng g^{-1} w.w.) Time did not significantly affect MC_{kidney} and the KSI, respectively, across treatments (F = 1.135, p = 0.362 and F = 0.815, p = 0.566, respectively) (Table S5). For juveniles, intracellular MC_{kidney} were detected between 24 h (32.0 ± 33.5 ng g^{-1} w.w.) and 96 h (14.6 ± 5.6 ng g^{-1} w.w.) (Figure 1d, Table 1). Extracellular MC_{kidney} increased from 24 h (73.6 ± 50.5 ng g^{-1} w.w.) to 96 h (101.0 ± 47.0 ng g^{-1} w.w.). Time and treatment as independent variables significantly increased MC_{kidney} (F = 3.451, p = 0.026 and F = 82.417, p < 0.001, respectively), and their joint effect significantly increased MC_{kidney}, as shown by the interaction term (F = 6.179, p < 0.001) (Table S5). In contrast, time did not significantly affect KSI across treatments (F = 0.486, p = 0.814) (Table S5). Both life stages accumulated intracellular and extracellular MC_{kidney}, with significantly higher levels toward extracellular MC_{kidney} and the highest amounts among all tissues analyzed.

3.2.3. Muscle. For adults, no intracellular and extracellular MC_{muscle} were detected above the MDL (Figure 1a, Table 1). For juveniles, intracellular MC_{muscle} were detected between 24 h (3.2 ± 0.6 ng g^{-1} w.w.) and 96 h (3.0 ± 3.0 ng g^{-1} w.w.)

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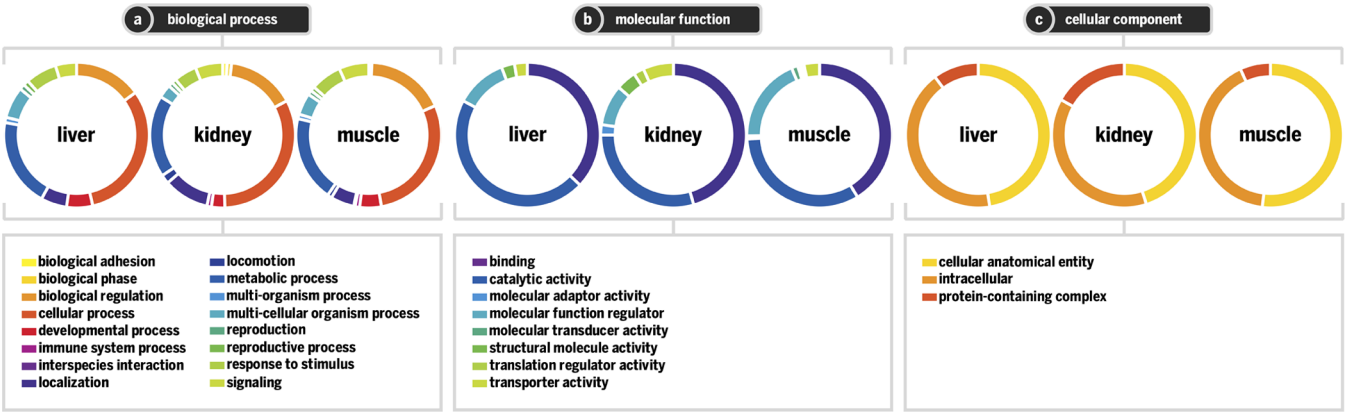

Figure 2. GO classification of significant differentially abundant proteins with functional annotation in the liver, kidney, and muscle of adult Rainbow Trout exposed to microcystins.

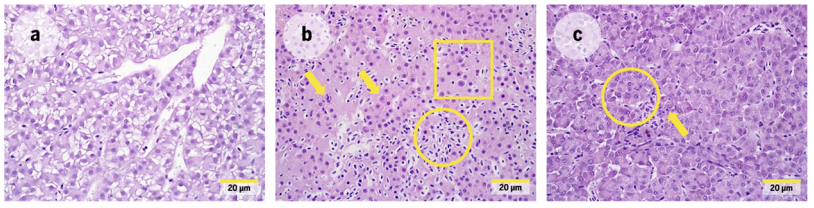

Figure 3. Liver histopathology in adult Rainbow Trout after 96 h of exposure to microcystins. Controls (a) are normal, while Rainbow Trout exposed to intracellular microcystins (b) has numerous single-cell isolated hepatocytes (square), intermixed with hemorrhage (circle) and necrotic and apoptotic hepatocytes (arrows). Rainbow Trout exposed to extracellular microcystins (c) had less severe lesions that were characterized primarily by shrunken basophilic hepatocytes (circle) but also some necrosis (arrow). Images are at 20 μm scale.

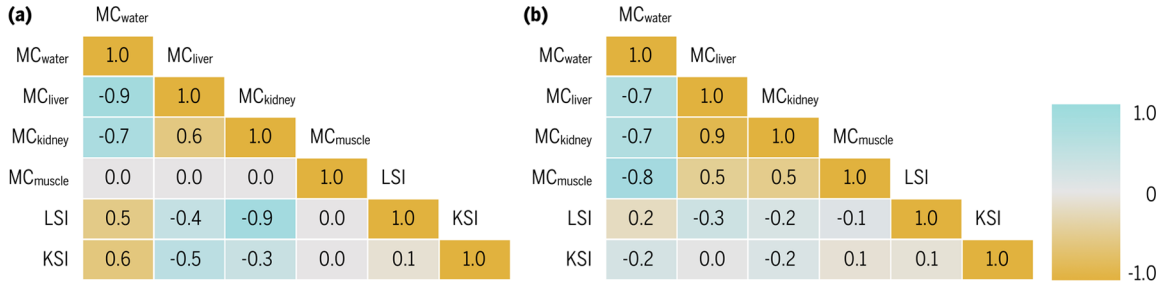

Figure 4. Correlation matrices for adult (a) and juvenile (b) Rainbow Trout after exposure to microcystins.

(Figure 1b, Table 1). Extracellular MC_{muscle} were also detected between 24 h (6.1 ± 6.9 ng g^{-1} w.w.) and 96 h (8.3 ± 6.9 ng g^{-1} w.w.). Time did not effect MC_{muscle} across treatments (F = 0.622, p = 0.711), and there was no statistical difference between microcystin treatments (Table S5).

3.3. Proteins Detected with Label-Free Proteomics in Adults. From livers, kidneys, and muscles, a total of 2183, 2466, and 1883 proteins were identified, respectively. After FDR-correction for each tissue (the same order as above), 49 (2.2%), 55 (2.3%), and 174 (9.2%) of these showed significant differential abundance from exposure to intracellular or extracellular microcystins compared to the control ($p \leq 0.05$; Tables S8−S10). Gene ontology (GO) bioinformatics identified major functions from these differentially expressed (DE) proteins and overlap among livers, kidneys, and muscles (Figure 2). For biological processes, proteins were mainly involved in cellular (29−32% of DE proteins), metabolic (18−20% of DE proteins), and biological regulation (15−18% of DE proteins) (Figure 2a). Molecular functions were mainly involved in catalytic activity (30−46% of DE proteins) and binding (37−46% of DE proteins) (Figure 2b). Classification by cellular components revealed proteins were strictly located in the cellular anatomical entity (45−52% of DE proteins), intracellular (38−42% of DE proteins), and protein-containing complex (7−17% of DE proteins) (Figure 2c). Top protein classes identified were metabolite interconversion enzyme (21−30% of DE proteins), gene-specific transcriptional regulator (9−16% of DE proteins), cytoskeletal protein (8−12% of DE proteins), and scaffold/adaptor protein (12% of DE proteins). GO enrichment analyses additionally revealed significant proteins were involved in pyruvate metabolic processes in livers (p = 0.004), anion binding in kidneys (p = 0.03), and myopathy in muscles (p = 0.03) (Figure S2, Table S11). Across all tissues, proteins were significantly dysregulated in both microcystin treatments, with a greater share that changed abundance in the extracellular treatment after 96 h of exposure.

3.4. Histopathology. Livers showed acute lesions (necrosis, apoptosis, and hemorrhage) in intracellular and extracellular treatments (Figure 3, Table S6). Lesions were either predominantly peribiliary or perivenular (pericentral). The character of the lesions varied somewhat between

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Environ. Sci. Technol. 2021, 55, 10422−10431

intracellular and extracellular treatments, with the former having more individualized hepatocytes and the latter having more basophilic shrunken hepatocytes. No other significant lesions were noted except for two eosinophilic altered foci present in replicate C from the intracellular treatment. Both the intracellular and extracellular treatments had a significant increase in dissociation/basophilia (Mann−Whitney Rank Sum Test, $p = 0.008$, $p = 0.03$, respectively) and necrosis/apoptosis (Mann−Whitney Rank Sum Test, $p = 0.024$, $p = 0.017$, respectively) compared with the control. Intracellular and extracellular treatments also had a significant increase in dissociation/basophilia (Kruskal−Wallis one-way ANOVA, $F = 6.153$, $p = 0.021$) and necrosis/apoptosis ($F = 5.74$, $p = 0.025$) compared with the control.

3.5. Correlation Analysis. For adults, significant negative correlations were observed between MC_{water} and MC_{liver} ($p = 0.001$) and MC_{kidney} ($p = 0.016$), respectively, suggesting both tissues accumulate microcystins and decrease the solution concentration (Figure 4, Table S7). Correlations with MC_{muscle} could not be measured because no microcystins were detected. For juveniles, a significant positive correlation was observed between MC_{liver} and MC_{kidney} ($p < 0.001$), indicating both tissues will increase together in the presence of microcystins. MC_{water} had a significant negative correlation with MC_{liver} ($p = 0.036$), MC_{kidney} ($p = 0.037$), and MC_{muscle} ($p = 0.003$), indicating all tissues accumulate microcystins and decrease the solution concentration (Figure 4, Table S7).

4. DISCUSSION

4.1. Bioavailability of Intracellular and Extracellular Microcystins. This study give strong indication that microcystin bioavailability in fish is influenced by whether it is present in its intracellular or extracellular state. Across all tissues, intracellular microcystins rarely exceeded 10 ng g^{-1} w.w. in tissues yet extracellular microcystins accumulated above 2- to 3-fold. This demonstrates microcystins will readily accumulate in fish tissues within 24 h regardless of their intracellular or extracellular state; yet, microcystins will preferentially accumulate at higher levels when they arer lysed from *M. aeruginosa* cells. Significant correlations between MC_{water} and MC_{liver} and MC_{kidney} in adults, and between MC_{water} and MC_{liver}, MC_{kidney}, and MC_{muscle} in juveniles broadly indicate all tissues can accumulate microcystins and decrease the solution concentration. These findings corroborate recent evidence from a meta-analysis demonstrating significant positive correlations between intracellular microcystin concentrations in water with fish tissue concentrations detected by the enzyme-linked immunosorbent assay ($p < 0.0001$) and the protein phosphatase inhibition assay ($p = 0.0006$).[42] Variability in the accumulation of intracellular and extracellular microcystins in fish tissues may also reflect differences in congener formation,[42] which can influence their bioavailability in tissues.[21] For example, microcystin-LR was not detected in the muscle of Silver Carp (*Hypophthalmichthys molitrix*) despite abundant accumulation in the intestine, yet microcystin-RR was measured in the liver and muscle.[43] These findings indicate microcystin-LR and [D-Asp^3]-microcystins-LR may not readily accumulate in muscles, observed in the present study. Differences in accumulation between adults and juveniles in the present study could further be a result of the different MC_{water} they were exposed to; as such, we limit comparison of observations between the two life stages.

4.2. Toxicity in Fish Tissues. GO enrichment analysis in Rainbow Trout livers revealed the largest cluster of significantly dysregulated proteins linked to pyruvate metabolic processes. Pyruvate is frequently measured to assess the toxicity of pollutants.[44] For example, intraperitoneal injection of a single dose of microcystin-LR (125 $\mu g\ kg^{-1}$ body weight) in the liver of Goldfish (*Carassius auratus*) significantly altered pyruvate activity by inducing hepatic tissue lesions within 96 h,[45] At sublethal doses used in the present study, histopathology in the liver revealed that intracellular and extracellular microcystins caused acute lesions at similar severity after 96 h. A significant decrease in the LSI of Rainbow Trout exposed to extracellular microcystins was additionally observed, which suggests cell loss or atrophy by apoptosis and/or necrosis.[46] Proteins linked to pyruvate metabolic processes (Aldoc, Eno1, Gpi, Me2, Pck1, and Pck2) are also of interest. For instance, Eno1 is an important biomarker of carcinogenicity and is highly expressed in liver cancer.[47] A proteomic investigation into pulmonary injuries in mice induced by microcystin-LR found Eno1 increase in abundance and contributed to protein phosphorylation and energy metabolism, which are defense-related mechanisms connected to PP2A inhibition caused by microcystin-LR.[48] For the present study, GO bioinformatics in the liver also indicates clusters of significantly dysregulated proteins may be connected to regulatory pathways responsible for cell replication (Dock8, Dpp3, Gbf1, Icmt, Mgat1, and Serpina10), cytoskeletal structure (Dctn3, Dnah8, and Spire1), stress responses (Aldoc, Eno1, Ethe1, Gpi, Mgat1, Me2, Nt5c3b, Pck1, Pck2, Ugdh, and Uprt), and DNA repair (Irx1, Myt1l, Vgll2, Zc3h4, Zc3h6, and Znf729). Members of the SLC family (Slc12a5 and Slc4a1ap), GST (Gstt1), Bcl (Bcl9), and MAPK (Map4k3) were also significantly dysregulated and give evidence to the hepatotoxicity that microcystins can inflict in fish regardless of its intracellular or extracellular state. Histopathological observations together with nontargeted proteomics substantiate that microcystins, in both states and at sublethal concentrations, can dysregulate proteins related to oxidative stress and carcinogenesis and cause noticeable liver damage in Rainbow Trout after an exposure period typical of a harmful algal bloom event.[49] GO bioinformatics classified the largest cluster of dysregulated proteins with hepatocyte metabolism (22% of DE proteins), corroborating previous literature, which suggests cellular damage can affect hepatocyte metabolism regulation and indirectly affect liver physiological activities.[50]

Microcystins are potent renal toxins to fish[22] and the recent discovery of OATPs in kidneys provides an empirical link for their nephrotoxicity.[15,51] Kidneys follow a pattern of histopathological alterations from exposure to microcystins including dilatations of Bowman's capsule, vacuolization, necrosis and pyknosis of tubular cells, and edema.[22,51−53] For the present study, GO enrichment analysis in Rainbow Trout kidneys revealed the largest cluster of significantly dysregulated proteins linked to anion binding (Actn2, Cacna1b, Ckm, Dnah3, Fchsd2, Gstp1, Hspe1, Krit1, Mthfd2, Nlrc3, P2rx1, Pde4d, Prkg1, Srpk3, St13, Ttn, Vcp, and Wee1), supporting the role of OATPs in nephrotoxicity. Among the cluster of proteins linked to anion binding was Gstp1, which plays an important role in the detoxification of microcystins and was linked to kidney alterations in the transcription of GST in Common Carp (*Cyprinids carpio*).[17] This indicates microcystins can interfere with metabolic pathways responsible for

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Environ. Sci. Technol. 2021, 55, 10422−10431

detoxification in kidneys. We further observed a significant dysregulation in flavin reductase (NADPH), an oxidoreductase linked to the production of reactive oxygen species (ROS) from microcystin exposure, which is typically expressed in the liver.[14,54] Recent findings using Zebrafish (*Danio rerio*) additionally uncovered significant histopathological lesions (apoptotic cells) in kidneys after 60 d of exposure to 25 $\mu g\ L^{-1}$ microcystin-LR that were linked to negative alterations in ROS levels, highlighting the role of apoptosis in nephrotoxicity.[53] GO bioinformatics in the kidney also indicates clusters of significantly dysregulated proteins connected to the same regulatory pathways as the liver, including cell replication (Dock7, Pkha1, Prkg1, Srpk3, and Wee1), cytoskeletal structure (Dnah3, Dync1i2, and Rtkn2), stress responses (Art5, Blvrb, Ckm, Gstp1, Mmut, Pde4d, and Tbxas1), and DNA repair (Lrrfip1, Twist2, and Zfp2). Altogether, the present study demonstrates high conformity between microcystin's nephrotoxicity and hepatotoxicity pathways in Rainbow Trout at sublethal concentrations in both intracellular and extracellular states.

Microcystin toxicity in muscle proteins is an unexplored area of research. Although we did not detect microcystins in the muscles of adult Rainbow Trout, likely due to the lower level of exposure, GO enrichment analysis revealed a large cluster of significantly dysregulated proteins linked to myopathy (Actn2, Cap2, Col12a1, Col6a3, Cryab, Dhx16, Gtf2ird1, Myh7, Ndufaf1, Neb, Nodal, Opa1, Svil, and Syne1). For instance, alpha-actins (Actn2) and nebulins (Neb) have been implicated in nemaline myopathy in Zebrafish, a skeletal muscle disease characterized by muscle weakness.[55] Evidence of myopathy in the present study suggests an indirect downstream effect that begins with hepatotoxicity and nephrotoxicity. Stress responses from the liver and kidney may have induced a whole organism stress response in Rainbow Trout, including the muscle. GO bioinformatics corroborated these findings with clusters of significantly dysregulated proteins connected to cell replication, stress responses, and DNA repair. Proteins linked to SLC (Slc12a5), MAPK (Pak6), and NADPH (Cyp1b1, eif4g1, Fmo5), typically associated with microcystin toxicity pathways in liver and kidney, were also dysregulated in Rainbow Trout muscle.

4.3. Cyanopeptides Beyond Microcystins. Notwithstanding the ecotoxicological concern of microcystins,[12,16] other bioactive metabolites (e.g., aerucyclamides and cyanopeptolins) may contribute to effects, which cannot be explained by microcystins alone.[27] Our study presents unusual results, prominently in the adult liver, where microcystins were only detected in the extracellular treatment yet histopathology and nontargeted proteomics revealed similar acute toxicity between intracellular and extracellular exposures.

Three possibilities can explain these findings. First, microcystins may have accumulated and caused biological impacts in tissues at levels below limits of detection. Second, the recent characterization of *M. aeruginosa* CPCC 300's metabolome revealed the production of aerucyclamides and cyanopeptolins alongside microcystins.[56] In one instance from batch culture studies, aerucyclamides dominated (63.1%) and surpassed the production of microcystins.[56] Aerucyclamides are toxic to freshwater crustaceans,[57] while cyanopeptolins are toxic to fish.[58] Subchronic toxicity studies in medaka (*Oryzias latipes*) further support the hypothesis that *M. aeruginosa* produce bioactive metabolites other than microcystins, after demonstrating histopathological cellular abnormalities in histopathology, proteomics, and metabolomics from livers that were exposed to both nonmicrocystin-producing and microcystin-producing strains of *M. aeruginosa*.[59] Third, exposure to a poor-quality diet that includes toxic *M. aeruginosa* may increase host resistance to parasitic infections. Research in zooplankton shows that high-quality phytoplankton diets can be beneficial to hosts and parasites, while lower-quality phytoplankton diets including *Microcystis* can, in some instances, increase host fitness and decrease parasitic infections.[60,61] This demonstrates the potential for cyanobacterial diets to mitigate parasitic infection.

4.4. Environmental Implications. Toxic *Microcystis* blooms are increasing globally[1] and possess enhanced mechanisms to endure and exploit variable pH, temperature, light, and nutrient conditions—mechanisms, which enable them to outcompete other planktons and gain dominance in freshwater ecosystems.[3] Climate change is also resulting in increased freshwater temperatures throughout the world,[62] which drives the persistence of harmful algal blooms and increases their threat to freshwater biodiversity.[63] Rainbow Trout, and other fish populations for that matter, which live in these freshwater ecosystems, may find it increasingly difficult to stay cool and evade the pervasive effects of toxic *Microcystis* blooms. The present study demonstrates that cyanotoxins from *M. aeruginosa* can adversely impact Rainbow Trout. Our findings are unique in that they differentiate the accumulation and molecular-level impacts in fish tissues from microcystins within and outside of *M. aeruginosa* cells. Interestingly, we show sublethal levels of intracellular and extracellular microcystins can accumulate in all tissues within 24 h, with greater levels from their extracellular state; yet, nontargeted proteomics and histopathology show microcystins in both states can, at similar severity, significantly dysregulate proteins linked to oxidative stress and carcinogenesis in all tissues and form acute lesions within 96 h. Our findings call attention to water- and fish-monitoring programs that can track intracellular and extracellular cyanotoxins during pre-to-postsenescent stages of a harmful algal bloom. To further our understanding of risks to fish populations, future work should measure and characterize the toxicity of cyanopeptides beyond microcystins in fish tissues in order to distinguish their effects, and examine whether an additive or synergistic relationship exists that can enhance cyanobacterial toxicity, which would not be possible if microcystins acted alone.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c01501.

M. aeruginosa CPCC 300 culturing methodology; microcystin water analysis methodology; microcystin fish tissue analysis methodology; database searches and analysis of identified proteins methodology; statistical analyses; physicochemical properties of the dechlorinated tap water used in all toxicity tests; growth parameters of Rainbow Trout used during toxicity tests; physicochemical properties of the solution during toxicity tests; method variables used for separation and detection by LC-QTOF MS; two-way analysis of variance measuring the interaction between time and treatment; histopathology scoring for liver of adult Rainbow Trout; correlations between microcystins in

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Environ. Sci. Technol. 2021, 55, 10422−10431

water (MC_{water}) and microcystins in liver (MC_{liver}), kidney (MC_{kidney}), and muscle (MC_{muscle}), and liver somatic index (LSI) and kidney somatic index (KSI); label-free proteins with differential abundance in Rainbow Trout adults' livers; label-free proteins with differential abundance in Rainbow Trout adults' kidneys; label-free proteins with differential abundance in Rainbow Trout adults' muscles; GO enrichment analysis for label-free proteins; total microcystins measured in water ($\mu g\ L^{-1}$) of intracellular and extracellular treatments; and heat maps of peak areas from key functions in GO enrichment analysis (PDF)

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R.S.S., S.P.B., and P.K.S. conceived and designed the experiment. R.S.S., G.H., K.H., P.K., M.S., D.S., and A.V. collected the data. R.S.S., X.O., J.S.L., and D.B.D.S. analyzed the data. R.S.S., A.V., G.H., K.H., P.K., M.S., D.S., S.P.B., and J.S.L. wrote the paper. All authors read, amended, and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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