Comparative Biochemistry and Physiology - Part D 33 (2020) 100656

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology - Part D

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

Shotgun proteomics analysis reveals sub-lethal effects in *Daphnia magna* exposed to cell-bound microcystins produced by *Microcystis aeruginosa*

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ARTICLE INFO

Keywords:
Daphnia
Harmful algal blooms
Microcystin
Proteomics
Life cycle tests

ABSTRACT

Microcystins that are cell-bound within *Microcystis* have demonstrated the ability to cause lethal and reproductive impairment in *Daphnia*, who constitute an important part of aquatic food chains and are known to feed on viable cyanobacterial cells. Recent advances in environmental toxicogenomics can be used to better understand the mechanistic effects from exposure to cell-bound microcystins in *Daphnia*; however, there remains a need to examine the effects of microcystins exposure as a function of dose and time in order to help elucidate the progression of (sub-)lethal effects. This study examines the effects of cell-bound microcystin exposure in *Daphnia magna* as a function of dose and time with shotgun proteomics in order to measure and provide insightful evidence describing functional mechanisms from, and relationships between, protein populations in response to toxic *Microcystis aeruginosa*. We further characterize the life-history fitness of *D. magna* in the presence of toxic exposure by measuring somatic growth rate. Chronic dietary exposure to cell-bound microcystins reduced the somatic growth rate of *D. magna*. Through proteomics analysis, we identified a significant increase in abundance of proteins related to reproductive success and development, removal of superoxide radicals, and motor activity in *D. magna* parents exposed to cell-bound microcystins at sub-lethal concentrations. We also identified a significant decrease in abundance of proteins related to apoptosis, metabolism, DNA damage repair, and immunity in *D. magna* neonates. This information will improve our understanding of the risks posed by cell-bound microcystins to cladocerans in freshwater ecosystems.

1. Introduction

Microcystis is the most common bloom-forming genus of freshwater cyanobacterium with high phenotypic plasticity (Lyu et al., 2016; Xiao et al., 2018). The frequency and intensity of *Microcystis* blooms continue to increase due to a variety of abiotic factors including nutrient additions from agricultural sources, rising ambient temperatures, and rising CO_2 levels associated with global heating (Scholten et al., 2005; Paerl et al., 2016; Visser et al., 2016; Xiao et al., 2018). Furthermore, *Microcystis* possess the ability to overwinter in the benthos of temperate climates and rise to the epilimnion during the summer to further expand its colonies when water temperatures exceed 15 °C (Reynolds and Rogers, 1976; Ibelings et al., 1991; Harke et al., 2016). The combination of these features makes *Microcystis* a cosmopolitan species, with blooms recently reported in 108 countries (Harke et al., 2016). *Microcystis* blooms can produce deleterious effects in the environment including increased turbidity of —and hypoxia within— freshwaters, threatening the existence of several macrophyte communities and causing aquatic (in)vertebrates to migrate or survive through adaptation (Paerl et al., 2016; Xiao et al., 2018). Several species of *Microcystis* are also toxic and can biosynthesize hundreds of cyanopeptides as a defensive mechanism to protect its colonies from grazers (Lürling, 2003; Xiao et al., 2018; Janssen, 2019). Microcystins, the most intensely studied class of cyanopeptides, are a family of potent hepatotoxins with over 100 structural variants routinely detected in freshwaters containing *Microcystis* (Harke et al., 2016; Janssen, 2019). It is well established that microcystins —and the most potent structural variant, microcystin-LR— can cause serious sickness, and even be lethal, to humans, mammals, birds, fish, mussels, and zooplankton (Yang et al., 2011; Sun et al., 2012; Huisman et al., 2018; Gene et al., 2019; Shahmohamadloo et al., 2019a). In vertebrates, microcystins suppress liver enzyme activities by inhibiting protein phosphatases (PP1 and PP2) that result in protein phosphorylation imbalance, oxidative stress, and finally liver failure (Janssen, 2019; Lyu et al., 2019). Microcystins are usually cell-bound within *Microcystis* at high concentrations and can harm pelagic invertebrates (e.g. *Daphnia* spp.) that feed on them

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https://doi.org/10.1016/j.cbd.2020.100656
Received 4 December 2019; Received in revised form 14 January 2020; Accepted 15 January 2020
Available online 29 January 2020
1744-117X/

(Rohrlack et al., 2005; Harke et al., 2016; Lyu et al., 2016; Ferrão-Filho et al., 2017; Xiao et al., 2018; Lyu et al., 2019).

Daphnia constitute an important part of aquatic food chains because they are primary feeders of phytoplankton and the prey of higher trophic level species (Davis et al., 2012; Lyu et al., 2019). Like most cladocerans, *Daphnia* are non-selective filter feeders and may not be able to differentiate toxic from non-toxic cells of *Microcystis* (Rohrlack et al., 2005). Consequently, two opposing hypotheses have been put forward to explain the interaction between *Daphnia* and cell-bound microcystins. The first proposes that *Daphnia* experience positive population growth when fed a diet containing *Microcystis* and can genetically adapt and phenotypically acclimate to maintain survival, growth, and reproduction (Hairston Jr et al., 2001; Gustafsson et al., 2005; Sarnelle and Wilson, 2005; Sarnelle et al., 2010). The second hypothesis posits that *Daphnia* experience abnormal development, mortality, and lower reproduction after the ingestion of cell-bound microcystins (Rohrlack et al., 2001; Rohrlack et al., 2005; Ferrão-Filho et al., 2014; Lyu et al., 2019; Shahmohamadloo et al., 2019a). Recent field and laboratory studies have demonstrated the main route of microcystin toxicity occurs in *Daphnia* after ingestion of viable *Microcystis* cells (Ferrão-Filho et al., 2014; Lyu et al., 2016; Ferrão-Filho et al., 2017; Lyu et al., 2019). A dose-response relationship measuring (sub-) lethal effects from exposure to cell-bound microcystins at environmentally relevant concentrations in freshwaters (0.5 to 300 μg L^{-1}) was determined in a previous study conducted by our research group (Shahmohamadloo et al., 2019a). Evidence from that study revealed a reduction in the growth of parents and number of neonates produced by *Daphnia magna* exposed to increasing concentrations of cell-bound microcystins, and the ability of surviving *D. magna* to gain tolerance to a diet of toxic *Microcystis aeruginosa*. Therefore, we propose that elements from both hypotheses occur within daphnid populations and that some clones can coexist within toxic *Microcystis* blooms, which is supported by evidence from another recent study (Lyu et al., 2019).

Recent advances in environmental toxicogenomics provide the potential to better understand the mechanistic basis for effects from exposure to cell-bound microcystins in *Daphnia*. Asselman et al. (2012) were among the first to implement microarray technology to measure gene expression response to a dietary mixture of toxic *M. aeruginosa* in *Daphnia pulex*. In their study, four pathways/gene networks were significantly overrepresented in *D. pulex* after chronic exposure to *M. aeruginosa*: first, differential regulation of the ribosome, which suggests an impact from *M. aeruginosa* on protein synthesis; second, oxidative phosphorylation, which suggests *D. pulex* were stressed and required additional energy to cope with microcystin exposure; third, mitochondrial dysfunction, which microcystins are known to affect by inducing reactive oxygen species that attack the mitochondria; and, fourth, protein export pathways, which are necessary for the overall health of *Daphnia* (Asselman et al., 2012). Asselman et al. (2017) later focused on genome-wide methylation patterns in *D. magna* exposed to toxic *M. aeruginosa* to identify mechanisms responsible for toxicity in *Daphnia*. In that study, results also suggested *Daphnia* allocate energy and resources to maintain normal protein expression and prevent the occurrence of misfolded proteins in the face of *Microcystis* stress, which may cause less energy to be available for reproduction and growth (Asselman et al., 2017). Similarly, Lyu et al. (2016) combined somatic growth rate (as an indicator of life-history fitness) with differential peptide labeling (iTRAQ)-based proteomics in *D. magna* exposed to toxic and non-toxic strains of *M. aeruginosa*. In that study, the somatic growth rate of *D. magna* was significantly reduced after exposure to toxic *M. aeruginosa*, and proteins associated with lipid, carbohydrate, amino acid, and energy metabolism —proteins that are related to the fitness of *Daphnia*— were dysregulated. Lyu et al. (2019) later conducted fitness and transcriptome analyses on two clones of *Daphnia similoides* —one clone sensitive and the other tolerant— in the presence of toxic *M. aeruginosa*. They observed a significant reduction in somatic growth rate in the sensitive clone compared to its tolerant counterpart, and metabolic processes linked to the fitness of *D. similoides* were significantly enhanced in the tolerant clone. These four studies demonstrate the important role that environmental toxicogenomics has played in advancing our understanding of the mechanistic effects from exposure to cell-bound microcystins in *Daphnia*. However, Lyu et al. (2019) identify the need for research that incorporates more treatments of microcystin exposure. Examining the effects of microcystins exposure as a function of dose and time at environmentally relevant concentrations will help to elucidate the gradual progression of (sub-)lethal effects in *Daphnia*. Proteins are the main determinants of biological functions within an organism (Lyu et al., 2016). By pairing ecotoxicological tests with proteomic analyses, the observer is better able to correlate effects at the organism level during exposure with biochemical functions that may be (in)directly the result of chemical stress, highlighting potential mechanisms of toxicity (Borgatta et al., 2015). Therefore, acquiring information from proteins within *Daphnia* through proteomic techniques can provide insightful evidence describing functional mechanisms from —and relationships between— protein populations in response to an environmental stressor such as cell-bound microcystins.

The present study addresses this research gap by analyzing changes in the proteomic profile of *D. magna* chronically exposed to cell-bound microcystins produced by *M. aeruginosa* at (sub-)lethal concentrations that are environmentally relevant in freshwaters (0.5 to 300 μg L^{-1}). Taking into consideration that *Microcystis* toxicity to *Daphnia* occurs via ingestion of viable cells, we examined the effects of dietary exposure to intracellular microcystins in viable *M. aeruginosa* cells. We use label-free shotgun proteomics to identify proteins in *D. magna* through a process of protein digestion that results in peptides that are then separated and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). These findings are further complimented by analyzing somatic growth rate to assess the life-history fitness of *D. magna* in the presence of increasing concentrations of microcystins. To our knowledge, the present study is the first to employ shotgun proteomic profiling on *D. magna* exposed to microcystins, and to compare the proteome of parents to neonates. Thus, the objectives of this study were to: 1) chronically expose *D. magna* parents and neonates to realistic low-to-high concentrations of toxic *M. aeruginosa*; 2) measure somatic growth rate in *D. magna* as an indicator of fitness in the presence of microcystins; 3) characterize proteome responses within *D. magna* using shotgun proteomics; and 4) identify relationships between (sub-)lethal concentrations of microcystins and differentially expressed proteins within *D. magna*.

2. Materials and methods

2.1. Animal and culture maintenance

A clonal culture of *D. magna* was provided by the Aquatic Toxicology Unit, Ministry of the Environment, Conservation and Parks (Ontario, Canada), and has been maintained in the laboratory for > 30 years (OMECP ATU, 2014). *D. magna* were cultured in municipal tap water and supplied a diet containing *Raphidocelis subcapitata* and *Chlorella fusca* that provided between 0.1 and 0.2 mg of organic carbon per *Daphnia* per day (OECD, 2012). *D. magna* were cultured under 400–800 lx cool-white fluorescent light at 20.0 ± 1 °C with a light:-dark cycle of 16:8 h. Water in culture vessels were renewed three times a week, and juveniles < 24 h old were used for the test.

M. aeruginosa strain CPCC 300 was supplied by the Canadian Phycological Culture Centre (University of Waterloo, Waterloo, Canada). This strain grows unicellular (1 to 2 μm diameter spheres) and was chosen for this study to prevent feeding inhibition observed in *D. magna* fed on colony-forming strains. Quantification by on-line solid phase extraction coupled to liquid chromatography-quadrupole time-of-flight high resolution mass spectrometry (Ortiz et al., 2017) confirmed *M. aeruginosa* CPCC 300 produces microcystin-LR and [D-Asp^3]-

microcystin-LR (Shahmohamadloo et al., 2019b). Cultures were maintained in a growth chamber under 600 ± 15 lx cool-white fluorescent light at 24.0 ± 1 °C with a light:dark cycle of 16:8 h. *M. aeruginosa* cultures were grown in BG-11 liquid media recipe for at least 1 mo before use in the experiment. Prior to testing, the cell concentration was measured by hemacytometer to be 1.68×10^7 cells mL^{-1}, and *M. aeruginosa* biomass was measured to be 0.55 mg d.w. mL^{-1}. The contribution of microcystins was approximately 2.0 μg per mg d.w. of *M. aeruginosa*.

2.2. Chronic toxicity test

The experimental design of this study is reported in Shahmohamadloo et al. (2019a) and is briefly described here. Test solutions were prepared by centrifuging *M. aeruginosa* CPCC 300 for 8 min at 2800 ×*g* followed by decanting 90% of BG-11 media and re-suspending the remaining 10% in dechlorinated municipal tap water. The test solution was stockpiled and stored in the dark at 6.0 ± 1 °C for at least 24 h prior to testing, to suspend growth of *M. aeruginosa*. Cell concentration was calculated using a hemacytometer.

D. magna were tested for survival and reproduction using the 21-d semi-static-renewal life-cycle test developed by the Organization for Economic Cooperation and Development (OECD, 2012), and is reported in Shahmohamadloo et al. (2019a). Results from Shahmohamadloo et al. (2019a) demonstrated that 0 to 100% mortality in juveniles occurred after exposure to concentrations between 0 and 302.9 μg L^{-1} microcystins. Therefore, to capture an exposure scenario both environmentally relevant and able to induce (sub-)lethal effects in *D. magna* for proteomics analysis, daphnids from four measured concentrations of 8.1, 15.2, 28.6, and 69.7 μg L^{-1} microcystins plus a blank control (water-only) and a BG-11 media control (0 μg L^{-1}) were collected from this test. Each treatment received 10 replicates, and each replicate received 1 daphnid. Each daphnid was placed in a 50-mL glass tube containing 50 mL of test solution and fed 1.0 mL of total food, subdivided to 0.5 mL *R. subcapitata* and 0.5 mL *C. fusca*, or 1:1 based on cells mL^{-1} mixture of the two algae. Solutions were changed 3 × wk by transferring adult *D. magna* from old to new glass tubes, after which each daphnid was provided 1.0 mL of total food. Mortality within parents and number of neonates were recorded during this time, and is reported in Shahmohamadloo et al. (2019a). Tubes were incubated under 400–800 lx cool-white fluorescent light at 20.0 ± 1 °C with a light:dark cycle of 16:8 h. Water chemistry parameters (temperature, pH, conductivity, dissolved oxygen) were measured at initiation, solution changes, and termination of the test, and is reported in Shahmohamadloo et al. (2019a). On day 14, broods that were < 24 h old were collected from each treatment, washed with distilled water, transferred to a 15-mL tube, and quick-frozen. At test termination on day 21, parents were collected following this same procedure. Parents and neonates were later subjected to protein extraction, followed by proteomic analyses.

2.3. Somatic growth rate

At test termination, triplicate samples of 21-d parent daphnids were randomly selected from each treatment and dried in an oven for 24 h at 70 °C to measure somatic growth rate using the following equation described by Effertz et al. (2015):

$$g = \frac{\ln(dw_t) - \ln(dw_0)}{d}$$

where *dw* is the body dry weight of a subsample of daphnids at the beginning (dw_0) and end (dw_t) of the experiment, and *d* is the length of the experiment in days. This equation was utilized in recent studies (Lyu et al., 2016; Lyu et al., 2019) to characterize the life-history fitness of *D. magna* in the presence of (non-)toxic *M. aeruginosa*.

2.4. Protein preparation

Frozen daphnids were thawed and pooled into 3 replicate groups for each treatment to ensure there was 50 mg of *D. magna* (wet weight) per pool (approximately 5–7 adults and 8–10 neonates) which we had previously determined as the ideal mass of whole daphnids (wet weight) to achieve a total protein concentration of 1 mg mL^{-1}. After, 100 μL of TEAB buffer was added to each pool and the samples homogenized using a Precellys 24 tissue homogenizer (Bertin Instruments, France) and Precellys CK14 – 2 mL soft tissue homogenizing kits. Homogenates were transferred to microcentrifuge tubes and centrifuged for 10 min at 10,000 ×*g* to remove suspended tissue. The liquid supernatents were then transferred to clean low-retention microcentrifuge tubes, and then reduced and alkylated prior to digestion by heating in formic acid using previously described methods (Simmons et al., 2012). Sample digests were then diluted so that the total protein concentration was 2 (± 0.2) mg mL^{-1}. Unlabeled peptide digests were separated and identified using the Waters ionKey/MS microflow LC-MS System tandem to the Xevo G2-XS QTof Quadrupole Time-of-Flight mass spectrometer. Separation was achieved by direct injection onto an iKey Peptide BEH C18 reverse phase separation device (300 Å, 1.7 μm, 150 μm × 50 mm) with a 75 min gradient (0–5 min 5% Solvent B, 5–35 min 5–40% solvent B, 35–45 min 40–60% solvent B, 45–50 min 85% solvent B, 50–75 min 5% Solvent B) using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The data was acquired in positive and sensitivity mode, with the following source settings: capillary voltage = 3.00 kV, sampling cone = 20 kV, and source offset = 80 kV, source temperature = 150 °C, desolvation gas temperature = 350 °C, desolvation gas flow = 135 l/h and cone gas flow = 140 l/h. Peptide spectra were acquired using DDA mode with a survey mass scan of 300–3000 Da. The threshold ion count to trigger tandem mass spectrometry acquisition was 2000. The gain time was 0.5 s, in continuum format, with 20 precursor ions per scan, and a scan rate of 0.5 s for each precursor. Scanning stopped after the accumulated TIC = 100,000 counts or after 5 s. Peak selection priority was based upon charge state: 2 > 3 > 4 > 5 > 6+ with no ions in the "include and exclude" list. Collision energy was ramped for each charge state, 25–40 V for *m/z* 400–1000.

2.5. Database searches and analysis of identified proteins

Spectral data was extracted, filtered, and searched against a Uniprot *D. magna* reference proteome (downloaded March 2018) using PEAKs Studio 8.5 (Bioinformatics Solutions Inc., Waterloo, Canada). Equivalent human ortholog gene symbols for each protein ID were found using a combination of the Uniprot retrieve/ID mapping and BLAST tools. Gene symbols and intensity counts for each sample were then exported from PEAKs for statistical analysis. Uncharacterized proteins were searched in Pfam 32.0 (European Bioinformatics Institute, Hinxton, United Kingdom) to identify the general family of proteins they may belong to.

2.6. Statistical analysis

Somatic growth rate data was tested for normality using the Shapiro-Wilk's test and equality of variance using the Levene's test. When assumptions for normal and equal variance passed, a one-way analysis of variance (ANOVA; $\alpha = 0.05$) was used to determine whether there was a significant difference in the somatic growth rate for *D. magna* among treatments. A Kruskal-Wallis one-way ANOVA on ranks ($\alpha = 0.05$) was employed when assumptions for normality and equal variance failed. When a significant difference between treatments was identified by ANOVA, Tukey's post-hoc test ($\alpha = 0.05$) was performed to compare all treatment means. ANOVAs were performed using Sigma Stat (Version 4.0, Systat Software, San Jose, CA, USA).

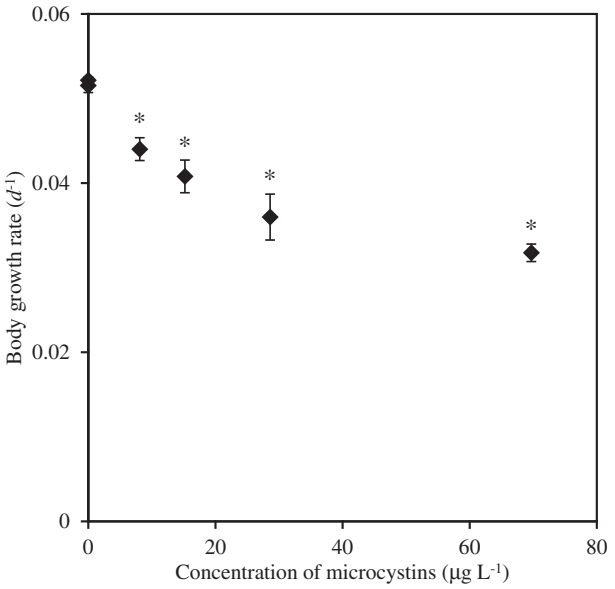

Fig. 1. Somatic growth rate (d^{-1}) of *Daphnia magna* (n = 3, ± SD) exposed to increasing concentrations of cell-bound microcystins (μg L^{-1}) from *Microcystis aeruginosa*. Mean triplicates are designated by a diamond symbol (◆). Significant differences between treatments and the blank control are indicated by an asterisk symbol (*).

Statistical analyses for label-free data (i.e. normalization, fold change, ANOVA, and tests of significance) were performed using Metaboanalyst 4.0, a comprehensive web-based tool for performing data analysis, visualization, and functional interpretation of proteins (Chong et al., 2018). Using this tool we: 1) uploaded peak intensity tables; 2) removed the missing value estimation; 3) filtered data using the interquartile range estimate; 4) normalized data using the median and Pareto scaling to achieve a normal distribution; and, 5) performed parametric statistics (i.e. volcano plots) to determine fold change compared to the blank control for each treatment, with a fold-change threshold of 1.0 and a p-value threshold of 0.1 in order to acquire all the data.

3. Results

3.1. Somatic growth rate in Daphnia magna

The somatic growth rate of *D. magna* was not significantly different between the blank control and the BG-11 media control, indicating the media did not cause growth impairment in parents (Fig. 1). Somatic growth rate, however, was significantly different between the blank control and microcystin treatments (one-way ANOVA, F = 27.320, p < .001), indicating *M. aeruginosa* caused growth impairment in parents exposed to increasing concentrations of microcystins (Fig. 1).

3.2. Proteins detected with label-free proteomics in parents

Using label-free LC-MS/MS quantification and after duplicate genes were removed, 974 proteins were identified, of which 346 were classified as uncharacterized (Supplemental data: Table 1). Among the identified proteins, 32 proteins were significantly different in abundance in at least one microcystins treatment compared to the blank control (Volcano analysis, $p \leq .10$; Fig. 2, Supplemental data: Table 2). Furthermore, 7 proteins were significantly dysregulated within two or more microcystins treatments compared to the blank control, namely: apolipophorin (Apolpp; lipid transport), aspartate aminotransferase (Ast; cellular amino acid metabolic process), di-domain hemoglobin (Ddm; oxygen carrier activity), fibronectin type-III domain-containing protein 3A (Fn3a; fertilization and development), myosin heavy chain (Myh; actin-binding and motor protein), polyubiquitin-B (Ubb; regulatory protein), and vitellogenin fused with superoxide dismutase A0A164EIE6 (Vtg-sod IE6; removal of superoxide radicals). The number of differentially abundant proteins in each of the microcystin exposures for parents were: 0 proteins increased or decreased in the BG-11 media control; 18 proteins increased and 2 proteins decreased in the 8.1 μg L^{-1} microcystins treatment; 15 proteins increased and 5 proteins decreased in the 15.2 μg L^{-1} microcystins treatment; 23 proteins increased and 4 proteins decreased in the 28.6 μg L^{-1} microcystins treatment; and, 15 proteins increased and 3 proteins decreased in the 69.7 μg L^{-1} microcystins treatment (Supplemental data: Table 2). Overall, protein abundance increased in parents as the concentration of microcystins increased. Specifically, 15 proteins increased in abundance over multiple treatments as the concentration of microcystins increased, namely: Ddm (oxygen carrier activity), Dnah17 (motor protein), 2 Fn3a proteins (fertilization and development), Macf1 (actin-binding), Myh (actin-binding and motor protein), and 9 Vtg-sod proteins (removal of superoxide radicals).

3.3. Proteins detected with label-free proteomics in neonates

Using label-free LC-MS/MS quantification and after duplicate genes were removed, 1174 proteins were identified, of which 413 were classified as uncharacterized (Supplemental data: Table 3). Among the identified proteins, 13 proteins were significantly different in abundance in at least one microcystins treatment compared to the blank control (Volcano analysis, $p \leq .10$; Fig. 3, Supplemental data: Table 4). Furthermore, 5 proteins were significantly dysregulated within two or more microcystins treatments compared to the blank control, namely: di-domain hemoglobin (Ddm; oxygen carrier activity), fasciclin-1 (Fas; cell adhesion domain), fibronectin type-III domain-containing protein 3A (Fn3a; fertilization and development), hemoglobin (Hb; oxygen-transport metalloprotein), and e3 ubiquitin-protein ligase HERC2 (Herc2; cellular response to DNA damage). The number of differentially abundant proteins in each of the microcystin exposures for neonates were: 0 proteins increased or decreased in the BG-11 media control; 5 proteins increased and 8 proteins decreased in the 8.1 μg L^{-1} microcystins treatment; 6 proteins increased and 6 proteins decreased in the 15.2 μg L^{-1} microcystins treatment; and, 5 proteins increased and 7 proteins decreased in the 28.6 μg L^{-1} microcystins treatment (Supplemental Data: Table 4). Overall, protein abundance decreased in neonates as the concentration of microcystins increased. Specifically, 5 proteins decreased in abundance over multiple treatments as the concentration of microcystins increased, namely: Aif1 (apoptosis-inducing factor), Egl (cellulase activity), Fas (cell adhesion domain), Fn3a (fertilization and development), and Herc2 (cellular response to DNA damage). Due to mortality in adults from toxicity to the 69.7 μg L^{-1} microcystins treatment, an insufficient number of neonates could be collected for proteomics analysis.

4. Discussion

We ran a life-cycle bioassay on *D. magna* to analyze changes in the proteomic profile of parents and neonates chronically exposed to cell-bound microcystins produced by *M. aeruginosa* at (sub-)lethal concentrations environmentally relevant to freshwaters (0.5 to 300 μg L^{-1}) during harmful algal bloom events. Our investigation into the proteomic profile of *D. magna* parents was further supported through utilizing the somatic growth rate formula to measure their life-history fitness in the face of toxic stress. Results from this study corroborate recent findings (Shahmohamadloo et al., 2019a) that demonstrate cell-bound microcystins can negatively impact the growth rate of *D. magna* parents at (sub-)lethal concentrations and cause reproductive stress (e.g. decline in number and size of offspring). These findings also agree with previous studies (DeMott, 1999; Schwarzenberger et al., 2014; Lyu

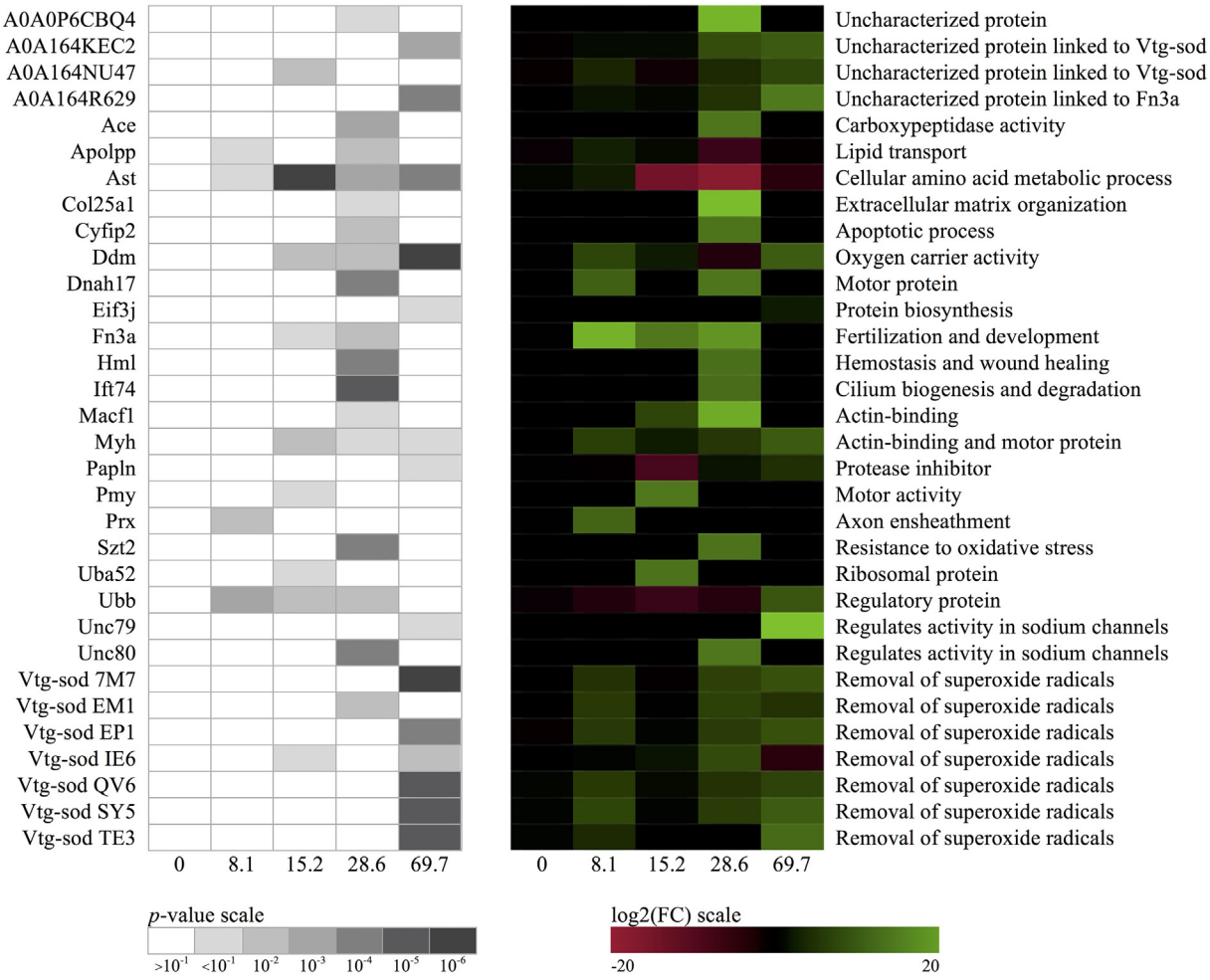

Fig. 2. Label-free proteins in *Daphnia magna* parents with significantly different abundances in at least one treatment of cell-bound microcystins (μg L^{-1}) from *Microcystis aeruginosa*, compared to the blank control (n = 3). Protein symbols are listed in the left column, while protein functions are described in the far right column.

et al., 2016; Lyu et al., 2019) that found a strong correlation between chronic dietary exposure to toxic *Microcystis* and a reduction in the somatic growth rate of *Daphnia*. Seeing that we supplied a 1:1 mixture of *R. subcapitata* and *C. fusca* in the microcystin treatments, and selected a unicellular strain of *M. aeruginosa* to avoid mechanical obstruction from consumption, we suspect the cause of decline in somatic growth rate in *D. magna* was a result of toxic microcystins rather than a lack of nutritious food. The possibility also exists that the passage of a toxic

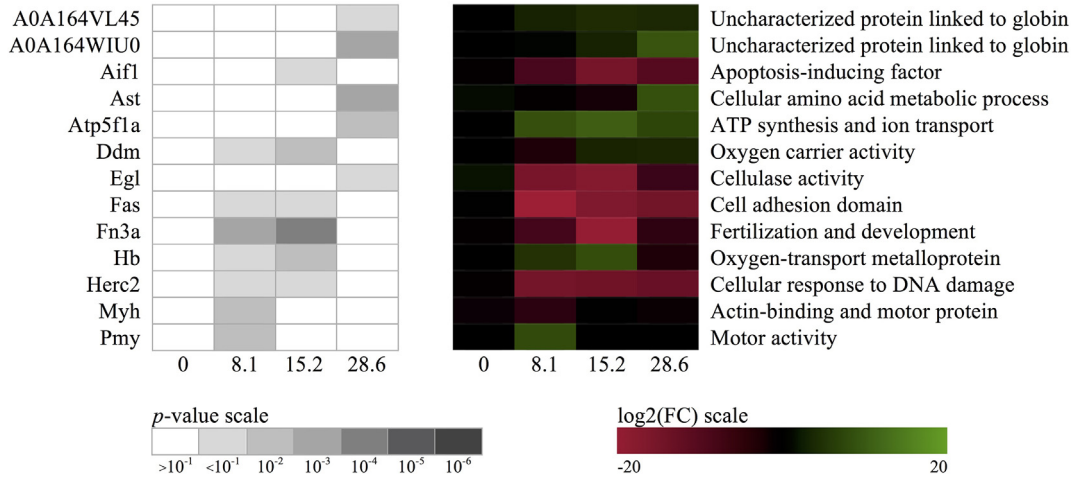

Fig. 3. Label-free proteins in *Daphnia magna* neonates with significantly different abundances in at least one treatment of cell-bound microcystins (μg L^{-1}) from *Microcystis aeruginosa*, compared to the blank control (n = 3). Protein symbols are listed in the left column, while protein functions are described in the far right column.

Microcystis cell through the gut of *Daphnia* can release microcystins into the water through digestive processes, thus mediating effects through waterborne exposure.

In the present study, the use of shotgun proteomics enabled us to see, for the first time, the gradual progression of sub-lethal effects in *Daphnia* chronically exposed to toxic *Microcystis*. The use of shotgun proteomics, however, comes with its advantages and disadvantages. In particular, use of data-dependent acquisition (DDA) mass-spectrometry with shotgun proteomics can result in bias towards higher abundance proteins, and also increased stochasticity in the overall dataset (Hu et al., 2016). We were limited to using DDA in the present study because we did not have access to bioinformatics software capable of searching spectral data captured using data-independent acquisition (DIA). It is likely that this reduced the overall number of proteins that were detected consistently among samples. This lack of consistency may also have affected what you might expect to see in terms of a traditional dose-response at the protein level. Nonetheless, shotgun proteomic methods, no matter the type of data acquisition, do provide an un-biased dataset overall, so that it is possible to identify novel mechanistic information and also conduct high-throughput measurements that increase efficiency over traditional gel-based protein methods.

To expand upon the current understanding of proteomic profile changes in *Daphnia* exposed to freshwater cyanobacteria, we discuss our results for parents and neonates separately. This choice was made to better grasp how *Daphnia* parents respond to chemical stress, and the impact this stress can cause to their offspring.

4.1. Interpretation of differentially expressed proteins in Daphnia magna parents

Evidence has shown microcystins can induce oxidative stress in aquatic organisms, leading to the production of reactive oxygen species, DNA damage, mitochondrial damage, and a decline in antioxidant defense systems (Amado and Monserrat, 2010). In the present study, the majority of significantly expressed proteins in *D. magna* increased in abundance over multiple treatments as the concentration of microcystins increased (Fig. 2, Supplemental Data: Table 2), suggesting parents invested more in growth, development, and survival in the face of toxic stress. The most frequently identified protein in the present study was vitellogenin fused with superoxide dismutase (Vtg-sod), a protein unique to *Daphnia* (Kato et al., 2004). Vitellogenin is the precursor egg yolk protein and plays a key role in the reproductive success and development of most oviparous species (Trapp et al., 2016), while superoxide dismutase is an antioxidant enzyme created to destroy reactive oxygen species (Lyu et al., 2013). Vtg-sod is the most abundant polypeptide in *D. magna* (Kato et al., 2004), and is regarded as a reliable biomarker for investigating the effects of chemicals on endocrine processes during daphnid development (Qi et al., 2018). Peng et al. (2018) recently collected six *D. similoides* clones from Lake Junshan (Jiangxi, China) to study the effects of toxic *M. aeruginosa* on the life history traits (e.g. body length, reproduction, number of offspring, and population growth) and superoxide dismutase activities in mothers and their offspring. Two from these six clones were selected to examine maternal effects. Results from their study demonstrated toxic *M. aeruginosa* significantly inhibited life history traits and increased superoxide dismutase activities in all six *D. similoides* clones. However, for the two clones selected for the maternal effect experiment, life history traits and superoxide dismutase activities in the offspring were varied —some greater and others lesser—than in their mothers. This indicates that developmental responses from neonates to maternal cues are clone-specific, with the possibility that mothers exposed to toxic *M. aeruginosa* can either enhance or decrease neonate fitness and ability to adapt in the face chemical stress. Similarly, Orsini et al. (2018) observed up-regulation of Vtg-sod in *D. magna* exposed to toxic *M. aeruginosa* in a study that characterized the early transcriptional response of daphnids to environmental perturbations, including biotic and abiotic stressors. Orsini et al. (2018) also discovered that approximately one-third of *Daphnia* genes —linked to metabolism, cell signaling, and stress response— drive early transcriptional response when faced with environmental stress, and is shared among genotypes (Orsini et al., 2018). In the present study, we identified 7 characterized Vtg-sod proteins and 2 uncharacterized proteins linked to Vtg-sod —the highest number of Vtg-sod proteins recorded to date in *D. magna*, all of which were significantly abundant in the presence of toxic *M. aeruginosa* at various concentrations. Our findings corroborate previous literature (Peng et al., 2018; Qi et al., 2018), and in particular Orsini et al. (2018) since we observed elevated expressions of Vtg-sod in treatments of increasing concentrations of microcystins, suggesting condition-specific responses in *D. magna* with a strong genotype-by-environment interaction.

The second set of proteins that significantly increased in abundance in *D. magna* exposed to microcystins concern fertilization and development, namely fibronectin type-III domain-containing protein 3A (Fn3a). Fibronectins are modular proteins that are typically expressed in humans and animals, and serve an important role in cell adhesion, growth, migration, and differentiation, as well as contributing to processes including wound healing, spermatogenesis, and embryonic development (Pankov and Yamada, 2002; Obholz et al., 2006). Fibronectins comprise three prototypical types of domains known as types I, II, and III (Petersen et al., 1983). Fibronectin type-III (Fn3) are both the largest and most common of the subdomains, and have been found in cell-surface receptors, enzymes, and muscle proteins (Bork and Doolittle, 1992). The amplification or increased expression of Fn3 has been associated with the formation of cancer, however the mechanistic functions of these proteins are not yet fully understood (Syed et al., 2019). Boucher et al. (2010) described the connection of Fn3 proteins in *Daphnia* to the insulin signaling pathway, which serves an importance function in carbohydrate metabolism and growth regulation. In a DNA microarray study, Hudder et al. (2007) also found altered profile expression of fibronectin genes in mice that received microcystin-LR via intraperitoneal injection at sub-lethal concentrations. In the present study, we identified 1 characterized Fn3 protein and 1 uncharacterized protein linked to Fn3. Given the limited information on Fn3 detection and expression in invertebrates, we suspect an increase in Fn3a expression in *D. magna* may be associated with a metabolic response to exposure of cell-bound microcystins.

The third set of proteins significantly expressed in *D. magna* concern the cytoskeleton, namely axonemal dynein heavy chain 17 (Dnah17), microtubule-actin cross-linking factor 1 (Macf1), myosin heavy chain (Myh), and paramyosin (Pmy). Dnah17 are cytoskeletal motor proteins of eukaryotic cells that convert energy derived from adenosine triphosphate hydrolysis (ATP) into force and move along microtubules in cells (Oiwa and Sakakibara, 2005). Between different cytoskeletal elements are bridges formed by Macf1, a family of proteins that play a role in cross-linking actin to other cytoskeletal proteins and binding to microtubules, which serve a key role in wound healing and epidermal cell migration (Amish et al., 2019). Myh and Pmy are motor proteins, abundant in the muscle thick filaments of invertebrates that are ATP-dependent and responsible for actin-linked motility (Matsumoto et al., 1988; Wells et al., 1996). Taking into account the abovementioned information on the third set of proteins, we noted a decrease in motor activity, and in some cases immobilization as early as 48 h in *D. magna* parents exposed to higher concentrations of toxic *M. aeruginosa* (data not shown). Our findings give support to an earlier study performed by Rohrlack et al. (2005), who exposed *Daphnia galeata* to cell-bound microcystins and recorded visible disturbances in neuromuscular communication (i.e. decline in the movement of thoracic legs, mandibles, foregut, and second antennae, and a rise in gut muscle activity). Microcystins are known to cause cytoskeleton disruption (Wickstrom et al., 1995) by inhibiting protein phosphatases 1 and 2A, which can cause hyperphosphorylation of the cytoskeletal protein tubulin (Lyu et al., 2016). Lyu et al. (2016) witnessed an up-regulation of tubulin

protein in *D. magna* exposed to *M. aeruginosa*, suggesting microcystins can affect quantitative changes in microtubules, which are considered the building blocks of tubulins and are responsible for movement in eukaryotic cells. Our findings give support to Lyu et al. (2016) by identifying and demonstrating a significant increase in abundance of Dnah17, Macf1, Myh, and Pmy in *D. magna* exposed to toxic *M. aeruginosa* at (sub-)lethal concentrations. Lyu et al. (2016) also describe a decrease in the abundance of trypsin-like serine proteases, an enzyme related to amino acid metabolism in *D. magna* that is a major protein in the digestive system of *Daphnia* gut and thought to defend against the cytotoxicity of microcystins. Interestingly, in the present study we observed a significant decrease in abundance of aspartate aminotransferase (Ast), an important enzyme in amino acid metabolism responsible for transferring an α-amino group between aspartate and glutamate. This further supports the possibility that exposure to toxic *M. aeruginosa* will impact cellular amino acid metabolism in *D. magna*.

The present study presents some potential interference with signals in the proteomics analyses which could have complicated dose-response that we would like to address. Firstly, since we worked with whole organisms, and *Daphnia* are parthenogenetic and have short generation life cycles, it is possible that some parents had brood pouches containing neonates, thus raising the potential for contributing signal in the proteomics analysis. While this is possible, the amount of signal contributed from neonates was likely negligible since there were no significant expressions of proteins in parents that matched with proteins expressed in neonates. Secondly, we further raise the possibility that *M. aeruginosa* cells may have been in the gut of *Daphnia*, thus raising the potential for proteins other than Daphnia to have been included in our analysis. While it is clear that most identified proteins are unlikely to be algal in nature, we conducted a BLAST search using NCBI protein BLAST to determine the similarity of our significantly different proteins identified in *D. magna* against those of *M. aeruginosa*. We found that three proteins, ubiquitin-60S ribosomal protein L40 (Uba52), polyubiquitin B (Ubb), and ATP synthase subunit alpha (Atp5f1a) had > 50% sequence similarity to *M. aeruginosa proteins*. This raises the potential that the significant expression of Uba52 in the 15.2 μg L^{-1} microcystins treatment and Ubb in the control of adults and increased expression of Atp5f1a in the neonates exposed to all concentrations of microcystins was due to the presence of *M. aeruginosa* in the gut. However we think that contributions from *M. aeruginosa* would be negligible since the abundance of those proteins would likely be much lower than our detection limit and that the false discovery rate and match scores would have been too low to pass our protein search filters. Finally, we raise the possibility that *D. magna* in the highest microcystins treatments may have been nutritionally better off than daphnids fed fewer *M. aeruginosa* cells due to higher organic carbon content. While this is possible, results from the present study measuring the somatic growth rate, and results from our previous work on mortality and reproduction (Shahmohamadloo et al., 2019a) demonstrate the size of parents and neonates decreased as the concentration of microcystins increased. Thus, it does not appear daphnids were nutritionally better off than those that were fed fewer *M. aeruginosa* cells. Overall, evidence from the present study suggests the presence of toxic *Microcystis* at sub-lethal levels of exposure can induce impairment in *Daphnia* parents, compromising the embryonic development of neonates.

4.2. Interpretation of differentially expressed proteins in Daphnia magna neonates

While the majority of significantly expressed proteins in *D. magna* parents increased in abundance over multiple treatments as the concentration of microcystins increased (Fig. 3; Supplemental data: Table 4), the reverse was observed for neonates, suggesting parents invested less towards the growth, development, and survival of their offspring in the face of toxic stress. This observation is in conformity with a previous study conducted by our research group where a decline in length of neonates was observed as the concentration of cell-bound microcystins increased (Shahmohamadloo et al., 2019a). Given that filter mesh size in *Daphnia* change as a function of size, it is possible that neonates could also observe toxicity related to parental exposure and/or waterborne exposure. The present study purposefully selected a strain of *M. aeruginosa* that produces cells between 1 and 2 μm in size to witness as best as possible toxic effects from dietary exposure. We measured a significant decrease in abundance of apoptosis-inducing factor 1 (Aif1), endoglucanase (Egl), fasciclin domain-containing protein (Fas), e3 ubiquitin-protein ligase HERC2 (Herc2), and Fn3a. We hereby explain four major adverse outcomes that could result from perturbations in these proteins, each protein's functional significance within invertebrates, and the implications from exposure to toxic *Microcystis*.

Firstly, mitochondria serve a key role in regulating apoptosis, and contain several proteins such as apoptosis-inducing factor (Aif) to facilitate this process (Susin et al., 1999). Deficiency in Aif causes severe mitochondrial dysfunction, producing muscle atrophy and neurodegeneration in organisms (Bano and Prehn, 2018). Chen et al. (2005) sought to determine how extra-cellular microcystins could affect mitochondria by exposing *D. magna* for 21 d to concentrations ranging from 0 to 2000 μg L^{-1}. In their study, no harmful effects or mortality were seen at low concentrations, however mitochondria were broken and blurry in the alimentary canal and epidermis of *D. magna* exposed to the highest microcystin concentration. To some degree, our results contrast the work of Chen et al. (2005) given we observed toxicity at sub-lethal concentrations far lower than 2000 μg L^{-1}. As the main route of microcystin exposure is generally accepted to be via ingestion of viable *Microcystis* cells (Ferrão-Filho et al., 2014; Lyu et al., 2016; Lyu et al., 2019; Shahmohamadloo et al., 2019a), mitochondrial damage may in fact be observed at lower, environmentally relevant concentrations such as those from the present study. Nonetheless, the molecular changes related to mitochondrial dysfunction —and decrease in abundance of Aif1— observed in the present study support the observations of Chen et al. (2005), suggesting neonates were stressed for survival in the presence of toxic *Microcystis*.

Secondly, fasciclin domain-containing protein (Fas) serves a key role in *Daphnia* by promoting cell adhesion, development, immune response, and apoptosis (Toumi et al., 2014). Toumi et al. (2014) measured proteomic changes in *D. magna* exposed to deltamethrin (pyrethroid insecticide), and revealed down-regulation of Fas and a subsequent promotion of apoptosis. Our observation that Fas decreased in abundance suggests the possibility of increased apoptosis in *D. magna* neonates.

Thirdly, gluconeogenesis serves a key role in providing energy to *Daphnia* by synthesizing glucose through utilizing endoglucanase (Egl) to breakdown cellulose, glycogen synthase, and glucose-6-phosphate (Connon et al., 2008). Connon et al. (2008) performed DNA microarrays to link molecular and population stress responses in *D. magna* exposed to cadmium, and found dysregulation in Egl activity linked to a reduction in somatic growth rate, ecdysis (i.e. moulting) and development. Lyu et al. (2019) similarly observed a significant decrease in number of moultings in a sensitive clone of *D. similoides* exposed to toxic *M. aeruginosa*, suggesting cell-bound microcystins interferes with the moulting cycle. Our findings support those of Connon et al. (2008) and Lyu et al. (2019) by measuring a significant decrease in abundance of Egl in *D. magna*, suggesting microcystins (i.e. cell-bound consumption, parental exposure, or waterborne exposure) interfered with metabolic processes, energy production, and moulting in neonates.

Fourthly, Herc2 serves a key role in cellular functions including DNA damage repair, cell growth, immune response, and neurological disorders (Sánchez-Tena et al., 2016). In mice, Herc2 was identified to be responsible for a syndrome known as runty, jerky, sterile (rjs) or juvenile development and fertility-2 (jdf2), which is marked by a reduction in viability, small size, sterility, and neuromuscular defects (Lehman et al., 1998; Walkowicz et al., 1999; Sánchez-Tena et al.,

2016). Herc2 is evolutionary highly conserved (i.e. its gene sequence has remained unchanged throughout natural selection) and shows between 41 and 62% identity between human and *Daphnia*, which is considered to be a high degree of homology between mammalian and invertebrate species (Ji et al., 2000; Uniprot, 2019). In the present study, the significant decrease in abundance of Herc2 indicates *D. magna* have reduced DNA repair and immunity, and potentially neurological effects, in the presence of sub-lethal concentrations of toxic *Microcystis*. This offers a possible explanation for the fifth protein, Fn3a, which, in contrast to *D. magna* parents, was significantly decreased in the presence of toxic *Microcystis*. Our results suggest *D. magna* neonates experienced a reduction in important functions related to carbohydrate metabolism, fertilization and development. Overall, evidence from the present study suggests *Daphnia* neonates can survive in the presence of toxic *Microcystis* at sub-lethal levels of exposure, at the expense of key functions related to growth and development of its own and, potentially, future generations.

Among the differentially regulated proteins was hemoglobin, which increased in abundance in the 15.2 μg L^{-1} microcystins treatment and decreased in abundance in the 28.6 μg L^{-1} microcystins treatment. Given the well-described association between the generation of harmful algal blooms and hypoxia, it is important to address whether an the *Daphnia* proteome was an indirect response to *Microcystis* density rather than a direct effect from microcystins. In the present study, dissolved oxygen levels maintained between 9.0 and 10.0 mg L^{-1} within treatments and the controls throughout the duration of the experiment (full data is available in Shahmohamadloo et al., 2019a). The stability in dissolved oxygen levels was likely due to the frequent solution changes that occurred during the test. Therefore, we have reason to believe that the fluctuating effects seen in hemoglobin were a result of microcystins exposure.

5. Conclusions

Through the application of shotgun proteomics, we identified proteins in *D. magna* parents and neonates that were significantly expressed in the presence of cell-bound microcystins at realistic concentrations to freshwater ecosystems. Our findings were further supported by assessing the somatic growth rate of *D. magna*, which demonstrated a significant decrease in the fitness of parents chronically exposed to toxic *Microcystis*. In *D. magna* parents, we identified a significant increase in abundance of proteins with key functions related to reproductive success and development, removal of superoxide radicals, and motor activity. These findings suggest parents' invest in growth, development, and survival when in the presence of cell-bound microcystins. In *D. magna* neonates, we identified the opposite —a significant decrease in abundance of proteins with key functions related to apoptosis, metabolism, DNA damage repair, and immunity. These findings suggest neonates have decreased ability to support key functions related to their own growth and development in the presence of cell-bound microcystins. Given the stress responses observed by *D. magna* in the present study, the potential exists for cell-bound microcystins to influence food web dynamics in freshwater ecosystems that experience harmful algal blooms dominated by *Microcystis*. Future work in the area of environmental toxicogenomics is needed to characterize the effects of dietary exposure and accumulation of microcystins in *Daphnia* over multiple generations, to assess whether the protein responses observed in the present study can be repaired or brought back to normal in *Daphnia* after exposure to a toxic *Microcystis* bloom.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

Funding provided for R.S. Shahmohamadloo and this project through an NSERC CREATE (2013-432269) program in Multiple Stressors and Cumulative Effects in the Great Lakes, a Canada-Ontario Agreement (2218) through the Ontario Ministry of the Environment, Conservation and Park, and a research grant from the Ontario Federation of Anglers and Hunters. We thank staff at the Aquatic Toxicology Unit (Ontario Ministry of the Environment, Conversation and Parks, Canada) for their technical expertise and support.

Author contributions

R.S.S., D.B.D.S., and P.K.S. conceived and designed the experiment. R.S.S. collected the data. R.S.S. and D.B.D.S. analyzed the data. R.S.S. wrote the paper. All authors read, amended, and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbd.2020.100656.

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