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2021

Cell free *Microcystis aeruginosa* spent medium affects *Daphnia magna* survival and stress response

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Abstract: Primary consumers in freshwater ecosystems, such as the zooplankton organism *Daphnia magna*, are highly affected by cyanobacteria, both as they may use it as a food source but also by cyanobacterial metabolites present in the water. Here, we investigate the impacts of cyanobacterial metabolites focussing on the environmental realistic scenario of the naturally released mixture without crushing cyanobacterial cells or their uptake as food. Therefore, *D. magna* were exposed to two concentrations of cell free cyanobacterial spent medium from *Microcystis aeruginosa* PCC 7806 to represent higher and lower ecologically-relevant concentrations of cyanobacterial metabolites. Including microcystin-LR, 11 metabolites have been detected of which 5 were quantified. Hypothesising concentration and time dependent negative impact, survival, gene expression marking digestion and metabolism, oxidative stress response, cell cycle and molting as well as activities of detoxification and antioxidant enzymes were followed for 7 days. *D. magna* suffered from oxidative stress as both catalase and glutathione S-transferase enzyme activities significantly decreased, suggesting enzyme exhaustibility after 3 and 7 days. Moreover, gene-expressions of the 4 stress markers (glutathione S-transferase, glutathione peroxidase, catalase and thioredoxin) were merely downregulated after 7 days of exposure. Energy allocation (expression of Glyceraldehyde-3-phosphate dehydrogenase) was increased after 3 days but decreased as well after 7 days exposure. Cell cycle was impacted time dependently but differently by the two concentrations, along with an increasing downregulation of myosin heavy chain responsible for cell arrangement and muscular movements. Deregulation of nuclear hormone receptor genes indicate that *D. magna* hormonal steering including molting seemed impaired despite no detection of microviridin J in the extracts. As a consequence of all those responses and presumably of more than investigated molecular and physiological changes, *D. magna* survival was impaired over time, in a concentration dependent manner. Our results confirm that besides microcystin-LR, other secondary metabolites contribute to negative impact on *D. magna* survival and stress response.

Keywords: zooplankton; cyanobacteria; secondary metabolites; PCC7806; oxidative stress; transcriptomics

Abbreviations

MC-LR: microcystin-LR
des-MC-LR: des-microcystin-LR
CP: cyanopeptolin
AC: aerucyclamide
CP-A: cyanopeptolin A
AC-A: aerucyclamide A
AC-D: aerucyclamide D
LC: cyanobacterial spent medium diluted with the BG11 in the ratio 1:400
HC: cyanobacterial spent medium diluted with the BG11 in the ratio 1:40

Acknowledgements

We are grateful to D. Azam and M. Coke from the PEARL INRA 1036 U3E system (The National Infrastructure in Biology and Health, France) for providing the *Daphnia magna* strain (originating from l'INERIS) and Bertrand Le Rouzic (University of Rennes 1) for providing us with the *Scenedesmus communis* culture (originating from the lake of Grand Lieu). We are thankful to Sophie Michon-Coudouel and Virginie Daburon who performed genetic analyses at the molecular ecology platform (UMR 6553 Ecobio, Rennes, CNRS/UR1), as well as Marion Chorin from Plateforme EcoChimie (EcoChim, UMS OSUR 3343) for help with enzyme analysis. We would like to thank Len Montgomery (Robert Gordon University) for proofreading the manuscript.

Funding

Gorenka Bojadzija Savic was supported by a Presidential scholarship from the University of Rennes 1. The project benefited from funding from the Centre National de la Recherche Scientifique (CNRS) Initiative Structurante Ecosphère continentale et côtière (EC2CO) under the Interactions métaboliques entre cyanobactéries et daphnies (MICYDA) project. Gorenka Bojadzija Savic was granted a mobility grant from the Doctoral School EGAAL (Bretagne-Loire, France) to visit the Robert Gordon University in Aberdeen, Scotland for metabolites analyses.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

1. INTRODUCTION

Due to eutrophication in freshwater ecosystems, cyanobacterial proliferation frequently results in blooms that potentially produce bioactive or toxic metabolites that harm the environment and humans (Heisler *et al*., 2008). *Microcystis* is one of the most widespread cyanobacterial genus in freshwaters, with *Microcystis aeruginosa* being one of the most commonly detected and investigated (Svirčev *et al*., 2019). Like other cyanobacteria, *Microcystis* produces a diverse range of secondary metabolites that have been shown to impact life traits and physiology of aquatic organisms including zooplankton such as *Daphnia* (Lürling and van der Grinten, 2003; Merwe and Sebbag, 2012). One of the most detected toxins produced by cyanobacteria is microcystin (MC), with more than 250 variants described so far (Mowe *et al*., 2015; Svirčev *et al*., 2019). Microcystins change the phosphorylation state of proteins by inhibiting 1 and 2A protein phosphatases, thus disrupting pathways involving phosphorylation (MacKintosh *et al*., 1990; Trinkle-Mulcahy and Lamond, 2006; Zurawell *et al*., 2005). Furthermore, microcystins trigger oxidative stress in aquatic organisms (Amado and Monserrat, 2010).

Besides microcystins, *Microcystis sp.* produces a wide range of intracellular and extracellular secondary metabolites such as aeruginosins, cyanopeptolins, cyclamides, microginins and microviridins (Welker and Von Döhren, 2006), that have various negative effects on *Daphnia* physiology (Rohrlack *et al*. 2001, Bister *et al*. 2004, Ishida *et al*. 2007, von Elert *et al*. 2012). Similar to microcystins, cyanopeptolins (CP) are synthesized through non-ribosomal metabolic pathways, by non-ribosomal peptide synthetases (NRPSs) and polyketide synthase (PKS) (Welker and Von Döhren, 2006). CP are widely distributed and diverse compounds with more than 82 variants described so far (Gademann *et al.* 2010). Aeruginosins are synthesized non-ribosomally and they can inhibit trypsin-type serine proteases (Ishida *et al.* 2000, 2007). CPs and aeruginosins are potent inhibitors of the serine proteases trypsin and chymotrypsin, that are among the main digestive enzymes in *Daphnia* (von Elert *et al*. 2005; Gademann and Portmann 2008; Elkobi-Peer *et al*. 2013). Microviridins are one of the largest oligopeptides produced in cyanobacteria, with 13 to 14 amino acids (1600-1900 Da), contrasting to the other groups, they are ribosomally synthesized tricyclic depsipeptides. They are produced by different cyanobacterial genera, including *Microcystis* sp., however, the natural

diversity of microviridins is still unknown (Gatte-Picchi *et al*., 2014; Ziemert *et al*., 2010). Microviridin J has been identified as causing fatal molting disruption in *Daphnia pulicaria* via inhibition of *Daphnia* proteases, thus leading to the death of the animals (Kaebernick *et al*., 2001; Rohrlack *et al*., 2004). Cyclamides are cyclic hexapeptides with cytotoxic properties to crustacea (Ishida *et al*., 2000; Portmann *et al*., 2008). Cyanopeptides can occur in high frequency comparable to MC concentrations in aquatic environments and thus may pose problems for drinking water purification from surface water reservoirs (Beversdorf *et al*., 2018, 2017; Janssen, 2019; Natumi and Janssen, 2020). Concentrations of cyanopeptolines (< 7 ug/L^{-1}), anabaenopeptins (< 1 ug/L^{-1}) and microginins (< 1 ug/L^{-1}) have been reported in surface waters (Beversdorf *et al*., 2017), however, more research is needed regarding their diversity and concentration in aquatic environments. Furthermore, their effect on organisms as a single compound or in a mixture are not investigated in detail.

Nevertheless, it has been suggested that in natural conditions some oligopeptides may be even more harmful than MCs and other cyanotoxins to certain zooplankton species, in particular as digestion inhibitors are active at much lower concentrations than the classical cyanotoxins (Von Elert *et al*., 2004). Digestive enzyme inhibition would cause starvation, impair growth and reproduction, eventually leading to slow death (Von Elert *et al*., 2004).

Among the freshwater zooplankton species, *Daphnia* graze on phytoplankton including cyanobacteria, thereby connecting the primary production to the consumers within the aquatic food web (Ger *et al*., 2016). Dominance of nutritionally inadequate phytoplankton, such as cyanobacteria, which lack important sterols and fatty acids necessary for *Daphnia* growth and development, can represent an obstacle in efficient carbon transfer to higher trophic levels (Martin-Creuzburg *et al*., 2008). During their lifetime, however, *Daphnia* are able to develop tolerance to cyanobacterial metabolites, as a physiological response to bioactive compounds (Gustafsson and Hansson, 2004; Ortiz-Rodríguez *et al*., 2012; Sarnelle and Wilson, 2005). Several mechanisms have been described so far, such as *Daphnia's* ability to remodel their digestive enzymes (Schwarzenberger *et al*. 2012, von Elert *et al*. 2012), or increase of antioxidant and biotransformation enzyme activity (Dao *et al*., 2013; Ortiz-Rodríguez and Wiegand, 2010; Sadler and von Elert, 2014a). When exposed to cyanobacterial metabolites that are chymotrypsin and trypsin inhibitors, *Daphnia* switch to

chymotrypsins and trypsin isoenzymes with smaller molecular mass, which are less inhibited (Schwarzenberger *et al*. 2012, von Elert *et al*. 2012). The antioxidative enzymatic defenses include catalase (CAT), superoxide dismutase (SOD), glutathione-peroxidase (GPx), while glutathione S-transferase (GST) is the detoxification mechanism (Ighodaro and Akinloye, 2018; Pflugmacher *et al*., 1998). *Daphnia* can reduce oxidative stress caused by cyanobacterial metabolites by increasing CAT activity (Ortiz- Rodríguez and Wiegand, 2010; Wojtal-Frankiewicz *et al*. 2014). Similar increased activity of antioxidant SOD and CAT enzyme providing cellular protection against ROS is found in a study where *Daphnia magna* was fed with *Microcystis* diet (Lyu *et al*., 2016b). *Daphnia* can reduce the toxicity of microcystin by increasing the activity of GST catalysing the biotransformation via conjugation to glutathione (Dao *et al*. 2010; Ortiz-Rodríguez *et al*., 2012; Miles *et al*. 2016).

Furthermore, studies in *Daphnia* have shown that cyanobacterial metabolites can affect expression of genes involved in digestive system (Asselman *et al*., 2014; Drugă *et al*., 2016; Schwarzenberger *et al*., 2012), cell cycle (De Coninck *et al*., 2014b; Giraudo *et al*., 2017; Tong *et al*., 2017), and oxidative stress (De Coninck et al., 2014b; Lyu et al., 2016a; Rhiannon et al., 2011). However, characterizing transcriptional responses of *Daphnia* to cyanobacterial toxins so far focussed merely on effect of microcystin or cyanobacterial media without considering other bioactive molecules that might be present apart from microcystin (Asselman *et al*., 2012; De Coninck *et al*., 2014a; Drugă *et al*., 2016; Lyu *et al*., 2016b).

Besides being affected by grazing on cyanobacteria, zooplankton can also be affected by naturally produced cyanobacterial metabolites released in water (Barrios *et al*., 2015; Ferrão-Filho *et al*., 2014; Smutná *et al*., 2014). Nevertheless, most of the studies investigate impact of cyanobacteria on *Daphnia* grazing on them, or exposure to extracts obtained by crushing cyanobacterial cells (from a culture or from a field sample), or to purified compounds (Dao *et al*., 2013; Esterhuizen-Londt *et al*., 2016; Peng *et al*., 2018). Hence, it is of interest to investigate if *Daphnia* are impaired by the presence of cyanobacterial metabolites naturally released during cyanobacterial normal growth, without necessarily feeding on them, or crushing cells. Furthermore, knowing the composition and concentration of metabolites present in the medium, naturally released as a product of cyanobacterial metabolic activity, would provide further understanding of *Daphnia's* response to cyanobacterial metabolites.

Therefore, the aim and novelty of this study is *Daphnia* exposure to cell free cyanobacterial spent medium obtained from a two weeks culture of exponentially growing *M. aeruginosa*, containing metabolites naturally released during that time. In addition, we detected and quantified cyanobacterial metabolites present in the medium, beside commonly investigated microcystin. We monitored markers of stress response in order to predict *Daphnia* physiological phenotypes as a response to cyanobacterial spent medium. We analyzed the effects of cell free cyanobacterial spent medium that contained a quantified mixture of compounds on i) *Daphnia* survival, ii) activity of enzymes involved in oxidative stress and detoxification and iii) expression of candidate genes involved in digestion, growth and development, detoxification and oxidative stress response. We hypothesize a negative impact on *Daphnia* survival, a trigger of CAT and GST activities, as well as expression of genes involved in *Daphnia* growth and development, digestion, detoxification and oxidative stress.

2. MATERIALS AND METHODS

2.1. Culture conditions

2.1.1. Microcystis aeruginosa PCC 7806

An axenic microcystin (MC) producing *M. aeruginosa* strain PCC 7806 was obtained from the Pasteur Culture collection of Cyanobacteria in Paris, France (https://research.pasteur.fr/en/team/collection-of-cyanobacteria/). The strain was grown in 100% cyanobacterial BG11 medium (SIGMA), under a 14h:10h light:dark regime using daylight white fluorescent tubes (Toshiba, 15 W, FL15D) with 20 μmol photons m^{-2} s^{-1} illumination at a constant temperature of 20±1°C (Sanyo incubator). The culture was maintained in exponential growth phase, while the axenicity was regularly evaluated as described in (Briand et al., 2012).

2.1.2. Daphnia magna

The *D. magna* clone was obtained from the PEARL INRA 1036 U3E. As the exposure to cyanobacteral metabolites was realized via *M. aeruginosa* PCC 7806 spent medium, the *D. magna* clone was acclimated to BG11 medium (SIGMA). Before performing experiments we compared osmolarity of BG 11 medium with the osmolarity of commonly used Artificial *Daphnia* medium, Elendt M4 and Elendt M7. Osmolarity of BG 11 medium was similar to that of these *Daphnia* media, thus not affecting *Daphnia* survival. During the first three weeks *D. magna* were slowly acclimated and adjusted to the cyanobacterial BG 11 medium that was used in all the experiments, by gradually increasing the % of BG11 medium until it reached 100% in the end of the acclimation period. After the acclimation period *D. magna* were grown in 100% of BG11 medium for a month, before neonates (< 36h old) were used in the experiment. During the acclimation and cultivation period no irregularities in *Daphnia* survival, eating, movement and growth were noticed, suggesting that *D. magna* were successfully acclimated to the medium (Bojadzija Savic *et al*., 2020). *D. magna* were grown and cultivated in the aquarium at a constant temperature of 20°C, light intensity of 15 μmol photons m^{-2} s^{-1} having a day/night cycle of 14h:10h (Sanyo MIR 154) and daily renewal of

20% of the medium. *Daphnia* were fed daily with a fresh sub-culture of the green algae *Scenedesmus communis* originating from lake Grand Lieu, France, that was isolated in our laboratory (University of Rennes 1).

Sufficient food that 100 *Daphnia* (According to OECD guidelines for testing of chemicals 202) that should be available daily in the medium is equivalent to 2.8 - $5.6x10^8$ cells/mL of *S. communis*. *Daphnia* are usually maintained in medium that is not appropriate for algal growth (Elendt M4, Elendt M7- OECD guidelines for testing of chemicals), therefore when *S. communis* are introduced to *Daphnia* medium, they are consumed before growing in high densities. However, in our cultures *Daphnia* were adjusted to BG11 medium that was also used for *S. communis* culture. Hence, it allowed *S. communis* to grow and reach high densities (10x higher than the initial one, within one week) while being consumed by *Daphnia*. As high density of *S. communis* disrupts normal *Daphnia* functioning; its concentration had to be adjusted to 2.8 - $5.6x10^7$ cells/mL to maintain equilibrium between growth and consumption. In order to keep the *S. communis* culture in more accessible form for consumption, in particular by neonates (i.e. unicellular /in pairs instead of four cells having long spikes), *Scenedesmus* culture was diluted every few days before feeding.

2.2. Experimental design

All experiments were performed in 5 replicates in 2 L aquariums and lasted for 3 and 7 days. All *Daphnia* were fed daily with *S. communis*, whose density was monitored and adjusted daily. For the control, 150 *D. magna* neonates (< 36h old) per aquarium were raised in BG11.

For the treatment, the cyanobacterial spent media was prepared in the following way: from an exponentially growing *M. aeruginosa* PCC 7806 culture, centrifuged cells were transferred in fresh sterile BG 11 medium to remove extracellular metabolites. From the pellet, a cyanobacterial culture of $2x10^5$ cells/mL initial density was grown for two weeks, reaching a cell density of $1x10^6$ cell/mL. From that culture, cell free cyanobacterial spent medium was collected by filtering through 0.2 µm sterile cellulose nitrate filter. *D. magna* were exposed to two concentrations of cyanobacterial spent medium: a) diluted with the BG11 in the ratio 1:40 (thereafter called HC for high concentration) and b) 1:400 (thereafter

called LC for low concentration). These 2 dilutions provided environmentally relevant concentrations of MC-LR in our experiments (final concentration of MC-LR: LC: 0.5 µg/L, HC: 5 µg/L), as dissolved MC ranges in the environment between 0.2 and 11 µg/L (Lahti *et al*., 1997; Pawlik-Skowrońska *et al*., 2008; Rastogi *et al*., 2015; Su *et al*., 2015).

D. magna neonates (150 per treatment) were exposed to 2 L of these two concentrations. At the end of the exposure (3 days and respectively 7 days), *D. magna* samples (biotransformation, oxidative stress, energetic profiles) were taken by collecting them over a mesh, briefly rinsed with fresh BG11 media, and after removal of the excess media, snap frozen in liquid nitrogen and stored at -80°C until analysis.

2.3. Cyanobacterial secondary metabolites analysis

Cyanobacterial secondary metabolites previously known to be produced by this particular strain PCC7806 (Briand *et al*., 2016; Rohrlack *et al*., 2004; Sadler and von Elert, 2014b) were monitored after 2 weeks growth phase from initially $2x10^5$ cells/mL to $1x10^6$ cells/mL. Cyanobacterial cells were separated from the supernatant by centrifugation. Cell free spent medium was filtered through 0.2 µm filter and 1 mL was lyophilized. Extraction of lyophilized material was done in 0.5 ml 50% methanol and processed as described in Bojadzija Savic *et al*., 2019. Waters Acquity Ultra-High Performance Liquid Chromatography coupled to a Xevo quadrupole time of flight mass spectrometer was used for the metabolites analysis. Cyanobacterial peptides were detected using extracted ion chromatograms for the respective specific masses of the different compounds (Bojadzija Savic *et al*., 2019). Microcystin-LR (MC-LR), Microcystin-des-LR (des-MC-LR), cyanopeptolin A (CP-A), and aerucyclamide A (AC-A) and D (AC-D) were quantified using linear relationship between peak area (MC-LR and des-MC-LR at 238 nm, CP-A at 220 nm, and AC-A at 237 nm and AC-D at 240 nm) and known concentrations of the toxin standards. The microcystin-LR standard was purified as previously described (Edwards *et al*., 1996). CP-A standard and AC-A and AC-D standard were purified using preparative HPLC (Biotage Parallex Flex, Cardiff, UK) and Flex V3 software for instrument control and data acquisition as described in (Bojadzija Savic *et al*., 2020). Detection and quantification of the cyanobacterial peptides was done by MassLynx v4.1 software.

2.3. *D.magna* survival

Survival of *D. magna* was monitored daily, by counting and removing dead *Daphnia* (identified as not moving and decaying *Daphnia* on the bottom of the aquarium) with results expressed in percentages of total individuals at the start of the exposure.

2.4. Enzyme extraction and measurement

Fifty *Daphnia* were resuspended and homogenized in 1 mL of ice cold extraction buffer (0.1 M phosphate buffer pH 6.5, glycerol, 1 mmol EDTA, and 1.4 mmol dithioerythritol) using Lysing Beads-Matrix E (MPbio) in the Vibro-mill MM200, (RETSCH) for 3 min at the frequency of 25 Hz to break the cells, followed by centrifugation (10,000 g, 10 min, 4°C, Sigma 3K18C). The supernatant was used for enzyme measurements using a spectrofluorometer (SAFAS Monaco Xenius XC, Monaco). Catalase (CAT) activity was assayed by measuring the rate of disappearance of H_2O_2 at 240 nm (Chang and Kao, 1997). GST was assayed at 340 nm using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. SOD activity was determined using a photochemical assay based on the reduction of nitro blue tetrazolium (NBT) according to total Superoxide Dismutase (T-SOD) assay kit (Hydroxylamine method, SIGMA KIT), however SOD activity in our experiments remained below the level of detection. All enzyme activities were related to the protein content in the extract, measured according to Bradford (1976).

2.5. Candidate function gene expression

2.5.1. RNA extraction and reverse transcription

RNA extraction and reverse transcription was done according to (Colinet *et al.* 2010) from the unexposed control and from *Daphnia* exposed to LC and HC after 3 and 7 days. For each condition and sampling time point, five RNA samples (i.e. biological replicates), each consisting of a pool of 50 *Daphnia*, were used. *Daphnia* were ground to fine powder in 1.5 mL tubes placed in liquid nitrogen. Samples were mixed with lysis buffer (containing 1% β-mercaptoethanol) from RNA extraction kits (Qiagen) and crushed for 10 min to complete homogenization. RNA extraction and purification was performed following the manufacturer's instructions (Qiagen). Total RNA was eluted in 40 µL of DEPC-treated water. RNA was quantified and quality-checked with a Nanodrop 1000 (Thermo Scientific, Waltham, MA). Three hundred nanograms of total RNA was used in the reverse transcription to cDNA, using the SuperScript ® III First-Strand Synthesis System for RT-PCR

320 (Invitrogen), according to the manufacturer's instructions. The undiluted cDNA was stored
321 at 20°C until use.
322
323 **2.5.2. Real-time PCR**
324
325 We used primers of candidate genes involved in *Daphnia* energy (glyceraldehyde-3-
326 phosphate dehydrogenase: *gapdh*), digestive system (protease: *ct383*), oxidative stress
327 (glutathione S-transferase: *gst*, glutathione peroxidase: *gpx*, catalase: *cat*, thioredoxin: *trx*,),
328 cell cycle (histone: *h2a*, myosin heavy chain: *mhcrt*) and molting (nuclear hormone receptor:
329 *hr3*, nuclear hormone receptor: *ftz-f1*) that potentially could be affected by cyanobacterial
330 metabolites. We used ***β***-actin as the reference gene. Oligonucleotide primers were obtained
331 from the previously published references (see **Table 1.** for details), having efficiency between
332 92% and 105% (Giraudo *et al*., 2017; Houde *et al*., 2013; Lyu *et al*., 2014; Rhiannon *et al*.,
333 2011; Schwarzenberger *et al*., 2010; Tong *et al*., 2017; Wang *et al*., 2016). Oligonucleotide
334 primers were made by Integrated DNA Technologies, BVBA.
335

Table 1. Oligonucleotide primers used in the experiments.

Gene name	Symbol	Primer sequence 5'-3'	Amplicon size	Reference
Glyceraldehyde-3-phosphate dehydrogenase	***gapdh***	F- TGCTGATGCCCCAATGTTTGTTGT R-GCAGTTATGGCGTGGACGGTTGT	132	(Giraudo *et al*., 2017)
Protease CT383	***ct383***	F- TTGGCACCTTCCACCGAAT R- TCATCAGGACTGGAGAAACGC	183	(Schwarzenberger *et al*., 2010)
Glutathione S-transferase (NCBI: No. EFX81634.1)	***gst***	F- GGGAGTCTTTTACCACCGTTTC R- TCGCCAGCAGCATACTTGTT	150	(Wang *et al*., 2016)
Glutathione peroxidase	***gpx***	F- AACGTTACGATGCCAGTTCC R- TCTTTCGAGCGGTTGAGATT	212	(Rhiannon *et al*., 2011)
Catalase	***cat***	F- AGGTGCCTTTGGATACTTTGA R- TTGCGTATTCCTTGGTCAGTC	495	(Lyu *et al*., 2014)
Thioredoxin	***trx***	F- GTATCCACGCCAGTCCTTGTT R- TCCTTCCACTTTTCCTCCCTTA	129	(Liu *et al*., 2019)
Histone 2A	***h2A***	F- CTGGTGCCCCTGTCTACCTA R- TAGGGAGGAGAACAGCCTGA	219	(Giraudo *et al*., 2017)
Myosin heavy chain	***mhcrt***	F - GATGCCGTTTCCGAGATGAG R - CTCGGCGGTCATGTGGTC	132	(Tong *et al*., 2017)

Nuclear hormone receptor HR3	***hr3***	F- AAGGTCGAGGATGAAGTGCG R- AAAGACGCTACTATCGGGCG	81	(Giraudo *et al*., 2017)
Nuclear hormone receptor FTZ-F1	***ftz-f1***	F- TCTTACCGGACATTCACGCC R- ACAGCCGTTGAGATGCTTGA	71	(Giraudo *et al*., 2017)
Beta-actin	***β-actin***	F- GCCCTCTTCCAGCCCTCATTCT R- TGGGGCAAGGGCGGTGATTT	189	(Houde *et al*., 2013)

Real-time PCRs were performed on the LightCycler 480 system. Reactions were performed in 384-well LightCycler plates, using LightCycler 480 High Resolution Melting Master Mix and the crossing point (Cp), equivalent to the cycle threshold (Ct), estimates were obtained using the absolute quantification module in the software package. The PCR reactions were performed in four replicates , containing 4 µL of cDNA sample, 2 µm each primer, and 6 µL of the High Resolution Melting Master Mix. After 10 min at 95°C, the cycling conditions were as follows: 60 cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. To validate the specificity of amplification, a post amplification melt curve analysis was performed. Amplicons were first denatured at 95°C for 1 min, and then cooled to 65 °C, and the temperature was then gradually raised to 95°C). Fluorescence data were recorded continuously during this period, and subsequently analyzed using the Tm calling module in the LightCycler 480 software.

$$R = \frac{(E_{target})^{\Delta CPtarget(control-treated\ sample)}}{(E_{reference})^{\Delta CPtarget(control-treated\ reference)}}$$

Relative expression ratios (R) (i.e. fold change) were calculated using the efficiency calibrated model of (Colinet et al., 2010; Pfaffl, 2001). In the Pfaffl model, CP is the crossing point (i.e. Ct) and E the efficiency of PCRs. qPCR CT values of all candidate genes have been provided in **Supplementary 1**. The ratio of the target gene is expressed in treated samples versus matched controls (calibrators), and normalized using the housekeeping reference gene.

2.7. Statistical analyses

R Core Team (2013) was used to access statistical analysis of the obtained data. All data are presented as mean ± standard deviation. Significant differences were determined at

$p<0.05$. We performed t-tests to determine the difference between CAT, GST activities, as well as gene expression between the control and treatment. Repeated-measures analysis of variance was done to determine the difference in *Daphnia* survival between Control/LC, Control/HC and LC/HC). Repeated-measures analysis of variance is a mixed linear model with day, treatment (Control vs Treatment) and interaction between day and treatment considering the repeated measures on replicates (random effect). Normality of residuals was tested via Shapiro test (residuals normaly distributed when $p>0.05$). Anova was performed to test the effects of the model. Pairwise comparison with correction for multiple comparison was performed to check significant differences between control and the two treatments, differences between LC and HC and if there was time dependency effect on control and treatment.

3. RESULTS

3.1. Composition of the two weeks old *M. aeruginosa* PCC7806 medium

In the media produced by exponentially growing *M. aeruginosa* PCC7806, 11 metabolites were detected after 2 weeks of cultivation. *M. aeruginosa* PCC 7806 produced MC-LR and des-MC-LR, cyanopeptolins, CP (963A, A and B), aerucyclamides, AC (A,B,C and D) and aeruginosins (684 and 602) (**Figure 1.**).

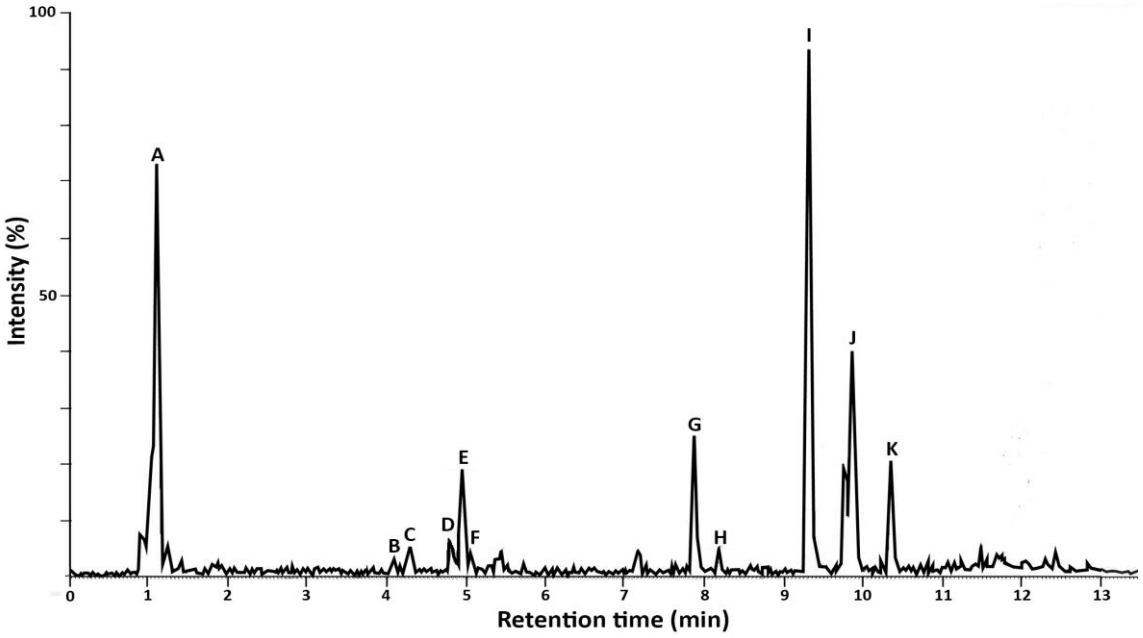

Figure 1. Secondary metabolites detected in *M. aeruginosa* PCC 7806. **(A)** aeruginosin 684, **(B)** cyanopeptolin B, **(C)** aeruginosin 602, **(D)** des-MCLR, **(E)** MC-LR, **(F)** CP A, **(G)** aerucyclamide D, **(H)** cyanopeptolin 963A, **(I)** aerucyclamide A, **(J)** aerucyclamide C, **(K)** aerucyclamide B.

The concentrations of five extracellular metabolites (MC-LR, des-MC-LR, CP-A, AC-D, AC-A) in *M. aeruginosa* PCC 7806 after 2 weeks growth phase from $2x10^5$ cells/mL initially to $1x10^6$ cells/mL, and in dilutions HC and LC are shown in **Table 2.**

Table 2. Concentrations of extracellular metabolites in original medium of the 2 weeks *M. aeruginosa* PCC 7806 and its HC and LC dilutions

Extracellular metabolite	Original spent *M. aeruginosa* PCC 7806 medium (µg/L)	HC (µg/L)	LC (µg/L)
MC-LR	**218±21**	**5.45±0.53**	**0.55±0.05**
Des-MC-LR	**61±1**	**1.53±0.03**	**0.15±0.01**
CP-A	**135±11**	**3.38±0.28**	**0.34±0.03**
AC-D	**93±10**	**2.33±0.25**	**0.23±0.02**

AC-A	**57±10**	**1.43±0.25**	**0.14±0.02**

3.1. *Daphnia* survival

The high concentration of the cyanobacterial medium (HC) caused a significant decrease in survival from day 2 onwards, compared with the control, while exposure to the low concentration (LC) caused significant decrease after day 3. Both concentration levels significantly decreased *Daphnia* survival over the course of the experiment. *Daphnia* survival was significantly higher in LC exposure from day 3 onwards (80% survival after 7 days) compared with the HC exposure (40% survival after 7 days) (**Figure 2**.)

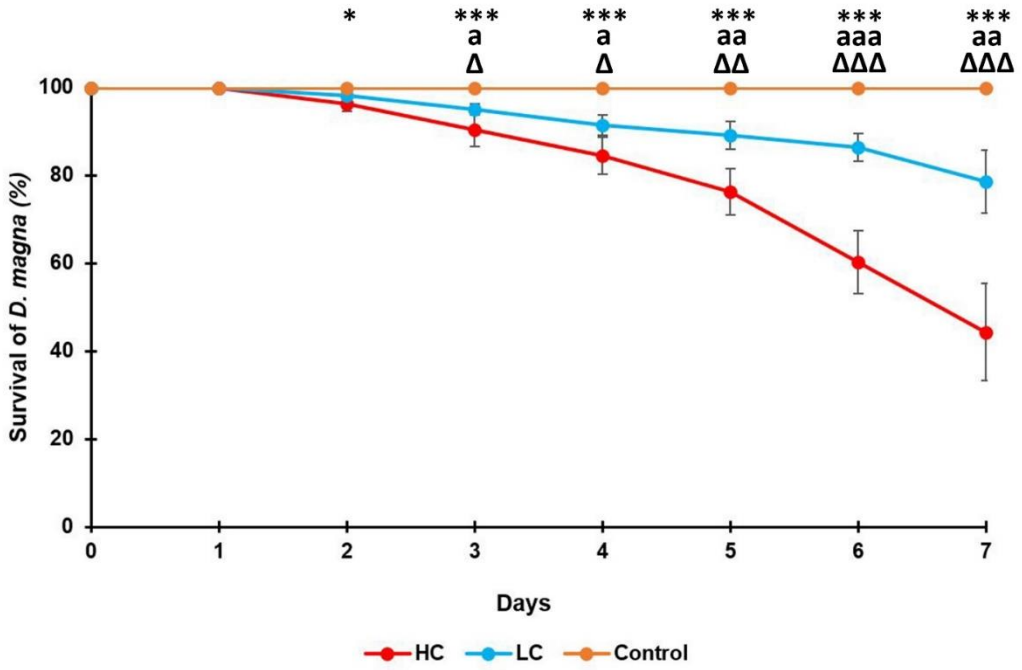

Figure 2. *D.magna* survival when exposed to HC and LC. Control vs HC: *; Control vs LC: a ; HC vs LC: Δ. *, a, Δ ($p < 0.05$), **, aa, ΔΔ ($p < 0.01$), ***, aaa, ΔΔΔ ($p < 0.01$); repeated-measures analysis of variance

3.2. Antioxidant and detoxification enzymes

CAT activity was significantly lower on day 3 and day 7 in the LC exposure, compared to the control. Similarly GST activity was significantly lower on day 3 and 7 in the treatment exposed to LC, compared to the control. Due to the high mortality in the HC exposure, the biomass of the remaining *Daphnia* was insufficient for enzyme analysis on both days (**Figure 3.**).

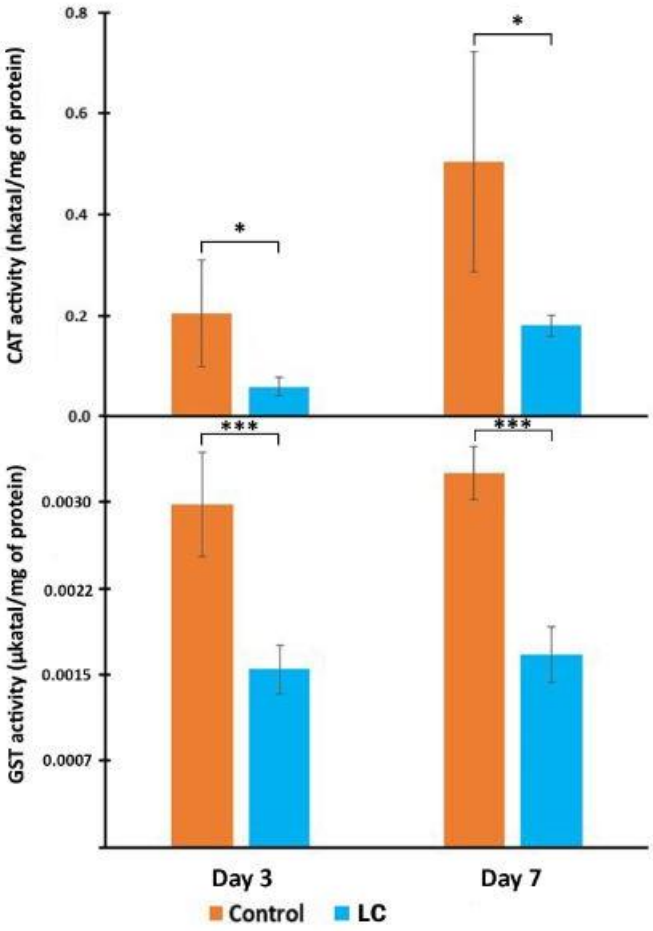

Figure 3. CAT and GST activity in *D.magna* exposed to LC * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.01$), t-test

3.5. Selected genes expression

Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) was significantly overexpressed on day 3 when *Daphnia* were exposed to both HC and LC, but downregulated after 7 days, significant for LC (**Figure 4. A**). The gene coding for digestive enzyme Protease CT383 (*ct383*) was significantly downregulated after 3 days exposure to LC (day 3) of *Microcystis* spent medium, but not in the other treatment (**Figure 4**. **B**). Concerning the oxidative stress and detoxification related genes, merely downregulation was observed. The only exception was the gene encoding for the detoxification enzyme Glutathione S-transferase (*gst*) which was significantly upgulated on day 3 when exposed to HC, while on day 7, *gst* was downregulated at LC treatment (**Figure 4. C**). Furthermore, Glutathione peroxidase (*gpx*) expression was significantly downregulated on day 7 after *Daphnia* exposure to LC (**Figure 4. D**). Genes responsible for oxidative stress enzymes Catalase (*cat*) (**Figure 4. E**) and Thioredoxin (*trx*) (**Figure 4. F**) were downregulated on day 3 (LC) and day 7 (HC and LC). The response of the histone 2A (*h2A*) gene was the opposite of this, when exposed to HC and

LC: *h2A* showed significant overexpression respectively downregulated on day 7 when exposed to HC or LC (**Figure 4. G**). HC and LC also had significant negative effect on gene expression of Myosin heavy chain (*mhcrt*) that was significantly underregulated on day 3 and 7 (**Figure 4. H**). Cyanobacterial secondary metabolites affected two genes involved in the molting cycle: the Nuclear hormone receptor HR3 (*hr3*) and FTZ-F1 (*ftz-f1*). While the *hr3* was significantly upregulated on day 3 when exposed to both HC and LC (**Figure 4. I**) *ftz-* was significantly downregulated on day 7 when exposed to both HC and LC (**Figure 4. J**).

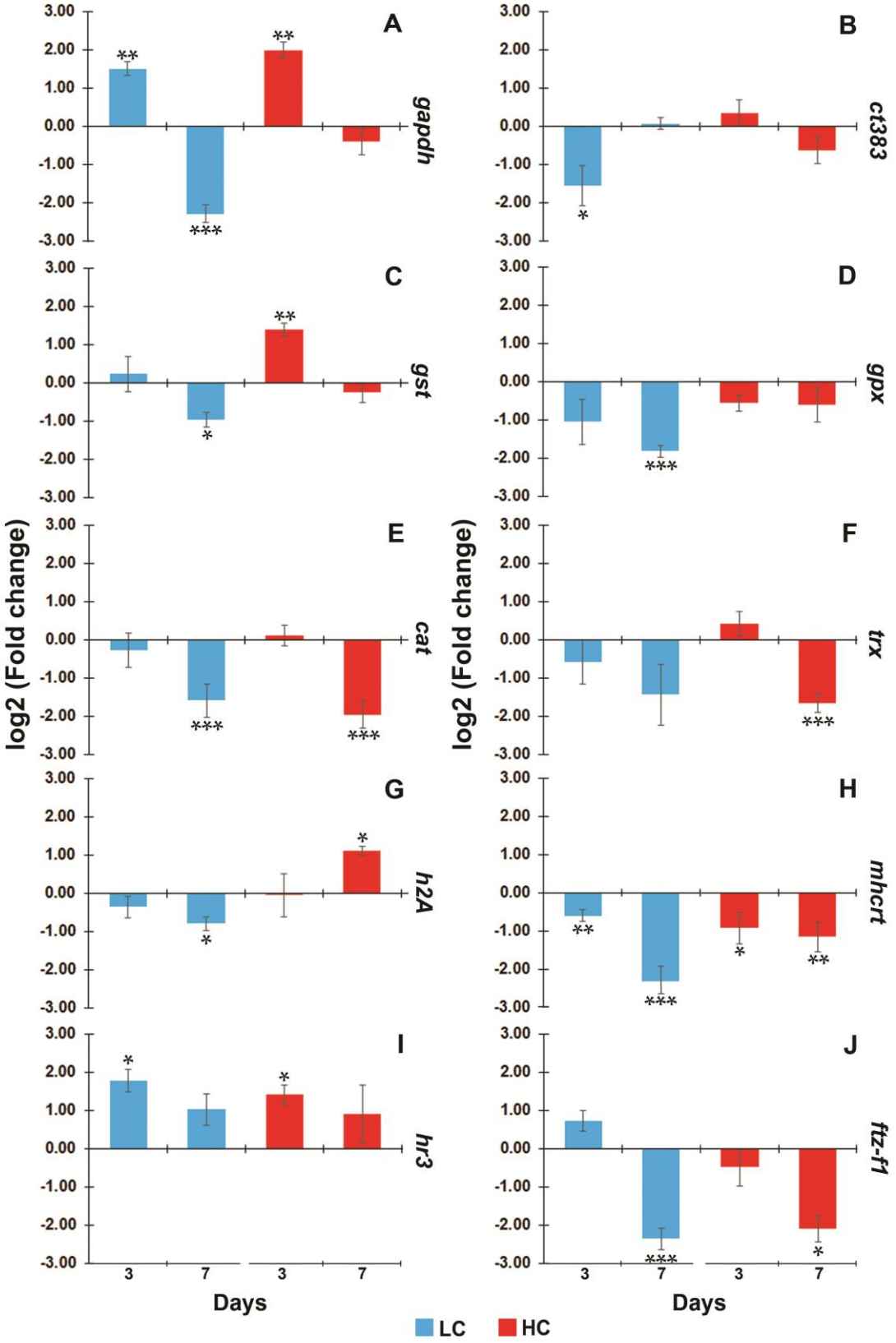

Figure 4. Effect of cyanobacterial media (LC and HC) on the relative gene expressions expressed as log2(fold change). Relative expressions in *D. magna* for: A) Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), B) Protease CT383 (*ct383*), C) Glutathione S-transferase (*gst*), D) Glutathione peroxidase (*gpx*) E) Catalase (*cat*), F) Thioredoxin (*trx*), G) histone 2A (*h2A*), H) Myosin heavy chain (*mhcrt*), I) Nuclear hormone receptor HR3 (*hr3*), J) Nuclear hormone receptor FTZ-F1 (*ftz-f1*). Gene expressions were normalized to the control condition for each sampling time. Replicates of culture are represented as a mean and error bars represent the standard deviations (n = 5). * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.01$), t-test

4. DISCUSSION

Daphnia are unselective phytoplankton filter feeders, and as such can be exposed to cyanobacterial metabolites by direct grazing on cyanobacteria as well as by uptake of metabolites released in the water. While previous studies have demonstrated the negative impacts of cyanobacterial medium (Lanaras and Cook 1994, Neumann *et al.* 2000, Dao *et al.* 2010, 2013, Esterhuizen-Londt *et al*. 2016, Peng et al. 2018) on *Daphnia*, the novelty of this study was to connect responses from selected markers on the gene-level to enzymatic activities and to consequences for the survival of *D. magna* exposed to cyanobacterial cell-free sprent medum. From the mixture of cyanobacterial metabolites in the medum we identified 11 and quantified 5.

As *M. aeruginosa* was grown in BG11, *D. magna* had to be acclimatised to this medium during several weeks before the experiment. Survival of the control group throughout the experiment was always high (>98%). These results are in line with the literature where non-treated *D. magna* survival was always high (close to 100%) in the first 8 days of experiments, although specific *D. magna* cultivation medium was used (Dao *et al*. 2010, Ortiz-Rodríguez *et al*. 2012), suggesting that BG 11 medium used in our study did not have impact on the *D. magna* survival. Apart from increased CAT activities no other changes were observed in the control group over the exposure time, we nevertheless suggest for further studies to compromise between both media by adding necessary minerals, e.g. calcium, using diluted BG 11, while keeping the osmolarity as we ensured in our exposures.

Cyanobacterial spent medium had a dose and time dependent negative impact on *D. magna* survival, verifying our hypothesis. The concentrations used (Table 2.) were detrimental thus preventing *D. magna* from acclimatisation during the exposure period. These detrimental effects within 7 days were not expected as these concentrations were chosen according to previous studies showing that survival of *D. magna* rapidly decreased during 3 weeks exposure to crude extract containing 50 µg/L of dissolved microcystin, while exposure to 5 µg/L did not impact *Daphnia* survival much (Dao *et al*., 2010). Similar results were observed by Lürling and van der Grinten (2003) where exposure to 3.5 µg/L of dissolved microcystin showed no significant decrease in survival over 7 days. When exposed to crude extract or artificial mixtures of pure microcystins containing 60 µg/L of total MC (MC-LR

21.1 ± 1.9 µg/L; MC-RR 37.2 ± 4.8 µg/L and MC-YR 6.1 ± 0.8 µg/L) it did not appear to have an impact on *D. pulex* survival for 6 days (Esterhuizen-Londt *et al*., 2016). Our results are, however, in line with studies exposing *D. magna* to *M. aeruginosa* containing 1.4–9 µg/L of MC, where feeding was inhibited, followed by increase of mortality in the first week of the treatment (DeMott, 1999; Demott *et al*., 1991; Ghadouani *et al*., 2004; Rohrlack *et al*., 2001). *D. laevis* (two clones) and *D. similis* exposed to five *Microcystis* extracts in which microcystins were detected (434 - 538 mg/g of lyophilized sample) suffered decreased survival, reproduction and disturbance in egg production after 48 h (Herrera *et al*. 2015). Crude extract obtained from *Microcystis* spp. had lethal effect on *D. magna* neonates, with LC50 (48 h) ranging from 168.3–442.7 mg microcystin (total) DW L^{-1} (Pham, 2018). Susceptibility to microcystin is, however, not only species-specific, but *Daphnia* species can also show different responses within their clones (DeMott, 1999; Hairston *et al*., 1999; Rohrlack *et al*., 2001).

As both concentrations in our experiment caused mortality (20% for LC and 60% for HC) within the 7 days exposure, we suggest a high *D. magna* sensitivity to the other cyanobacterial metabolites present in the spent medium, as besides MC-LR and desmethyl MC-LR, cyanopeptolin-A, aerucyclamides A and D were quantified, and aeruginosins (602 and 684), cyanopeptolins (B and 963) and aerucyclamides (B and C) detected. HC and LC of *Microcystis* spent medium downregulated a gene encoding the digestive enzyme protease, *ct383*, thus potentially interfered with *D. magna* digestion and as a consequence could contribute to the increasing mortality in a concentration and time dependent manner. Similarly, when *D. magna* were fed with MC-producing strain of PCC 7806 wild type, *ct383* was downregulated after 6 days, leading to reduced total chymotrypsin activity (Schwarzenberger *et al*., 2010).

When fed with *Microcystis* strain UWOCC MRC (a non-microcystin producing strain) a lethal molting disruption in *Daphnia* spp. has been observed, suggesting that cyanobacterial proteases, other than microcystin, could interfere with the molting cycle (Kaebernick *et al*., 2001). Despite the absence of microviridins J in our medium, known to impair the molting cycle in *Daphnia* via protease inhibition (Rohrlack *et al*., 2004), upregulation of a gene coding for nuclear hormone receptor (*hr3*) followed by significant downregulation of *ftz-f1*

transcripts involved in crustacean molting and development (Street *et al*., 2019) has been observed. Our results suggest that other metabolites have a role in impairment of these pathways which may affect *Daphnia* molting.

Activity of anti-oxidative stress enzymes is a crucial defense mechanism against the induction of oxidative stress by microcystins (Amado and Monserrat, 2010). Through increased oxidative stress enzyme activities and detoxication, *D. magna* are able to enhance acclimation to cyanobacterial metabolites (Ortiz-Rodríguez *et al*., 2012). CAT is a highly efficient antioxidant enzyme, responsible for reduction of H_2O_2 concentrations in the cells (Fridovich, 1998). Elevated CAT activity can prevent oxidative damage caused by cyanotoxins thus provides oxidative protection for *Daphnia* (Wojtal-Frankiewicz *et al*., 2013). Increased CAT activity was observed in adults and neonates after 24 h when exposed to 100 µg/L pure MC-LR, while exposure to 5 µg/L MC-LR or less, CAT activity was similar to control (Ortiz-Rodríguez *et al*., 2012). In our experiments, genes of antioxidant enzymes, *cat* and *trx,* were downregulated, confirming the results of the enzyme activity. The significant decrease in CAT activity on days 3 and 7, suggests that these enzymes were exhausted due to the presence of the mixture of cyanobacterial compounds in the concentrations applied. Similarly, CAT decreased in *D. magna* after being exposed to cyanobacterial crude extract (containing 60 µg/L total MC) during the whole exposure period of 72 h while the pure toxin MC-LR increased its activity (Esterhuizen-Londt *et al*. 2016). Even when *D. magna* were exposed to extracts from non-microcystin and non-cylindrospermopsin medium, their CAT decreased after day 1 and 7 (Dao *et al*., 2013). In combination with the observed lethality, our results suggest that *D. magna* oxidative defence response was repressed in such way that oxidative damages may have occurred resulting in lethal effects.

In *D. magna and D. longispina,* increased GST activity can detoxify microcystin up to a certain concentration or exposure duration, which can also be transferred to the next generation (Ortiz-Rodrigues *et al*., 2012; Wojtal-Frankiewicz *et al*. 2013, 2014). Despite the upregulation of *gst* within the first 3 days, our exposures, however, decreased GST activity, compared with the control on day 3 and day 7, where expression of *gst* gene was downregulated as well. Our GST results concern one or at maximum two out of on average

539 7 GST isoenzymes present in an organism, hence genes regulating other GST isoenzymes
540 may have had a different response. Asselman *et al*. (2012) showed that sigma-class *gst* genes
541 in *D. pulex* were upregulated when fed on MC-producing *M. aeruginosa* after 16 days.
542 However, our results are in line with previous studies where short term exposure (48 h) to
543 microcystin-producing *M. aeruginosa* PCC7806 increased *delta-gst* transcript levels, while
544 long term exposure (15 days) downregulated them (Lyu et al., 2016a). Similar decrease in
545 GST activities were observed in *D. magna* exposed to cyanobacterial medium (containing 60
546 µg/L total MC) after 24 h and 72 h (Esterhuizen-Londt *et al*., 2016). Furthermore, GST
547 activity in *D. magna* decreased with increased MC-LR concentrations from 10 to 2000 µg/L
548 (Chen *et al*., 2005), suggesting enzymes exhaustibility as also seen in (Dao *et al*., 2013).
549 Our results suggest that even lower concentrations of MC in the cyanobacterial medium,
550 along with the other cyanobacterial metabolites can affect enzyme activity in a similar way
551 like cyanobacterial medium containing higher microcystin concentrations. Besides
552 cyanobacterial metabolites that were detected, cyanobacterial medium could contain
553 undetected compounds that could potentially interfere with overall ecotoxicity (Smutná *et*
554 *al*., 2014) or inhibit enzymes activity, such as microcin SF608 that was shown to have
555 inhibitory impact on GST (Wiegand *et al*., 2002).
556 When *Daphnia* are in toxic environments, mobilization of detoxification and antioxidant
557 defense mechanisms, as well as growth and development, comes with energetic cost (Calow,
558 1991; McKee and Knowles, 1986; Pane *et al*., 2004). For instance, *D. magna* exposed to
559 nickel (Pane *et al*., 2004) or the fungicide tebuconazole (McKee and Knowles, 1986) showed
560 increased energy consumption, as glycogen and lipids levels were decreased. In our
561 experiments energetic resouces were instantly used, visible by the induction of *gapdh*
562 (involved in glycolysis) at day 3. Upregulation of *gapdh* has been observed in *D. magna* in
563 response to diet containing microcystins after 4 days of the experiment, suggesting that
564 ingestion of microcystin induces glycolysis and protein catabolism (Schwarzenberger *et al*.,
565 2009). Our results further suggest that severe depletion of energy affected also the muscular
566 activity, as the myosin heavy chain was immediately and significantly downregulated in both
567 experimental exposures (HC and LC). Depletion of energy in combination with
568 downregulation of the myosin heavy chain could provide a mechanistic explanation of the

569 locomotory inhibition (feeding and swimming) observed in previous studies (e.g. Rohrlack
570 *et al*., 2001; Ghadouani *et al*., 2004).

571 The response of the histone 2A gene (*h2A*) (involved in normal cell cycle progression)
572 may indicate two different cellular pathways, depending on the exposure concentration. The
573 downregulation at LC hints on apoptotic processes, while the upregulation at HC exposure
574 may imply cellular dysfunction occurring during necrotic processes, indicating a stronger
575 negative effect of HC medium on *Daphnia,* that is in line with the higher mortality in this
576 exposure. Activation of apoptotic pathways are positively correlated with the decline in *mhcrt*
577 (Tong *et al*., 2017) that was also observed in our study, suggesting apoptotic processes in *D.*
578 *magna*, caused by cyanobacterial metabolites. Used concentrations of cyanobacterial
579 metabolites in our exposures may have been too high for *Daphnia* to adapt, therefore use of
580 lower concentration in future studies would provide deeper understading of *Daphnia*'s
581 molecular response to the cyanobacterial metabolites.
582

583 # 5. CONCLUSION
584

585 In conclusion, *D. magna* survival was strongly affected by cyanobacterial spent medium
586 at both LC and HC, due to the combined effect of MC and the other secondary metabolites
587 present. *D. magna* were not able to acclimate during the short exposure period. Medium with
588 the highest tested concentration of cyanobacterial metabolites was the most detrimental as
589 expected. Exposure to cyanobacterial compounds in the *M. aeruginosa* spent medium
590 affected *D. magna* genes involved in i) digestion, thus mobilization of the limited internal
591 energetic resources, ii) oxidative stress and detoxification, iii) muscular activity and iv) cell
592 regulation including the molting process. We also observed consequences of cyanobacterial
593 medium on detoxification and antioxidant capacities which were dose and in particular time-
594 dependent indicating an exhaustion of the enzymes with high concentration or long exposure
595 duration. Despite this, the concentrations were chosen based on available data of pure MCs
596 or MCs in crude extracts causing low lethality, for following sublethal effects an even lower
597 concentration is recommended.
598
599

6. BIBLIOGRAPHY

Amado, L.L., Monserrat, J.M., 2010. Oxidative stress generation by microcystins in aquatic animals: Why and how. Environ. Int. doi:10.1016/j.envint.2009.10.010

Asselman, J., De Coninck, D.I.M., Glaholt, S., Colbourne, J.K., Janssen, C.R., Shaw, J.R., De Schamphelaere, K.A.C., 2012. Identification of pathways, gene networks, and paralogous gene families in daphnia pulex responding to exposure to the toxic cyanobacterium Microcystis aeruginosa. Environ. Sci. Technol. 46, 8448–8457. doi:10.1021/es301100j

Asselman, J., Hochmuth, J.D., De Schamphelaere, K.A.C., 2014. A comparison of the sensitivities of Daphnia magna and Daphnia pulex to six different cyanobacteria. Harmful Algae 39, 1–7. doi:10.1016/j.hal.2014.06.008

Barrios, C.A.Z., Nandini, S., Sarma, S.S.S., 2015. Effect of crude extracts of Dolichospermum planctonicum on the demography of Plationus patulus (Rotifera) and Ceriodaphnia cornuta (Cladocera). Ecotoxicology 24, 85–93. doi:10.1007/s10646-014-1358-8

Beversdorf, L.J., Rude, K., Weirich, C.A., Bartlett, S.L., Seaman, M., Kozik, C., Biese, P., Gosz, T., Suha, M., Stempa, C., Shaw, C., Hedman, C., Piatt, J.J., Miller, T.R., 2018. Analysis of cyanobacterial metabolites in surface and raw drinking waters reveals more than microcystin. Water Res. 140, 280–290. doi:10.1016/j.watres.2018.04.032

Beversdorf, L.J., Weirich, C.A., Bartlett, S.L., Miller, T.R., 2017. Variable cyanobacterial toxin and metabolite profiles across six eutrophic lakes of differing physiochemical characteristics. Toxins (Basel). 9. doi:10.3390/toxins9020062

Bister, B., Keller, S., Baumann, H.I., Nicholson, G., Weist, S., Jung, G., Süssmuth, R.D., Jüttner, F., 2004. Cyanopeptolin 963A, a chymotrypsin inhibitor of Microcystis PCC 7806. J. Nat. Prod. 67, 1755–1757. doi:10.1021/np049828f

Bojadzija Savic, G., Bormans, M., Edwards, C., Lawton, L., Briand, E., Wiegand, C., 2020. Cross talk: Two way allelopathic interactions between toxic Microcystis and Daphnia. Harmful Algae 94, 101803. doi:10.1016/j.hal.2020.101803

Bojadzija Savic, G., Edwards, C., Briand, E., Lawton, L., Wiegand, C., Bormans, M., 2019. Daphnia magna Exudates Impact Physiological and Metabolic Changes in Microcystis aeruginosa. Toxins (Basel). 11, 421. doi:10.3390/toxins11070421

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. doi:10.1016/0003-2697(76)90527-3

Briand, E., Bormans, M., Gugger, M., Dorrestein, P.C., Gerwick, W.H., 2016. Changes in secondary metabolic profiles of Microcystis aeruginosa strains in response to intraspecific interactions. Environ. Microbiol. 18, 384–400. doi:10.1111/1462-2920.12904

Briand, E., Bormans, M., Quiblier, C., Salençon, M.J., Humbert, J.F., 2012. Evidence of the

cost of the production of microcystins by Microcystis aeruginosa under differing light and nitrate environmental conditions. PLoS One 7, e29981. doi:10.1371/journal.pone.0029981

Calow, P., 1991. Physiological costs of combating chemical toxicants: ecological implications. Comp. Biochem. Physiol. C. 100, 3–6.

Chang, C.J., Kao, C.H., 1997. Plant growth regulation., Plant growth regulation. Kluwer Academic Publishers.

Chen, W., Song, L., Ou, D., Gan, N., 2005. Chronic toxicity and responses of several important enzymes in Daphnia magna on exposure to sublethal microcystin-LR, in: Environmental Toxicology. pp. 323–330. doi:10.1002/tox.20108

Colinet, H., Lee, S.F., Hoffmann, A., 2010. Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult Drosophila melanogaster. FEBS J. 277, 174–185. doi:10.1111/j.1742-4658.2009.07470.x

Dao, T.S., Do-Hong, L.C., Wiegand, C., 2010. Chronic effects of cyanobacterial toxins on Daphnia magna and their offspring. Toxicon 55, 1244–1254. doi:10.1016/j.toxicon.2010.01.014

Dao, T.S., Ortiz-Rodríguez, R., Do-Hong, L.C., Wiegand, C., 2013. Non-microcystin and non-cylindrospermopsin producing cyanobacteria affect the biochemical responses and behavior of Daphnia magna. Int. Rev. Hydrobiol. 98, 235–244. doi:10.1002/iroh.201301626

De Coninck, D.I.M., Asselman, J., Glaholt, S., Janssen, C.R., Colbourne, J.K., Shaw, J.R., De Schamphelaere, K.A.C., 2014a. Genome-wide transcription profiles reveal genotype-dependent responses of biological pathways and gene-families in daphnia exposed to single and mixed stressors. Environ. Sci. Technol. 48, 3513–3522. doi:10.1021/es4053363

De Coninck, D.I.M., Asselman, J., Glaholt, S., Janssen, C.R., Colbourne, J.K., Shaw, J.R., De Schamphelaere, K.A.C., Pfrender, M.E., Lopez, J.A., De Coninck, D.I.M., Janssen, C.R., Shaw, J.R., De Schamphelaere, K.A.C., 2014b. Conserved transcriptional responses to cyanobacterial stressors are mediated by alternate regulation of paralogous genes in Daphnia. Mol. Ecol. 48, 1844–1855. doi:10.1021/es4053363

DeMott, W.R., 1999. Foraging strategies and growth inhibition in five daphnids feeding on mixtures of a toxic cyanobacterium and a green alga. Freshw. Biol. 42, 263–274. doi:10.1046/j.1365-2427.1999.444494.x

Demott, W.R., Zhang, Q.-X., Carmichael, W.W., 1991. Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of Daphnia. Limnol. Ocean. 36, 1346–357. doi:10.4319/lo.1991.36.7.1346

Drugă, B., Turko, P., Spaak, P., Pomati, F., 2016a. Cyanobacteria Affect Fitness and Genetic Structure of Experimental Daphnia Populations. Environ. Sci. Technol. 50, 3416–3424. doi:10.1021/acs.est.5b05973

Drugă, B., Turko, P., Spaak, P., Pomati, F., 2016b. Cyanobacteria Affect Fitness and Genetic Structure of Experimental *Daphnia* Populations. Environ. Sci. Technol. 50,

681 3416–3424. doi:10.1021/acs.est.5b05973

682 Edwards, C., Lawton, L.A., Coyle, S.M., Ross, P., 1996. Laboratory-scale purification of
683 microcystins using flash chromatography and reversed-phase high-performance liquid
684 chromatography, in: Journal of Chromatography A. Elsevier B.V., pp. 163–173.
685 doi:10.1016/0021-9673(95)01004-1

686 Elkobi-Peer, S., Singh, R.K., Mohapatra, T.M., Tiwari, S.P., Carmeli, S., 2013.
687 Aeruginosins from a microcystis sp. bloom material collected in Varanasi, India. J.
688 Nat. Prod. 76, 1187–1190. doi:10.1021/np4001152

689 Esterhuizen-Londt, M., von Schnehen, M., Kühn, S., Pflugmacher, S., 2016a. Oxidative
690 stress responses in the animal model, Daphnia pulex exposed to a natural bloom
691 extract versus artificial cyanotoxin mixtures. Aquat. Toxicol. 179, 151–157.
692 doi:10.1016/j.aquatox.2016.09.003

693 Esterhuizen-Londt, M., von Schnehen, M., Kühn, S., Pflugmacher, S., Oxidative stress
694 responses in the animal model, Daphnia pulex exposed to a natural bloom extract
695 versus artificial cyanotoxin mixturesEsterhuizen-Londt, M., von Schnehen, M., Kühn,
696 S., Pflugmacher, S., 2016b. Oxidative stress responses in the animal model, Daphnia
697 pulex exposed to a natural bloom extract versus artificial cyanotoxin mixtures. Aquat.
698 Toxicol. 179, 151–157. doi:10.1016/j.aquatox.2016.09.003

699 Ferrão-Filho, A.S., Herrera, N.A., Echeverri, L.F., 2014. Microcystin accumulation in
700 cladocerans: First evidence of MC uptake from aqueous extracts of a natural bloom
701 sample. Toxicon 87, 26–31. doi:10.1016/j.toxicon.2014.05.015

702 Fridovich, I., 1998. Oxygen toxicity: a radical explanation. J. Exp. Biol. 201.

703 Gademann, K., Portmann, C., 2008. Secondary Metabolites from Cyanobacteria: Complex
704 Structures and Powerful Bioactivities. Curr. Org. Chem. 12, 326–341.
705 doi:10.2174/138527208783743750

706 Gatte-Picchi, D., Weiz, A., Ishida, K., Hertweck, C., Dittmann, E., 2014. Functional
707 analysis of environmental DNA-derived microviridins provides new insights into the
708 diversity of the tricyclic peptide family. Appl. Environ. Microbiol. 80, 1380–1387.
709 doi:10.1128/AEM.03502-13

710 Ger, K.A., Urrutia-Cordero, P., Frost, P.C., Hansson, L.A., Sarnelle, O., Wilson, A.E.,
711 Lürling, M., 2016. The interaction between cyanobacteria and zooplankton in a more
712 eutrophic world. Harmful Algae 54, 128–144. doi:10.1016/j.hal.2015.12.005

713 Ghadouani, A., Pinel-Alloul, B., Plath, K., Codd, G. a., Lampert, W., 2004. Effects of
714 Microcystis aeruginosa and purified microcystin-LR on the feeding behavior of
715 Daphnia pulicaria. Limnol. Oceanogr. 49, 666–679. doi:10.4319/lo.2004.49.3.0666

716 Giraudo, M., Douville, M., Cottin, G., Houde, M., 2017. Transcriptomic, cellular and life-
717 history responses of Daphnia magna chronically exposed to benzotriazoles:
718 Endocrinedisrupting potential and molting effects. PLoS One 12, e0171763.
719 doi:10.1371/journal.pone.0171763

720 Gustafsson, S., Hansson, L.-A., 2004. Development of tolerance against toxic cyanobacteria
721 in Daphnia. Aquat. Ecol. 38, 37–44. doi:10.1023/B:AECO.0000020985.47348.5e

Hairston, N.G., Lampert, W., Cáceres, C.E., Holtmeier, C.L., Weider, L.J., Gaedke, U., Fischer, J.M., Fox, J. a., Post, D.M., 1999. Lake ecosystems: Rapid evolution revealed by dormant eggs. Nature 401, 446. doi:10.1038/46731

Hayes, J.D., Flanagan, J.U., Jowsey, I.R., 2005. Glutathione transferases. Annu. Rev. Pharmacol. Toxicol. 45, 51–88. doi:10.1146/annurev.pharmtox.45.120403.095857

Heisler, J., Glibert, P.M., Burkholder, J.M., Anderson, D.M., Cochlan, W., Dennison, W.C., Dortch, Q., Gobler, C.J., Heil, C.A., Humphries, E., Lewitus, A., Magnien, R., Marshall, H.G., Sellner, K., Stockwell, D.A., Stoecker, D.K., Suddleson, M., 2008. Eutrophication and harmful algal blooms: A scientific consensus. Harmful Algae 8, 3–13. doi:10.1016/j.hal.2008.08.006

Herrera, N.A., Echeverri, L.F., Ferrão-Filho, A.S., 2015. Effects of phytoplankton extracts containing the toxin microcystin-LR on the survival and reproduction of cladocerans. Toxicon 95, 38–45. doi:10.1016/j.toxicon.2014.12.016

Houde, M., Carter, B., Douville, M., 2013. Sublethal effects of the flame retardant intermediate hexachlorocyclopentadiene (HCCPD) on the gene transcription and protein activity of Daphnia magna. Aquat. Toxicol. 140–141, 213–219. doi:10.1016/j.aquatox.2013.06.008

Ighodaro, O.M., Akinloye, O.A., 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria J. Med. 54, 287–293. doi:10.1016/j.ajme.2017.09.001

Ishida, K., Christiansen, G., Yoshida, W.Y., Kurmayer, R., Welker, M., Valls, N., Bonjoch, J., Hertweck, C., Börner, T., Hemscheidt, T., Dittmann, E., 2007. Biosynthesis and Structure of Aeruginoside 126A and 126B, Cyanobacterial Peptide Glycosides Bearing a 2-Carboxy-6-Hydroxyoctahydroindole Moiety. Chem. Biol. 14, 565–576. doi:10.1016/j.chembiol.2007.04.006

Ishida, K., Nakagawa, H., Murakami, M., 2000. Microcyclamide, a cytotoxic cyclic hexapeptide from the cyanobacterium Microcystis aeruginosa. J. Nat. Prod. 63, 1315–1317. doi:10.1021/np000159p

Janssen, E.M.-L., 2019. Cyanobacterial peptides beyond microcystins – A review on co-occurrence, toxicity, and challenges for risk assessment. Water Res. 151, 488–499. doi:10.1016/J.WATRES.2018.12.048

Kaebernick, M., Rohrlack, T., Christoffersen, K., Neilan, B.A., 2001. A spontaneous mutant of microcystin biosynthesis: Genetic characterization and effect on Daphnia. Environ. Microbiol. 3, 669–679. doi:10.1046/j.1462-2920.2001.00241.x

Lahti, K., Rapala, J., Färdig, M., Niemelä, M., Sivonen, K., 1997. Persistence of cyanobacterial hepatotoxin, microcystin-LR in particulate material and dissolved in lake water. Water Res. 31, 1005–1012. doi:10.1016/S0043-1354(96)00353-3

Lanaras, T., Cook, C.M., 1994. Toxin extraction from an Anabaenopsis milleri — dominated bloom. Sci. Total Environ. 142, 163–169. doi:10.1016/0048-9697(94)90324-7

763 Liu, S., Ding, R., Nie, X., 2019. Assessment of oxidative stress of paracetamol to Daphnia
764 magna via determination of Nrf1 and genes related to antioxidant system. Aquat.
765 Toxicol. 211, 73–80. doi:10.1016/j.aquatox.2019.03.014

766 Lürling, M., van der Grinten, E., 2003. Life-history characteristics of *Daphnia* exposed to
767 dissolved microcystin-LR and to the cyanobacterium *Microcystis aeruginosa* with and
768 without microcystins. Environ. Toxicol. Chem. 22, 1281–1287.
769 doi:10.1002/etc.5620220614

770 Lyu, K., Gu, L., Li, B., Lu, Y., Wu, C., Guan, H., Yang, Z., 2016a. Stress-responsive
771 expression of a glutathione S-transferase (delta) gene in waterflea Daphnia magna
772 challenged by microcystin-producing and microcystin-free Microcystis aeruginosa.
773 Harmful Algae 56, 1–8. doi:10.1016/j.hal.2016.04.009

774 Lyu, K., Guan, H., Wu, C., Wang, X., Wilson, A.E., Yang, Z., 2016b. Maternal
775 consumption of non-toxic Microcystis by Daphnia magna induces tolerance to toxic
776 Microcystis in offspring. Freshw. Biol. 61, 219–228. doi:10.1111/fwb.12695

777 Lyu, K., Wang, Q., Li, Z., Chen, R., Zhu, C., Liu, J., Yang, Z., 2014. Age-dependent
778 survival and selected Gene expression in Daphnia magna after short-term exposure to
779 low dissolved oxygen. J. Plankton Res. 37, 66–74. doi:10.1093/plankt/fbu097

780 MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P., Codd, G.A., 1990. Cyanobacterial
781 microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A
782 from both mammals and higher plants. FEBS Lett. 264, 187–92.

783 Martin-Creuzburg, D., von Elert, E., Hoffmann, K.H., 2008. Nutritional constraints at the
784 cyanobacteria—Daphnia magna interface: The role of sterols. Limnol. Oceanogr. 53,
785 456–468. doi:10.4319/lo.2008.53.2.0456

786 McKee, M.J., Knowles, C.O., 1986. Effects of fenvalerate on biochemical parameters,
787 survival, and reproduction of Daphnia magna. Ecotoxicol. Environ. Saf. 12, 70–84.
788 doi:10.1016/0147-6513(86)90007-2

789 Merwe, D. Van der, Sebbag, L., 2012. Investigation of a Microcystis aeruginosa
790 cyanobacterial freshwater harmful algal bloom associated with acute microcystin
791 toxicosis in a dog. J. Vet. ….

792 Miles, C.O., Sandvik, M., Nonga, H.E., Ballot, A., Wilkins, A.L., Rise, F., Jaabaek, J.A.H.,
793 Loader, J.I., 2016. Conjugation of Microcystins with Thiols Is Reversible: Base-
794 Catalyzed Deconjugation for Chemical Analysis. Chem. Res. Toxicol. 29, 860–870.
795 doi:10.1021/acs.chemrestox.6b00028

796 Mowe, M.A.D., Mitrovic, S.M., Lim, R.P., Furey, A., Yeo, D.C.J., 2015. Tropical
797 cyanobacterial blooms: A review of prevalence, problem taxa, toxins and influencing
798 environmental factors. J. Limnol. doi:10.4081/jlimnol.2014.1005

799 Natumi, R., Janssen, E.M.L., 2020. Cyanopeptide Co-Production Dynamics beyond
800 Mirocystins and Effects of Growth Stages and Nutrient Availability. Environ. Sci.
801 Technol. 54, 6063–6072. doi:10.1021/acs.est.9b07334

802 Neumann, U., Campos, V., Cantarero, S., Urrutia, H., Heinze, R., Weckesser, J., Erhard,
803 M., 2000. Co-occurrence of non-toxic (cyanopeptolin) and toxic (microcystin)

804 peptides in a bloom of Microcystis sp. from a Chilean lake. Syst. Appl. Microbiol. 23,
805 191–197. doi:10.1016/S0723-2020(00)80004-1

806 Ortiz-Rodríguez, R., Dao, T.S., Wiegand, C., 2012. Transgenerational effects of
807 microcystin-LR on Daphnia magna. J. Exp. Biol. 215, 2795–2805.
808 doi:10.1242/jeb.069211

809 Ortiz-Rodríguez, R., Wiegand, C., 2010. Age related acute effects of microcystin-LR on
810 Daphnia magna biotransformation and oxidative stress. Toxicon 56, 1342–1349.
811 doi:10.1016/j.toxicon.2010.07.020

812 Pane, E.F., McGeer, J.C., Wood, C.M., 2004. Effects of chronic waterborne nickel
813 exposure on two successive generations of Daphnia magna. Environ. Toxicol. Chem.
814 23, 1051–6.

815 Pawlik-Skowrońska, B., Pirszel, J., Kornijów, R., 2008. Spatial and temporal variation in
816 microcystin concentrations during perennial bloom of Planktothrix agardhii in a
817 hypertrophic lake. Ann. Limnol. 44, 145–150. doi:10.1051/limn:2008015

818 Peng, S., Deng, D., He, P., Xu, X., Zhang, C., Cao, J., Liu, Q., Zhang, T., 2018. Effects of
819 Microcystis aeruginosa on the life history traits and SOD activity of Daphnia
820 similoides sinensis. Environ. Sci. Pollut. Res. 25, 30696–30707. doi:10.1007/s11356-
821 018-3040-9

822 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-
823 PCR, Nucleic Acids Research.

824 Pflugmacher, S., Wiegand, C., Oberemm, A., Beattie, K.A., Krause, E., Codd, G.A.,
825 Steinberg, C.E.W.W., 1998. Identification of an enzymatically formed glutathione
826 conjugate of the cyanobacterial hepatotoxin microcystin-LR: The first step of
827 detoxication. Biochim. Biophys. Acta - Gen. Subj. 1425, 527–533.
828 doi:10.1016/S0304-4165(98)00107-X

829 Pham, T.L., 2018. First report of microcystin-producing Microcystis (Chroococales,
830 Cyanobacteria) in a central highland Vietnam lake. Fundam. Appl. Limnol. 191, 189–
831 197. doi:10.1127/fal/2018/1124

832 Portmann, C., Blom, J.F., Gademann, K., Jüttner, F., Jüttner, F., Jüttner, F., 2008.
833 Aerucyclamides A and B: Isolation and synthesis of toxic ribosomal heterocyclic
834 peptides from the cyanobacterium Microcystis aeruginosa PCC 7806. J. Nat. Prod. 71,
835 1193–1196. doi:10.1021/np800118g

836 Rastogi, R.P., Madamwar, D., Incharoensakdi, A., 2015. Bloom dynamics of cyanobacteria
837 and their toxins: Environmental health impacts and mitigation strategies. Front.
838 Microbiol. doi:10.3389/fmicb.2015.01254

839 Rhiannon, D.M., Dakic, V., Williams, T.D., Winter, M.J., Chipman, J.K., 2011.
840 Transcriptional responses in neonate and adult Daphnia magna in relation to relative
841 susceptibility to genotoxicants. Aquat. Toxicol. 104, 192–204.
842 doi:10.1016/j.aquatox.2011.04.016

843 Rohrlack, T., Christoffersen, K., Kaebernick, M., Neilan, B.A., 2004. Cyanobacterial
844 protease inhibitor microviridin J causes a lethal molting disruption in Daphnia

845 pulicaria. Appl. Environ. Microbiol. 70, 5047–5050. doi:10.1128/AEM.70.8.5047-
846 5050.2004

847 Rohrlack, T., Dittmann, E., Börner, T., Christoffersen, K., 2001. Effects of Cell-Bound
848 Microcystins on Survival and Feeding of Daphnia spp. Appl. Environ. Microbiol. 67,
849 3523–3529. doi:10.1128/AEM.67.8.3523-3529.2001

850 Sadler, T., von Elert, E., 2014a. Physiological interaction of Daphnia and Microcystis with
851 regard to cyanobacterial secondary metabolites. Aquat. Toxicol. 156, 96–105.
852 doi:10.1016/j.aquatox.2014.08.003

853 Sadler, T., von Elert, E., 2014b. Dietary exposure of Daphnia to microcystins: No in vivo
854 relevance of biotransformation. Aquat. Toxicol. 150, 73–82.
855 doi:10.1016/j.aquatox.2014.02.017

856 Sarnelle, O., Wilson, A.E., 2005. Local adaptation of Daphnia pulicaria to toxic
857 cyanobacteria. Limnol. Oceanogr. 50, 1565–1570. doi:10.4319/lo.2005.50.5.1565

858 Schwarzenberger, A., Courts, C., von Elert, E., 2009. Target gene approaches: Gene
859 expression in Daphnia magna exposed to predator-borne kairomones or to
860 microcystin-producing and microcystin-free Microcystis aeruginosa. BMC Genomics
861 10, 527. doi:10.1186/1471-2164-10-527

862 Schwarzenberger, A., Kuster, C.J., Von Elert, E., 2012. Molecular mechanisms of tolerance
863 to cyanobacterial protease inhibitors revealed by clonal differences in Daphnia magna.
864 Mol. Ecol. 21, 4898–4911. doi:10.1111/j.1365-294X.2012.05753.x

865 Schwarzenberger, A., Zitt, A., Kroth, P., Mueller, S., Von Elert, E., 2010. Gene expression
866 and activity of digestive proteases in Daphnia: Effects of cyanobacterial protease
867 inhibitors. BMC Physiol. 10, 6. doi:10.1186/1472-6793-10-6

868 Smutná, M., Babica, P., Jarque, S., Hilscherová, K., Maršálek, B., Haeba, M., Bláha, L.,
869 2014. Acute, chronic and reproductive toxicity of complex cyanobacterial blooms in
870 Daphnia magna and the role of microcystins. Toxicon 79.
871 doi:10.1016/j.toxicon.2013.12.009

872 Street, S.M., Eytcheson, S.A., LeBlanc, G.A., 2019. The role of nuclear receptor E75 in
873 regulating the molt cycle of Daphnia magna and consequences of its disruption. PLoS
874 One 14, e0221642. doi:10.1371/journal.pone.0221642

875 Su, X., Xue, Q., Steinman, A.D., Zhao, Y., Xie, L., 2015. Spatiotemporal dynamics of
876 microcystin variants and relationships with environmental parameters in lake Taihu,
877 China. Toxins (Basel). 7, 3224–3244. doi:10.3390/toxins7083224

878 Svirčev, Z., Lalić, D., Bojadžija Savić, G., Tokodi, N., Drobac Backović, D., Chen, L.,
879 Meriluoto, J., Codd, G.A., 2019. Global geographical and historical overview of
880 cyanotoxin distribution and cyanobacterial poisonings. Arch. Toxicol. 1–53.
881 doi:10.1007/s00204-019-02524-4

882 Tong, Q., Zhang, M., Cao, X., Xu, S., Wang, D., Zhao, Y., 2017. Expression and activation
883 of Daphnia pulex Caspase-3 are involved in regulation of aging. Gene 634, 37–46.
884 doi:10.1016/j.gene.2017.08.035

885 Trinkle-Mulcahy, L., Lamond, A.I., 2006. Mitotic phosphatases: no longer silent partners.

886 Curr. Opin. Cell Biol. doi:10.1016/j.ceb.2006.09.001

887 Von Elert, E., Agrawal, M.K., Gebauer, C., Jaensch, H., Bauer, U., Zitt, A., 2004. Protease
888 activity in gut of Daphnia magna: Evidence for trypsin and chymotrypsin enzymes.
889 Comp. Biochem. Physiol. - B Biochem. Mol. Biol. 137, 287–296.
890 doi:10.1016/j.cbpc.2003.11.008

891 Von Elert, E., Oberer, L., Merkel, P., Huhn, T., Blom, J.F., 2005. Cyanopeptolin 954, a
892 chlorine-containing chymotrypsin inhibitor of Microcystis aeruginosa NIVA Cya 43.
893 J. Nat. Prod. 68, 1324–1327. doi:10.1021/np050079r

894 von Elert, E., Zitt, A., Schwarzenberger, A., 2012. Inducible tolerance to dietary protease
895 inhibitors in Daphnia magna. J. Exp. Biol. 215, 2051–2059. doi:10.1242/jeb.068742

896 von Elert, Eric, Zitt, A., Schwarzenberger, A., 2012. Inducible tolerance to dietary protease
897 inhibitors in *Daphnia magna*. J. Exp. Biol. 215, 2051–2059. doi:10.1242/jeb.068742

898 Wang, L., Peng, Y., Nie, X., Pan, B., Ku, P., Bao, S., 2016. Gene response of CYP360A,
899 CYP314, and GST and whole-organism changes in Daphnia magna exposed to
900 ibuprofen. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 179, 49–56.
901 doi:10.1016/j.cbpc.2015.08.010

902 Welker, M., Von Döhren, H., 2006. Cyanobacterial peptides - Nature's own combinatorial
903 biosynthesis. FEMS Microbiol. Rev. doi:10.1111/j.1574-6976.2006.00022.x

904 Wiegand, C., Peuthert, A., Pflugmacher, S., Carmeli, S., 2002. Effects of microcin SF608
905 and microcystin-LR two cyanotobacterial compounds produced by Microcystis sp., on
906 aquatic organisms. Environ. Toxicol. 17, 400–406. doi:10.1002/tox.10065

907 Wiegand, C., Pflugmacher, S., Oberemm, A., Steinberg, C., 2000. Activity development of
908 selected detoxication enzymes during the ontogenesis of the zebrafish (Danio rerio).
909 Int. Rev. Hydrobiol. 85, 413–422. doi:10.1002/1522-2632(200008)85:4<413::AID-
910 IROH413>3.0.CO;2-3

911 Wojtal-Frankiewicz, A., Bernasi??ska, J., Jurczak, T., Gwo??dzi??ski, K., Frankiewicz, P.,
912 Wielanek, M., 2013. Microcystin assimilation and detoxification by Daphnia spp. in
913 two ecosystems of different cyanotoxin concentrations. J. Limnol. 72, 154–171.
914 doi:10.4081/jlimnol.2013.e13

915 Wojtal-Frankiewicz, A., Bernasińska, J., Frankiewicz, P., Gwoździński, K., Jurczak, T.,
916 2014. Response of daphnia' s antioxidant system to spatial heterogeneity in
917 cyanobacteria concentrations in a lowland reservoir. PLoS One.
918 doi:10.1371/journal.pone.0112597

919 Ziemert, N., Ishida, K., Weiz, A., Hertweck, C., Dittmann, E., 2010. Exploiting the natural
920 diversity of microviridin gene clusters for discovery of novel tricyclic depsipeptides.
921 Appl. Environ. Microbiol. 76, 3568–3574. doi:10.1128/AEM.02858-09

922 Zurawell, R.W., Chen, H., Burke, J.M., Prepas, E.E., 2005. Hepatotoxic cyanobacteria: a
923 review of the biological importance of microcystins in freshwater environments. J.
924 Toxicol. Environ. Health. B. Crit. Rev. 8, 1–37. doi:10.1080/10937400590889412

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Supplementary 1. qPCR CT values for all *Daphnia* genes observed in the study under different conditions (HC and LC treatment) and control observed after 3 and 7 days

Gene name	Sample name	CT
Glyceraldehyde-3-phosphate dehydrogenase		
	REPLICATE_1_HC_7days	25.16
	REPLICATE_2_HC_7days	26.31
	REPLICATE_3_HC_7days	25.11
	REPLICATE_4_HC_7days	25.28
	REPLICATE_5_HC_7days	27.22
	REPLICATE_1_HC_3days	26.32
	REPLICATE_2_HC_3days	26.36
	REPLICATE_3_HC_3days	26.52
	REPLICATE_4_HC_3days	24.95
	REPLICATE_5_HC_3days	26.6
	REPLICATE_1_LC_7days	27.7
	REPLICATE_2_LC_7days	27.04
	REPLICATE_3_LC_7days	25.76
	REPLICATE_4_LC_7days	27.93
	REPLICATE_5_LC_7days	27.52
	REPLICATE_1_LC_3days	
	REPLICATE_2_LC_3days	25.46
	REPLICATE_3_LC_3days	24.93
	REPLICATE_4_LC_3days	24.96
	REPLICATE_5_LC_3days	26.38
	REPLICATE_1_Control_7days	26.56
	REPLICATE_2_Control_7days	24.89
	REPLICATE_3_Control_7days	24.12
	REPLICATE_4_Control_7days	22.88
	REPLICATE_5_Control_7days	22.73
	REPLICATE_1_Control_3days	27.65
	REPLICATE_2_Control_3days	28.46
	REPLICATE_3_Control_3days	26.68
	REPLICATE_4_Control_3days	28.4
	REPLICATE_5_Control_3days	
Protease_CT383		
	REPLICATE_1_HC_7days	28.58

	REPLICATE_2_HC_7days	29.18
	REPLICATE_3_HC_7days	29.61
	REPLICATE_4_HC_7days	28.56
	REPLICATE_5_HC_7days	28.49
	REPLICATE_1_HC_3days	27.9
	REPLICATE_2_HC_3days	28.45
	REPLICATE_3_HC_3days	28.10
	REPLICATE_4_HC_3days	29.14
	REPLICATE_5_HC_3days	29.04
	REPLICATE_1_LC_7days	28.37
	REPLICATE_2_LC_7days	27.97
	REPLICATE_3_LC_7days	26.58
	REPLICATE_4_LC_7days	29.31
	REPLICATE_5_LC_7days	27.60
	REPLICATE_1_LC_3days	
	REPLICATE_2_LC_3days	29.35
	REPLICATE_3_LC_3days	27.57
	REPLICATE_4_LC_3days	29.88
	REPLICATE_5_LC_3days	28.59
	REPLICATE_1_Control_7days	28.99
	REPLICATE_2_Control_7days	26.55
	REPLICATE_3_Control_7days	26.77
	REPLICATE_4_Control_7days	26.88
	REPLICATE_5_Control_7days	26.19
	REPLICATE_1_Control_3days	28.15
	REPLICATE_2_Control_3days	29.49
	REPLICATE_3_Control_3days	27.09
	REPLICATE_4_Control_3days	27.88
	REPLICATE_5_Control_3days	
Glutathione S-transferase		
	REPLICATE_1_HC_7days	27.64
	REPLICATE_2_HC_7days	27.74
	REPLICATE_3_HC_7days	27.13
	REPLICATE_4_HC_7days	28.36
	REPLICATE_5_HC_7days	29.34
	REPLICATE_1_HC_3days	27.49
	REPLICATE_2_HC_3days	27.88
	REPLICATE_3_HC_3days	
	REPLICATE_4_HC_3days	27.18

	REPLICATE_5_HC_3days	
	REPLICATE_1_LC_7days	
	REPLICATE_2_LC_7days	28.67
	REPLICATE_3_LC_7days	27.72
	REPLICATE_4_LC_7days	28.39
	REPLICATE_5_LC_7days	27.72
	REPLICATE_1_LC_3days	27.74
	REPLICATE_2_LC_3days	28.61
	REPLICATE_3_LC_3days	27.18
	REPLICATE_4_LC_3days	26.60
	REPLICATE_5_LC_3days	
	REPLICATE_1_Control_7days	29.16
	REPLICATE_2_Control_7days	26.68
	REPLICATE_3_Control_7days	26.61
	REPLICATE_4_Control_7days	25.59
	REPLICATE_5_Control_7days	24.96
	REPLICATE_1_Control_3days	29.27
	REPLICATE_2_Control_3days	29.43
	REPLICATE_3_Control_3days	28.32
	REPLICATE_4_Control_3days	28.52
	REPLICATE_5_Control_3days	
Glutathione peroxidase		
	REPLICATE_1_HC_7days	
	REPLICATE_2_HC_7days	28.08
	REPLICATE_3_HC_7days	
	REPLICATE_4_HC_7days	27.06
	REPLICATE_5_HC_7days	28.80
	REPLICATE_1_HC_3days	28.87
	REPLICATE_2_HC_3days	
	REPLICATE_3_HC_3days	29.56
	REPLICATE_4_HC_3days	
	REPLICATE_5_HC_3days	28.85
	REPLICATE_1_LC_7days	28.87
	REPLICATE_2_LC_7days	29.14
	REPLICATE_3_LC_7days	27.46
	REPLICATE_4_LC_7days	28.50
	REPLICATE_5_LC_7days	28.56
	REPLICATE_1_LC_3days	

	REPLICATE_2_LC_3days	28.21
	REPLICATE_3_LC_3days	28.88
	REPLICATE_4_LC_3days	27.32
	REPLICATE_5_LC_3days	28.43
	REPLICATE_1_Control_7days	28.92
	REPLICATE_2_Control_7days	25.99
	REPLICATE_3_Control_7days	26.55
	REPLICATE_4_Control_7days	24.38
	REPLICATE_5_Control_7days	24.29
	REPLICATE_1_Control_3days	28.46
	REPLICATE_2_Control_3days	28.86
	REPLICATE_3_Control_3days	26.62
	REPLICATE_4_Control_3days	28.13
	REPLICATE_5_Control_3days	
Catalase		
	REPLICATE_1_HC_7days	27.95
	REPLICATE_2_HC_7days	26.92
	REPLICATE_3_HC_7days	27.83
	REPLICATE_4_HC_7days	26.73
	REPLICATE_5_HC_7days	27.45
	REPLICATE_1_HC_3days	26.61
	REPLICATE_2_HC_3days	28.75
	REPLICATE_3_HC_3days	28.38
	REPLICATE_4_HC_3days	25.9
	REPLICATE_5_HC_3days	27.41
	REPLICATE_1_LC_7days	28.19
	REPLICATE_2_LC_7days	27.34
	REPLICATE_3_LC_7days	25.49
	REPLICATE_4_LC_7days	25.65
	REPLICATE_5_LC_7days	25.75
	REPLICATE_1_LC_3days	27.69
	REPLICATE_2_LC_3days	26.3
	REPLICATE_3_LC_3days	25.84
	REPLICATE_4_LC_3days	25.48
	REPLICATE_5_LC_3days	
	REPLICATE_1_Control_7days	27.35
	REPLICATE_2_Control_7days	24.56
	REPLICATE_3_Control_7days	24.62
	REPLICATE_4_Control_7days	22.34
	REPLICATE_5_Control_7days	22.30

	REPLICATE_1_Control_3days	27.28
	REPLICATE_2_Control_3days	28.09
	REPLICATE_3_Control_3days	26.47
	REPLICATE_4_Control_3days	26.87
	REPLICATE_5_Control_3days	
Thioredoxin		
	REPLICATE_1_HC_7days	27.7
	REPLICATE_2_HC_7days	28.10
	REPLICATE_3_HC_7days	28.88
	REPLICATE_4_HC_7days	27.45
	REPLICATE_5_HC_7days	28.99
	REPLICATE_1_HC_3days	27.56
	REPLICATE_2_HC_3days	27.35
	REPLICATE_3_HC_3days	28.62
	REPLICATE_4_HC_3days	28.08
	REPLICATE_5_HC_3days	27.98
	REPLICATE_1_LC_7days	25.74
	REPLICATE_2_LC_7days	29.09
	REPLICATE_3_LC_7days	27.62
	REPLICATE_4_LC_7days	27.38
	REPLICATE_5_LC_7days	
	REPLICATE_1_LC_3days	27.68
	REPLICATE_2_LC_3days	29.36
	REPLICATE_3_LC_3days	25.95
	REPLICATE_4_LC_3days	26.81
	REPLICATE_5_LC_3days	
	REPLICATE_1_Control_7days	28.03
	REPLICATE_2_Control_7days	25.74
	REPLICATE_3_Control_7days	25.66
	REPLICATE_4_Control_7days	23.89
	REPLICATE_5_Control_7days	23.55
	REPLICATE_1_Control_3days	28.51
	REPLICATE_2_Control_3days	29.02
	REPLICATE_3_Control_3days	26.89
	REPLICATE_4_Control_3days	27.54
	REPLICATE_5_Control_3days	
Histone 2A		
	REPLICATE_1_HC_7days	28.48
	REPLICATE_2_HC_7days	26.48
	REPLICATE_3_HC_7days	26.61

	REPLICATE_4_HC_7days	26.83
	REPLICATE_5_HC_7days	
	REPLICATE_1_HC_3days	28.93
	REPLICATE_2_HC_3days	
	REPLICATE_3_HC_3days	
	REPLICATE_4_HC_3days	27.43
	REPLICATE_5_HC_3days	27.26
	REPLICATE_1_LC_7days	28.52
	REPLICATE_2_LC_7days	27.82
	REPLICATE_3_LC_7days	27.76
	REPLICATE_4_LC_7days	26.58
	REPLICATE_5_LC_7days	
	REPLICATE_1_LC_3days	
	REPLICATE_2_LC_3days	27.84
	REPLICATE_3_LC_3days	27.43
	REPLICATE_4_LC_3days	27.29
	REPLICATE_5_LC_3days	28.22
	REPLICATE_1_Control_7days	27.74
	REPLICATE_2_Control_7days	28.01
	REPLICATE_3_Control_7days	27.8
	REPLICATE_4_Control_7days	24.86
	REPLICATE_5_Control_7days	24.78
	REPLICATE_1_Control_3days	27.71
	REPLICATE_2_Control_3days	
	REPLICATE_3_Control_3days	28.35
	REPLICATE_4_Control_3days	28.52
	REPLICATE_5_Control_3days	
Histone 2A		
	REPLICATE_1_HC_7days	28.48
	REPLICATE_2_HC_7days	26.48
	REPLICATE_3_HC_7days	26.61
	REPLICATE_4_HC_7days	26.83
	REPLICATE_5_HC_7days	
	REPLICATE_1_HC_3days	28.93
	REPLICATE_2_HC_3days	
	REPLICATE_3_HC_3days	
	REPLICATE_4_HC_3days	27.43
	REPLICATE_5_HC_3days	27.26
	REPLICATE_1_LC_7days	28.52

	REPLICATE_2_LC_7days	27.82
	REPLICATE_3_LC_7days	27.76
	REPLICATE_4_LC_7days	26.58
	REPLICATE_5_LC_7days	
	REPLICATE_1_LC_3days	
	REPLICATE_2_LC_3days	27.84
	REPLICATE_3_LC_3days	27.43
	REPLICATE_4_LC_3days	27.29
	REPLICATE_5_LC_3days	28.22
	REPLICATE_1_Control_7days	27.74
	REPLICATE_2_Control_7days	28.01
	REPLICATE_3_Control_7days	27.8
	REPLICATE_4_Control_7days	24.86
	REPLICATE_5_Control_7days	24.78
	REPLICATE_1_Control_3days	27.71
	REPLICATE_2_Control_3days	
	REPLICATE_3_Control_3days	28.35
	REPLICATE_4_Control_3days	28.52
	REPLICATE_5_Control_3days	
Myosin heavy chain		
	REPLICATE_1_HC_7days	26.38
	REPLICATE_2_HC_7days	24.79
	REPLICATE_3_HC_7days	26.14
	REPLICATE_4_HC_7days	25.18
	REPLICATE_5_HC_7days	25.68
	REPLICATE_1_HC_3days	25.61
	REPLICATE_2_HC_3days	25.97
	REPLICATE_3_HC_3days	26.57
	REPLICATE_4_HC_3days	26.86
	REPLICATE_5_HC_3days	27.14
	REPLICATE_1_LC_7days	
	REPLICATE_2_LC_7days	27.46
	REPLICATE_3_LC_7days	25.04
	REPLICATE_4_LC_7days	27.51
	REPLICATE_5_LC_7days	26.35
	REPLICATE_1_LC_3days	25.29
	REPLICATE_2_LC_3days	24.82
	REPLICATE_3_LC_3days	23.99
	REPLICATE_4_LC_3days	24.42

	REPLICATE_5_LC_3days	
	REPLICATE_1_Control_7days	26.50
	REPLICATE_2_Control_7days	23.16
	REPLICATE_3_Control_7days	23.13
	REPLICATE_4_Control_7days	21.78
	REPLICATE_5_Control_7days	21.84
	REPLICATE_1_Control_3days	25.11
	REPLICATE_2_Control_3days	27.08
	REPLICATE_3_Control_3days	23.37
	REPLICATE_4_Control_3days	25.02
	REPLICATE_5_Control_3days	
Nuclear hormone receptor HR3		
	REPLICATE_1_HC_7days	27.62
	REPLICATE_2_HC_7days	29.08
	REPLICATE_3_HC_7days	30.45
	REPLICATE_4_HC_7days	26.97
	REPLICATE_5_HC_7days	27.17
	REPLICATE_1_HC_3days	28.18
	REPLICATE_2_HC_3days	28.39
	REPLICATE_3_HC_3days	28.22
	REPLICATE_4_HC_3days	29.15
	REPLICATE_5_HC_3days	28.23
	REPLICATE_1_LC_7days	30.52
	REPLICATE_2_LC_7days	27.39
	REPLICATE_3_LC_7days	27.75
	REPLICATE_4_LC_7days	27.28
	REPLICATE_2_LC_3days	27.32
	REPLICATE_3_LC_3days	25.24
	REPLICATE_4_LC_3days	26.3
	REPLICATE_5_LC_3days	27.03
	REPLICATE_1_Control_7days	28.18
	REPLICATE_2_Control_7days	29.00
	REPLICATE_3_Control_7days	26.17
	REPLICATE_4_Control_7days	27.15
	REPLICATE_5_Control_7days	29.44
	REPLICATE_1_Control_3days	29.11
	REPLICATE_2_Control_3days	30.13

	REPLICATE_4_Control_3days	27.28
	REPLICATE_5_Control_3days	29.90
Nuclear hormone receptor FTZ-F1		
	REPLICATE_1_HC_7days	28.63
	REPLICATE_2_HC_7days	27.32
	REPLICATE_3_HC_7days	27.97
	REPLICATE_4_HC_7days	26.26
	REPLICATE_5_HC_7days	29.01
	REPLICATE_1_HC_3days	26.78
	REPLICATE_2_HC_3days	27.45
	REPLICATE_3_HC_3days	29.20
	REPLICATE_4_HC_3days	26.46
	REPLICATE_5_HC_3days	29.74
	REPLICATE_1_LC_7days	28.28
	REPLICATE_2_LC_7days	29.15
	REPLICATE_3_LC_7days	27.16
	REPLICATE_4_LC_7days	27.62
	REPLICATE_5_LC_7days	27.84
	REPLICATE_1_LC_3days	
	REPLICATE_2_LC_3days	26.06
	REPLICATE_3_LC_3days	26.57
	REPLICATE_4_LC_3days	25.09
	REPLICATE_5_LC_3days	26.33
	REPLICATE_1_Control_7days	27.47
	REPLICATE_2_Control_7days	24.40
	REPLICATE_3_Control_7days	24.70
	REPLICATE_4_Control_7days	23.75
	REPLICATE_5_Control_7days	24.60
	REPLICATE_1_Control_3days	27.63
	REPLICATE_2_Control_3days	
	REPLICATE_3_Control_3days	26.25
	REPLICATE_4_Control_3days	27.37
	REPLICATE_5_Control_3days	
β actin		
	REPLICATE_1_HC_7days	25.55
	REPLICATE_2_HC_7days	25.55
	REPLICATE_3_HC_7days	26.10
	REPLICATE_4_HC_7days	26.09

REPLICATE_5_HC_7days	26.79
REPLICATE_1_HC_3days	24.94
REPLICATE_2_HC_3days	25.79
REPLICATE_3_HC_3days	26.25
REPLICATE_4_HC_3days	24.56
REPLICATE_5_HC_3days	25.51
REPLICATE_1_LC_7days	25.86
REPLICATE_2_LC_7days	25.55
REPLICATE_3_LC_7days	24.78
REPLICATE_4_LC_7days	25.60
REPLICATE_5_LC_7days	25.49
REPLICATE_1_LC_3days	
REPLICATE_2_LC_3days	24.21
REPLICATE_3_LC_3days	23.21
REPLICATE_4_LC_3days	24.11
REPLICATE_5_LC_3days	25.27
REPLICATE_1_Control_7days	25.33
REPLICATE_2_Control_7days	24.50
REPLICATE_3_Control_7days	24.54
REPLICATE_4_Control_7days	24.91
REPLICATE_5_Control_7days	21.56
REPLICATE_1_Control_3days	26.08
REPLICATE_2_Control_3days	24.91
REPLICATE_3_Control_3days	23.82
REPLICATE_4_Control_3days	25.40
REPLICATE_5_Control_3days	