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An Examination of Cyanotoxins and Environmental Estrogens in the Middle and Upper Potomac River Watershed, USA

Ryan Patrick Braham

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**An examination of cyanotoxins and environmental estrogens in the middle
and upper Potomac River watershed, USA**

Ryan Patrick Braham

Dissertation submitted to the
Davis College of Agriculture, Natural Resource and Design
at West Virginia University
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy in
Forest Resources Science

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Abstract

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Potomac River watershed, USA

Ryan Patrick Braham

The Potomac River is a large tributary of the Chesapeake Bay. Large fish die-offs were reported in the Potomac watershed (specifically the South Branch of the Potomac) in 2002 and 2005, as well as smaller episodic events in recent years which have no clear correlation to any one pathogen or contaminant. Although a variety of pathogens have been identified (such as trematodes, myxozoans, *Aeromonas hydrophila*, *A. salmonicida*, *Flavobacterium columnare*, and largemouth bass virus) in recent die-offs of smallmouth bass *Micropterus dolomieu*, no clear cause has been derived. Additionally, reproductive endpoints were reported in smallmouth bass and likely associated with instream estrogenic compounds. There has been increasing support for estrogenic compounds associated with agricultural runoff as one of the causes. However, there are little data on the contribution from primary producers such as cyanobacteria. Harmful algal blooms are of increasing concern to human health worldwide and their effects on the aquatic community within the South Branch Potomac watershed is largely unknown. To address knowledge gaps associated with fish health in the South Branch Potomac watershed, an assessment of the temporal and spatial distribution of algal toxins and environmental estrogens was required. This dissertation addresses: 1) the spatial and temporal distribution of cyanobacterial toxins in water, periphyton, and fish tissue; 2) the transcriptional response of smallmouth bass hepatocytes and leukocytes challenged with the cyanobacterial toxin microcystin-LR; 3) the spatial and temporal distribution of environmental estrogens in water and

periphyton and their association with phytoestrogen presence and cyanobacterial toxins; and 4)
an assessment of reporter interference in water and periphyton samples.

Dedication

For Missy

Acknowledgements

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Foreword

This dissertation is comprised of five chapters and has been written in the style of manuscripts associated with journals. Chapter two is written in the format of Environmental Monitoring and Assessment. Chapter three is written in the format of Journal of Comparative Biochemistry and Physiology: Part D. Chapter four is written in the style of Water Research. Chapter five is written in the format of Cytometry Part A.

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Chapter One. Introduction and Literature Review

Microcystins in the aquatic environment and fish tissue

Cyanobacteria are found throughout marine, freshwater, and terrestrial habitats. Some genera of cyanobacteria produce chemical toxins (cyanotoxins) that have been implicated in human and animal illness and death (Codd et al. 2005; Lopez et al. 2008). Toxins may also be produced by eukaryotic taxa (Landsberg 2002). The generic term for these compounds produced by both prokaryotic and eukaryotic organisms is algal toxins. Algal toxins can be sorted according to their primary effects which include neurotoxins, hepatotoxins, cytotoxins and irritants (Sivonen 2009). A variety of taxa have been identified that produce these toxins (Carmichael 1992b; Carmichael 1997; Dittmann and Wiegand 2006; Leflaive and Ten-Hage 2007). Under the current climate change projections, there is a growing global concern over the increased distribution and frequency of harmful algal blooms (Paerl and Huisman 2009).

Hepatotoxins are a family of cyanotoxins that are characterized by their toxic effects on hepatic tissue originating from various cyanobacteria taxa. The hepatotoxin microcystin (MC) is likely the most prevalent cyanotoxin in the environment comprising up to 1% dry weight of the total cyanobacteria biomass (Blaha et al. 2009; Pavagadhi and Balasubramanian 2013).

Microcystin is a cyclic heptapeptide with over 80 known structural variants (Welker and von Dohren 2006). Microcystins contain 7 distinct regions with the aromatic β -amino acid side chain 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA) being highly conserved among all 89 known variants at position 5 (Carmichael 1997). The ADDA side chain facilitates transport into hepatic tissue (Campas and Marty 2007). This likely occurs via organic anion transporting peptides (Sierlslawska 2010). Microcystins impair fishes as a potent inhibitor of serine/threonine-specific protein phosphatases 1 and 2A (PP1 and PP2A, respectively;

Runnegar et al. (1995)). Research with the MC structural variant, MCLR (containing the amino acids L-leucine and L-arginine at the variable positions 2 and 4, respectively) found that the inhibition of the PP1 and PP2A resulting from exposure was similar to the known PP1 and PP2A inhibitor and tumor promoter effects of okadaic acid suggesting that MCLR toxin may also act as a tumor promoter similar to the okadaic acid pathway (Carmichael 1992a; Carmichael 1997; Nishiwaki-Matsushima et al. 1992). The specific inhibition of PP1 and PP2A by MCLR has been refined to specifically describe the inhibition of the catalytic subunits of PP1 and PP2A, as well as the ATP-synthase beta-subunit (Mikhailov et al. 2003). Inhibition is mediated by the ADDA side chain and the glutamyl carboxyl located at positions 5 and 6, respectively. Covalent linkage of the thiol group of the cysteine-273 or -266 (for PP1 and PP2A, respectively) is bound with the α , β -unsaturated carbonyl of the methylthioalanine (Mdha) group located at position 7 (Dawson 1998).

Additional effects (generally in the liver) have been observed resulting from exposure to MC toxin. For example, disruption of PP1 is believed to cause a disruption in intracellular glucose levels (Dawson 1998). When PP1 is in an inactive state, phosphorylase a remains tightly bound in an active, phosphorylated state and thus drives the conversion of glycogen to glucose (Goldberg et al. 1995). In a normal cell, under the influence of insulin, PP1 activates and phosphorylase a is deactivated into phosphorylase b which terminates the production of glucose. Microcystins bind PP1 in an inactive state which antagonizes the feedback from insulin. Over time, this will result in the loss of intracellular glycogen stores and may cause a downstream cascade to compensate for the increased levels of glucose. A second effect of MC toxin involves the protein actin (Ghosh et al. 1995). Specifically, MC appears to reorganize α -actinin and talin. This reorganization is believed to be responsible for the breakdown of microtubules and

intermediate filaments. It is believed that this restructuring is responsible for the collapse of sinusoidal capillaries which results in hemorrhaging (Dittmann and Wiegand 2006).

Although much of the documented effects from MC exposure focuses on acute toxicity, there is other research suggesting that MC exposure may be genotoxic (reviewed by Zegura et al. (2011)). Although the authors describe minimal evidence of genotoxicity of bacterial cells, they provide a thorough review of genotoxicity in mammalian cell lines. Furthermore, they review a suite of genes associated with DNA repair, tumor suppression, and tumor promotion which are modulated by exposure to MC's. The specific mode-of-action for the observed genotoxicity of MCLR is still debated. A likely scenario is that damage results not from MC directly but rather by the downstream effects from inhibition of PP1 and PP2A (Dias et al. 2014). Specifically, they theorize that the breakdown of microtubules results in strand breakage.

Microcystins have widely variable presence in the environment. O'Neil et al. (2012) provide a thorough review of the status of cyanobacterial blooms specifically from those genera which produce cyanotoxins. They describe in detail the difficulties in attributing blooms to water quality metrics such as eutrophication because the ability to either fix nitrogen or readily uptake phosphorous (P) is variable among species. This can be confounded by rapid changes in species composition resulting from a high flow event. Among MC-producing cyanobacteria, the shift in species richness can be tracked via the variants observed as presented in Pawlik-Skowrońska et al. (2013). They observed an inverse correlation with total P in the water column and total intracellular MC among cyanobacteria. They also observed a slight shift away from the variants –LA, –LY, and –LF to variant –LR which they believe was associated with an increased abundance of *Microcystis* spp. in the water column. Along with variability associated with species richness and nutrient availability, MC concentrations in the water column have been

shown to fluctuate seasonally (Billam et al. 2006). They observed a reduction in total MC's in the warmer months with elevated concentrations in the cooler months. The authors suggest that the conditions in the warmer months were above the thermal threshold for the likely MC-producing cyanobacteria present in their system (*Microcystis* spp).

Much of the environmental research on MC's has focused on pelagic-oriented genera (*Anabaena*, *Microcystis*, *Aphanizomenon*, etc.). However, the presence of MC toxins in benthic cyanobacterial mats has also been documented and introduced as a potential health risk to pets and livestock (Codd et al. 1992; Mez et al. 1996; Mez et al. 1997). The genera identified were *Oscillatoria*, *Phormidium*, *Tolypothrix* and *Stigonema*. Mez et al. (1997) were able to detect protein phosphatase inhibition believed to be associated with MC in all 4 of these genera in Switzerland. However, only the species *O. limosa* and *P. konstantinosum* were able to cause mortalities via intraperitoneal injection (IP) in mice challenges. *P. autumnale* was not toxic to mice nor induced protein phosphatase inhibition but did have detectable concentrations of MC. Mohamed et al. (2006) identified a suite of benthic cyanobacterial species in Egyptian irrigation canals, of which 5 species produced MC as detected by an enzyme-linked immunosorbent assay (ELISA; *Anabaena subcylindrica*, *Anabaena variables*, *Nostoc spongiaeforme*, *Plectonema boryanum*, *P. corium*). Izaguirre et al. (2007) detected positive MC samples from southern California reservoirs. The isolates investigated were similar to the genera *Oscillatoria*, *Lyngbya*, and *Phormidium*. Although they did not make any final determination on the exact taxonomy, they did add to the phylogenetic tree of benthic-oriented MC-producing cyanobacteria. In New Zealand, MC variants were identified as likely responsible for dog deaths (Wood et al. 2010). The species implicated were morphologically similar to *Phormidium* but genetically similar to *Planktothrix* spp. Overall, benthic-oriented cyanobacteria have been shown to contribute

significantly to the total MC load of aquatic systems and should be of interest to resource managers.

The presence and effects of MC's are well documented in the aquatic food web. Ferrao-Filho Ada and Kozlowsky-Suzuki (2011) provide a review of the presence of MC's among functional feeding groups of aquatic organisms. They provide a review of invertebrates (55 studies grouped by crustaceans, bivalves, gastropods, and zooplankton), as well as fish (28 studies grouped by carnivores, herbivores, omnivores, and planktivores). Overall, the zooplankton and planktivorous groups had the highest MC concentrations while crustaceans and carnivores had the lowest; however the data varied greatly among the representative groupings. Within fish, MC has been shown to accumulate in a variety of tissues such as the liver, intestine, kidney, etc. (e.g. Mohamed et al. 2003). In general, liver and kidneys had the most consistent MC concentrations across study sites while muscle tissue had the lowest in the omnivorous planktivore *Oreochromis niloticus*. Microcystin concentrations in the gut were variable across their sites. This would likely suggest that liver and kidneys are a more informative tissue of MC exposure while the intestine may help identify source (water vs. diet). Xie et al. (2005) provide a comprehensive analysis across multiple tissues in various fish species in China. They analyzed fish according to their functional feeding group (phytoplanktivorous, herbivorous, omnivorous, and carnivorous). Overall, carnivorous species had the highest MC concentrations in the liver and muscle. There was low reporting of MC in the blood and inconsistent data in the kidney. They observed high MC concentrations in the intestine of the phytoplanktivorous species similar to Mohamed et al. (2003). Interestingly, they also reported MC in the bile across taxa which were believed to be the first field study to report those results. Lei et al. (2008) evaluated the temporal distribution of MC in *Carassius auratus* after IP injection. After one hour, they

detected MC in all ten tissues (heart, spleen, gill, gonad, liver, brain, gallbladder, kidney, intestine, and muscle) with the highest concentrations reported in the heart, liver, and kidney. The brain, liver, gallbladder, and kidney all reported relatively higher concentrations of MC after 48 hours with the concentrations in the kidney increasing. This is likely the result from the detoxification of the toxin through the organs. Finally, Chen et al. (2009) quantified MC across tissues (intestinal contents, intestinal wall, liver, muscle, and gonad) and across aquatic species including periphyton (scum), four species of fish (*Hypophthalmichthys molitrix* (phytoplanktivorous), *Cyprinus carpio* (benthivorous), the *C. auratus* (omnivorous), and the *Culter ilishaeformis* (carnivorous)), a turtle (*Pelodiscus sinensis*), a piscivorous heron (*Nycticorax nycticorax*), and a duck (*Anas platyrhynchos*). Similar to earlier research, they observed MC in most tissues with the highest concentrations in the intestinal contents and walls of the phytoplanktivorous *Hypophthalmichthys molitrix*. In general, the liver and muscle reported the most consistent MC concentrations across taxa with the gonad yielding variable concentrations. Based on the literature, the liver is the most likely organ to determine MC exposure across taxa and across functional feeding groups.

Cyanobacterial Toxins Research Objectives

Cyanobacterial blooms (specifically *Microcystis aeruginosa*) have been reported in the lower Potomac River in 1983 (Kroghmann et al. 1986). They also provide a summary of additional blooms of cyanobacteria reported as early as 1930. The specific cause of these blooms is unclear; however it is believed to be partially driven by low water years (Kroghmann et al. 1986). The evaluation of cyanotoxins, specifically the molecular effects observed within fish tissue from individuals collected within the Potomac River drainage may yield insight as to the overall health of both fish populations and the accompanying ecosystem. Our research objective

is to quantify the abundance and distribution of MC's in the water column and benthos, as well as the bioaccumulation in hepatic tissues from fishes within the upper and middle Potomac River watershed.

Evaluation of transcript abundance and possible association with total microcystins

Much of the transcriptional MC research involves either the indirect effects from its metabolic detoxification or the downstream effects of unregulated phosphorylation. For example, Pflugmacher et al. (1998) focused on the effects of glutathione (GSH) and its conjugation enzyme glutathione-S-transferase (GST) which is believed to be the first step in MC detoxification. The antioxidant GSH is well documented as an oxidizer of compounds (Jones et al. 1978). However, this oxidization can lead to damage termed oxidative stress. Bouaicha and Maatouk (2004) evaluated rat hepatocytes for the effects of oxidative stress resulting from exposure to MCLR. Glutathione increased significantly 3 hours post exposure, however, it was not different than the control 24 and 48 hours post exposure. This was mirrored by the increase in reactive oxygen species (ROS) 0-60 minutes post-exposure. These data suggest a sharp increase in oxidative stress resulting from exposure to MCLR as GSH is a powerful antioxidant. Jayaraj et al. (2006) showed a decrease in GSH over time resulting from exposure to 38.31 μ g/kg and 76.62 μ g/kg MCLR. This was theorized to result from an exhaustion of stores of intracellular GSH. A similar decrease in the enzymes glutathione peroxidase (GPX) and superoxide dismutase (SOD) was observed and associated with oxidative stress. They did observe a time-dependent increase in the genes that encode heat shock protein-70 (HSP70) and glutathione reductase (GR) at the high dose which would correspond with a response to oxidative stress. Microcystin-LR has also been shown to affect genes associated with signaling, metabolism, and apoptosis. Clark et al. (2007) examined a microarray on a suite of genes in mice livers associated

with a variety of cellular activities. Real-time PCR results verified an upregulation of the genes *cyst B*, *gss*, *gstm3*, *nqo1*, and *jun* following chronic exposure (IP) to 40 µg/kg body weight MCLR. Using a similar study design, Clark et al. (2008) exposed p53 deficient mice to the same concentration of MCLR. Analysis of similar genes showed a similar upregulation of the above-mentioned genes; however most genes had a much higher fold-increase among the p53 deficient mice. This would suggest that p53 is an important modulator of MCLR within the liver. Interestingly, they also observed an upregulation of the gene that encodes the protein p21 (a cyclin-dependant kinase inhibitor). The protein p21 is generally assumed to be under the control of p53. The increase in the mutant mice would suggest that p21 can act independent of p53 following exposure to MCLR.

Microcystin-LR has also been evaluated on more primitive forms of life such as fishes *in vivo*. Liang et al. (2007) examined the expression of GST genes in the livers of three fish species; the silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idellus*), and Nile tilapia (*Oreochromis nilotica*). Specifically, they examined both the α class GST gene which corresponds to the cytosolic GST in mammals and a unique ρ class GST gene. Interestingly, all three species had different responses following eight and 24 hours post-IP injection. Silver carp expressed a significant increase in both GST classes eight hours post-exposure. After 24 hours, the α class was still significantly elevated while the ρ class was expressed below control levels. In the grass carp, the α class expressed a significant reduction at both eight and 24 hours post-injection while there was no change in the ρ class GST. The α class GST in Nile tilapia significantly decreased 8 hours post-injection, then significantly increased 24 hours post-injection. The ρ class GST changed expression in the same trend as the silver carp. The authors postulate that the differential species-level response to detoxification of MCLR within tissue

among these species may result from evolutionary tolerance to MC exposure. He et al. (2010) evaluated these three species for the expression of HSP70 in the liver. The chaperonin HSP70 was chosen as the authors were interested in evaluating the possible mechanisms present within each species to protect against oxidative stress which would result (for example) from exposure to MCLR. Each individual received a 50 µg/kg IP injection of MCLR. While both the grass carp and Nile tilapia both showed an increase in HSP70 expression 8 hours post-exposure, only the Nile tilapia was significant at the $\alpha = 0.05$ level. They observed no change in the silver carp. This may suggest a genus-, if not species-level mechanism to metabolize the toxic effects of MCLR. Similar to Liang et al. (2007), the authors postulate an evolutionary link to MC tolerance among these species.

Microcystin-LR has been shown to interfere with the expression genes associated with exposure to endocrine disrupting compounds (Sychrová et al. 2012). Specifically, low doses on MCLR interferes with the gene that encodes the enzyme luciferase in the mammalian MELN cell line (Oziol and Bouaicha 2010). Following exposure, the luciferase gene was significantly upregulated. The authors suggest that this disruption results from the interference to the estrogen receptor by MCLR. Rogers et al. (2011) observed a similar upregulation in the gene that encodes vitellogenin (*vtg*, *vtg1*, *vtg2*, and *vtg3*) in zebrafish (*Danio rerio*). Larvae were exposed 72 hours post fertilization to 100 µg/L and 1000 µg/L MCLR, as well as lyophilized *Microcystis aeruginosa* cells. Interestingly, the observed upregulation was only reported in individuals exposed to *M. aeruginosa* and not those exposed to MCLR. Marie et al. (2012) conducted a similar experiment on medaka (*Oryzias latipes*). Individuals were exposed (oral gavage) to an extract of the cyanobacteria *Planktothrix agardhii* which contained 2.5 µg of MCLR. Using a microarray, 15 genes that encode proteins associated with oxidative stress were identified and

differentially expressed among challenged fish. The gene that encodes vitellogenin was also upregulated among the challenged fish. Although there are likely a variety of compounds capable of influencing the genes that encode vitellogenin, this would suggest that MCLR may act as an endocrine disruptor. The effects on MCLR was elucidated *in vivo* by Zhao et al. (2015). The authors observed a positive correlation in a suite of genes associated with the hypothalamic-pituitary-gonadal axis, ovarian maturation, and vitellogenin. This correlation generally broke down at their high dose (likely due to cytotoxicity). Wei et al. (2008) identified genes associated with mitogen-activated protein kinases (MAPK) and immune pathways in zebrafish exposed to purified MCLR. Although the specific mechanism is not clear, MCLR or the cyanobacteria which metabolize them should be considered contributors to endocrine disruption in aquatic systems.

Influence of microcystin on the transcriptional leukocyte response of fishes to a bacterial insult *ex vivo*

Chronic mortality of fish has been reported in a variety of species within the Chesapeake Bay drainage. Some of these fish kill events have been linked to specific pathogens. Examples include *Aphanomyces invadans* which afflicts Atlantic menhaden (*Brevoortia tyrannus*; Blazer et al. (2002)) and *Mycobacterium* spp. which afflicts striped bass (*Morone saxatilis*; Gauthier et al. (2008)). There are, however reported fish kill events which have no clear correlation to any one specific pathogen or contaminant. An example of such events is reported by Blazer et al. (2010). Although a variety of pathogens were identified (such as trematodes, myxozoans, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Flavobacterium columnare*, and largemouth bass virus), no clear correlation to any fish kill event could be derived. This is likely due to a variety of contributing factors which lead to diminished overall fish health.

Microcystin toxins have been reported to affect immunological endpoints in fishes (Palíková et al. 1998). The researchers highlight the differential response among similar species (common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*)). Consistent among both species was the inverse correlation among total leukocytes with MC concentration. Among silver carp, there was an inverse correlation among phagocytic activity and MC concentration. The authors more closely evaluated individual cell types which yielded variable results. Overall, they concluded a general immunosuppression by MCLR. These initial findings of immunomodulation were expounded upon by Rymuszka et al. (2010) in common carp. They noted an increase in respiratory burst activity at low doses (0.01 µg/mL) but a decrease at higher concentrations of MCLR (>0.5 µg/mL). They theorize this was due to the specific cytotoxicity among granulocytes at these concentrations. Lymphocytes showed a decreased proliferative response to lipopolysaccharide (LPS) at higher concentrations (>0.5 µg/mL); however there was no affect from concanavalin A (ConA). The authors explain this as ConA is known to stimulate T-cells (among other cell types) while LPS stimulates B-cells (among other cell types). This is important given the function of B-cells (in part) producing immunoglobulins (Ig; specifically in teleost fishes IgM, IgD, IgT, and the light chain Igλ, Igκ, Igσ, and Igσ-2) as reviewed by Sunyer (2012) and Mashoof and Criscitiello (2016). The B cells are critical for antibody development as part of the adaptive humoral immunity.

Rymuszka and Adaszek (2012) reviewed a suite of genes in response to MC stimulation. Specifically, they quantified the pro-inflammatory genes interleukin-1β (IL-1β) and tumor necrosis factor- α (TNF-α), as well as the anti-inflammatory genes interleukin-10 (IL-10) and transforming growth factor- β (TGF-β) in common carp cultured leukocytes (isolated both from blood and anterior kidney). The authors observed a positive dose response among the two anti-

inflammatory cytokines. There was a positive response among both pro-inflammatory cytokines at the low dose (0.01 $\mu\text{g/mL}$) and an inverse correlation at the high dose (0.1 $\mu\text{g/mL}$) of MCLR. Their results begin to shed light on the observed immunomodulation as previously mentioned. They document an upregulation of certain pro-inflammatory cytokines at low doses of MC and a nominal upregulation of anti-inflammatory cytokines at these concentrations. However at higher concentrations the anti-inflammatory transcripts are significantly higher and may be overwhelming the pro-inflammatory cytokines. It should be noted that this was only at 10% of World Health Organization (WHO) guideline for MCLR in drinking water (1 $\mu\text{g/L}$; WHO 1998). Rymuszka and Adaszek (2013) followed up their earlier results *in vitro* and *in vivo*, again with common carp using aqueous exposure. Similar to previous studies, this study showed the cytotoxicity among phagocytic cells at higher concentrations of MCLR ($>0.5\mu\text{g/mL}$). A major change in this study was that MCLR did not yield the same transcriptional results as the *in vitro* study. However when they used MC-producing cyanobacterial extract, they observed a dose-dependent increase in both pro-inflammatory cytokines and the anti-inflammatory cytokine IL-10. This elucidated the fact that not only the purified toxin but also cyanobacterial lysate can have immunomodulatory effects.

Chen et al. (2017) quantified endpoints of oxidative stress (the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX)), as well as pro-inflammatory cytokines in common carp following IP injection. The specific transcriptional endpoints were the pro-inflammatory IL-1 β , TNF- α , interferon type I (IFN-I), as well as the chaperonins HSP70, and heat shock protein 90 (HSP90). They observed an upregulation in all of the oxidative stress endpoints immediately following MCLR exposure. Most of these enzymes returned to pre-challenge levels by the end of their experiment. Similarly, both chaperonins were

upregulated immediately following MCLR exposure but returned to baseline levels over time. All three pro-inflammatory cytokines were downregulated following exposure across MCLR treatments. Overall, the response of oxidative stress and chaperonin endpoints suggest detoxification and clearing of the toxin. What is quite interesting is the downregulation of the pro-inflammatory cytokines. The authors postulate that this response may occur to minimize an excessive response within the cell. An unintended downstream effect of this downregulation may be to dampen the immune response to a bacterial insult (specifically the toll-like receptor 2 (TLR2) signaling cascade (Rebl et al. 2010).

Li et al. (2009) quantified a broad swath of genes associated with immunoregulation in zebrafish (*Danio rerio*) larvae under an aqueous challenge with MCLR. Among their analysis were 6 genes associated with T and B cell development (recombination activation gene-1 and -2 (RAG1 and RAG2), Ikaros, erythroid transcription factor (GATA1), lymphocyte-specific protein tyrosine kinase (Lck), and T-cell receptor α (TCR α). They also quantified 4 heat shock proteins (HSP27, HSP60, HSP70, and HSP90) as they are commonly associated with exposure to antigens. They observed reduced transcript abundance of both RAG genes during the first 48 hours followed by an increased abundance up to 168 hours. There were variable responses among Ikaros, GATA1, Lck, and TCR α . Most notable was a significant increase in Lck abundance after 96 hours and a nearly consistent increased abundance of Ikaros and GATA1. The gene TCR α was only significantly upregulated in the high dose group (800 $\mu\text{g/L}$). They provide a thorough review of the function of each of these genes which, taken as a whole provide a more detailed example of immunomodulation by MCLR. For example, the upregulation of the non-chaperonin genes could lead to proliferation of T- and B-cells.

Smallmouth Bass Hepatic and Leukocyte Transcriptional Abundance Research Objectives

The effects of MCLR on hepatocytes are well documented. However, to our knowledge there are no data on the molecular effects of MCLR on SMB. Our research objective is to evaluate SMB hepatocytes stimulated with MCLR *ex vivo*. To our knowledge, there are no data characterizing the leukocyte transcriptome of SMB. We are unaware of any data describing the transcriptional response of SMB leukocytes to MC toxins. Thus, our specific research objective is to investigate the effects of MCLR toxin on the transcriptional response of leukocytes to the immunostimulant peptidoglycan.

Associations among estrogenic activity, select chemical compounds and microcystin toxins

A variety of endocrine disrupting compounds (EDC's) have been identified in surface waters throughout the United States (Kolpin et al. 2002; Snow et al. 2010). Of these compounds, a subset has been shown to act directly or indirectly as estrogenic compounds in living organisms. The exact sources of these compounds are diverse. Young et al. (2014) provided a landscape analysis of estrogenic activity within a large part of the upper Potomac River basin. They identified agricultural practices (confined animal feedlot operations), as well as urban impacts (wastewater inputs) as positively correlated to estrogenic endpoints. Campbell et al. (2006) provide a review of the transport of estrogenic EDC's through the environment. They reviewed the suite of biological assays which identify those effects in organisms. Blazer et al. (2012) and Iwanowicz et al. (2009) have used many of these techniques to quantify exposure to estrogenic compounds in the environment. Taken together, these studies highlight the complex distribution of estrogenic compounds in the environment and the difficulty in correlating biological effect to the presence of estrogenic activity across endpoints.

A less understood source of estrogenic compounds is the contribution from photosynthetic organisms. Liu et al. (2010) reviewed a suite of phytoestrogens and their association with wastewater facilities. They determined that phytoestrogens were positively correlated to the excretion rates of human urea associated with wastewater facility inflow. Kang and Price (2009) quantified phytoestrogenic compounds associated with wastewater facilities and streams. They identified a suite of phytoestrogens present in wastewater inflow but very little in the outflow. They also quantified a suite of phytoestrogens in the environment. Specifically, the compounds present (daidzein, enterolactone, enterodiol, genistein and formononetin) are associated with agricultural systems. These results are supported by Kolpin et al. (2010). They quantified a variety of phytoestrogens (as well as mycotoxins and atrazine) in Iowa that were associated with agricultural systems. A thorough review of current phytoestrogen research is provided by Jarosova et al. (2015). They highlight the correlation of phytoestrogen presence to agricultural systems and wastewater treatment facilities (WWTP's). They also reported the data gaps associated with estrogenic potency, the effect on biological systems as compared to animal sterols, and the potential contribution from cyanobacteria as a possible source for phytoestrogens in the environment.

The purified secondary cyanobacterial metabolite MCLR has been shown to cause an increase in estrogenic activity in the MELN cell line. Oziol and Bouaicha (2010) reported a small increase in activity of the luciferase gene. This effect was shown to be estrogen receptor-mediated as it was inhibited via an estrogen receptor antagonist (although dose-dependency curves were likely skewed from the cytotoxicity of MCLR). They concluded that at low concentrations MCLR had an indirect relationship with the estrogen receptor. Along with purified MCLR, extracts from cyanobacteria and algal taxa have been found to contribute

environmental estrogens *in vitro*. Štěpánková et al. (2011) reported detectable estrogenic equivalency from cyanobacterial extracts using the MVLN cell line from *Planktothrix agardhii*, *Aphanizomenon gracile*, and *Microcystis aeruginosa*. Sychrová et al. (2012) expanded upon this research and showed appreciable estrogenic potential in extracts from the cyanobacteria *Aphanizomenon gracile* and *Microcystis aeruginosa* and the algae *Cylindrospermopsis raciborskii* and *Chlorella kessleri*. They also showed that extracts from the cyanobacterial species *Aphanizomenon flos-aquae* and the algae *Scenedesmus quadricauda* were found to be estrogen receptor antagonists. Based on this research, it is important to consider the contribution of phytoestrogens when evaluating the total estrogenicity of surface waters.

The source of phytoestrogens in the environment is likely correlated to land use practices. Many of these compounds are present either directly or as a metabolite from legumes (Franke et al. 1994). They quantified the phytoestrogens coumestrol, biochanin A, daidzein, formononetin, and genistein in a variety of legumes using high-performance liquid chromatography. Kuhnle et al. (2009) further described additional non-legume sources of phytoestrogens sorted by isoflavones (biochanin A, daidzein, formononetin, genistein and glycitein), lignans (matairesinol and secoisolariciresinol), as well as coumestrol. Jarosova et al. (2015) provide a review of the distribution and concentrations of known phytoestrogens in the literature. Along with providing a comprehensive list of compounds and sources, they also include the relative estrogenic equivalency (cEEQ) which they define as the concentration multiplied by the relative potency as compared to 17 β -estradiol (E2) specific to the assay conducted. Although the relative potencies of each compound is generally $>10^2$ less than that of E2, the ubiquity of sources is concerning. Although many of the plants which produce these compounds may appear in the wild, agribusiness can increase their occurrence on the landscape by encouraging the growth of plants

which contain higher concentrations of phytoestrogens (e.g. legumes). However the presence of phytoestrogens is not strictly confined to agricultural run-off. For example, equol is a metabolite of intestinal bacteria derived from the breakdown of the soy isoflavones daidzin and daidzein (Setchell and Clerici 2010). It is released in the waste of animals containing these bacteria and can occur in humans, livestock, and in the wild. Thus, phytoestrogen occurrence in the environment may be linked both to point- and non-point source pollution.

Phytoestrogen exposure has been correlated to biological markers of endocrine disruption. Bagheri et al. (2014) observed an increase in plasma E2 as a result of a long-term feed study with goldfish (*Carassius auratus*) supplemented with genistein and daidzein. Kiparissis et al. (2003) observed an increase in testicular oocytes (TO) when they exposed *Oryzias latipes* to the isoflavones equol and genestein. They observed TO occurrence rates of 12% at 1 g/L genestein and 10% at 0.4 µg/L equol. They also reported that equol caused a dose-dependent impairment of spermatogenesis. Wang et al. (2016) also observed a higher occurrence of TO in *O. latipes* as a result of exposure to equol (35% at 145.7 ng/L). They also observed an inverse dose-response in 11-ketotestosterone suggesting that equol may be antiandrogenic. Phytoestrogens have been shown to induce changes in transcript abundance. Cleveland and Manor (2015) exposed rainbow trout (*Oncorhynchus mykiss*) IP to genestein and daidzein and compared the transcriptional response to E2 and the control. They observed a significant upregulation of the gene which encodes the egg yolk precursor protein vitellogenin among both genestein and daidzein. They also observed mixed effects among a suite of other genes within the liver which (among other functions) are associated with endocrine disruption and cellular regulation.

Environmental Estrogens Research Objectives

The presence of biologically relevant estrogenic compounds is becoming better documented. However, the change in these estrogenicity data over time (monthly and yearly) is less understood. My research objective is to monitor the distribution of estrogenic compounds in the middle and upper Potomac River watershed among various land cover/land use areas. This estrogenic signal can be compared to the occurrence of both steroidal and phytoestrogens. This signal can also be compared to both physical and nutrient parameters.

Evaluation of possible interference as reported by the yeast strain *Saccharomyces cerevisiae*

BLYR

Biological assays (bioassays) are designed to quantify an effect in the context of a living organism. Estrogenic endocrine disrupting compounds (EEDCs) are a group of compounds which have been shown to modulate estrogen receptor-mediated pathways in aquatic organisms (Campbell et al. 2006). Many of these compounds (which include both steroidal and non-steroidal chemicals) have been correlated to deleterious biological effects in fishes (Segner et al. 2013; Senthilkumaran 2015). Routledge and Sumpter (1996) described a yeast (*Saccharomyces cerevisiae*) bioassay which incorporates human estrogen receptor (hER) into the yeast genome, as well as the reporter gene *Lac-Z*. This allows for the identification of compounds which activate this specific pathway and is termed the yeast estrogen screen assay (YES). Sanseverino et al. (2005) further developed the strain *S. cerevisiae* and instead of the colorimetric *Lac-z* reporter, they incorporate the bioluminescent *luxA/B* reporter. This strain is termed bioluminescent yeast estrogen screen (BLYES). The new strain *S. cerevisiae* BLYES yielded a similar detection range of 17 β -estradiol standards; however shortened the duration of the assay

from 72 hours to 6 hours. This strain was used to determine the estrogenicity of samples in Chapter Four.

An important component of bioassays is the determination of cellular viability both before and after a challenge experiment. This ensures that the observed effect is not due to cell death (termed toxicity) but from the experimental agent. The strain *S. cerevisiae* BLYR(a) was first published by Eldridge et al. (2007) and was designed to constitutively express the *luxA/B* bioluminescent reporter. This was done to report cytotoxicity among (in this example) potentially androgenic compounds (incorporating human androgen receptor instead of hER and termed the bioluminescent yeast androgen screen (BLYAS)). The authors identified toxicity by a reduction in the bioluminescent reporter as compared to a control. Sanseverino et al. (2009) analyzed a comprehensive suite of compounds and quantified the estrogenic and androgenic potential using the BLYES and BLYAS assays as well as the toxicity as reported by BLYR.

Cell Viability Research Objective

To our knowledge, there are no data reporting the environmental trends in water or periphyton with regards to strain *S. cerevisiae* BLYR. Interference is defined as a significant deviation from the control luminescence. The specific objective of this study was to determine whether the interference (specifically the lower output luminescence) determined by the BLYR assay constitutes dead cells as reported by flow cytometry. The second objective was to quantify the variability in strain BLYR among environmental samples. These results were compared to both the microcystin data as reported in Chapter Two and estrogenicity data reported in Chapter Four.

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Chapter Two. Temporal associations among cyanotoxins in water and fish tissue at various sites in the upper and middle Potomac River drainage, USA

Abstract

Microcystin (MC) toxins are exclusively produced by cyanobacteria. Historically, blooms of pelagic-oriented cyanobacteria have been documented in the Potomac River watershed. We investigated the occurrence of MCs in the upper and middle Potomac River watershed. We sampled both the filtered and whole (unfiltered) water fractions to determine the relative contribution of pelagic-oriented MC-producing taxa; as well as the periphyton to determine the contribution from benthic-oriented MC-producing taxa. Samples were collected at sites throughout Maryland, Virginia, and West Virginia monthly (or bi-monthly) for 28 months. We compared these MC concentrations to both in-stream physical metrics, as well as nutrient parameters. We also sampled smallmouth bass (*Micropterus dolomieu*) and golden redhorse sucker (*Moxostoma erythrurum*) at 19 time points to quantify the accumulation of MCs in hepatic tissue. All MC data were quantified using an enzyme-linked immunosorbent assay (ELISA). We observed relatively low concentrations of MCs in water, periphyton, and livers at all of the sites sampled as compared to acutely toxic concentrations reported in literature and the World Health Organization guideline for microcystin-LR in drinking water (1.0 µg/L). We observed seasonal variability of MCs in the water column that positively correlated to stream temperature. We observed seasonal variability of MCs in hepatic tissue similar to those observed in the water column. Overall, although the concentrations of MCs are low, we believe further investigation into the specific taxa present in each fraction, as well as overall biomass may yield a fuller picture to the initial research presented here.

Introduction

Cyanobacteria are found throughout marine, freshwater, and terrestrial habitats. Some genera of cyanobacteria produce chemical toxins (cyanotoxins) that have been implicated in human and animal illness and death (Codd et al. 2005; Lopez et al. 2008). Toxins may also be produced by eukaryotic taxa (Landsberg 2002). The generic term for these compounds produced by both prokaryotic and eukaryotic organisms is algal toxins. Algal toxins can be grouped according to their primary effects which include neurotoxins, hepatotoxins, cytotoxins and irritants (Sivonen and Jones 1999). A number of taxa have been identified that produce these toxins (Carmichael 1992, 1997; Dittmann and Wiegand 2006; Leflaive and Ten-Hage 2007). Under the current climate change projections, there is an increased growing global concern over the increased distribution and frequency of harmful algal blooms (Paerl and Huisman 2009).

Cyanobacterial-derived hepatotoxins are a family of cyanotoxins that are characterized by their toxic effects on liver tissue. The hepatotoxin microcystin (MC) is an extensively studied cyanotoxin in the environment comprising up to 1% dry weight of the total cyanobacteria biomass (Blaha et al. 2009; Pavagadhi and Balasubramanian 2013). Microcystin is a cyclic heptapeptide with over 80 known structural variants (Welker and von Dohren 2006). Microcystins are potent inhibitors of serine/threonine-specific protein phosphatases 1 and 2A (Runnegar et al. 1995). The inhibition of these enzymes causes the dysregulation of intracellular glycogen, as well as the collapse of sinusoidal capillaries which results in hemorrhaging (Dawson 1998; Dittmann and Wiegand 2006). The presence and effects of MCs are well documented in the environment (O'Neil et al. 2012; Pawlik-Skowrońska et al. 2013; Quiblier et al. 2013) and the distribution of MC concentrations have been shown to fluctuate seasonally (Billam et al. 2006). Although much of the documented effects from MC exposure focuses on

acute toxicity, there is some research correlating chronic MC exposure to carcinogenesis (Zegura et al. 2011), immune dysfunction (Liu et al. 2015) and other sublethal effects. Cyanotoxins have also been shown to bioaccumulate (Ferrao-Filho Ada and Kozlowsky-Suzuki 2011) and consequently are important when assessing ecosystem health.

Krogmann et al. (1986) documented cyanobacterial blooms in the lower Potomac River in 1983 and reports suspected blooms in other areas of the watershed as early as 1930. The causes of these blooms are unclear; however it is suggested that they were in part driven by excess nutrients and low water years. Between 2002 and 2009, various mortality events were documented involving smallmouth bass (SMB; *Micropterus dolomieu*). Blazer et al. (2010) conducted an exhaustive analysis and identified a variety of stressors which may have driven those events. Despite no correlation to a specific pathogen, there is considerable interest among fisheries managers to prevent such events in the future. The occurrence and distribution of cyanotoxins within the Potomac River watershed is poorly understood. This data gap is of interest given that cyanotoxins are known to bioaccumulate in fish tissue (Ferrao-Filho Ada and Kozlowsky-Suzuki 2011). The evaluation of cyanotoxins, specifically the accumulation of MCs within fish tissue from individuals collected within the Potomac River drainage may yield insights into the risk factors associated with degraded fish health observed in certain areas of the watershed (Blazer et al. 2010). Our objective was to quantify the abundance and distribution of MCs in the water column and periphyton, as well as the bioaccumulation in hepatic tissues from fishes within the Potomac River drainage.

Methods

Field Collection

We selected 15 sites from streams located in the Potomac River watershed in West Virginia, Maryland, and Virginia (Figure 1; Reconnaissance Sites). Each site was sampled once-monthly beginning June 26, 2013 through June 18, 2014, resulting in 11 sampling periods (we were unable to collect samples in October 2013 due to the Federal government shutdown). Due to spatial logistics, all 15 sites were sampled in the same sample period which was conducted approximately within the same week. Nine sites were sampled in the South Branch of the Potomac River basin, one site on the North Branch of the Potomac River, three sites in the Shenandoah River basin, one site on the mainstem of the Potomac River upstream from the confluence with the Shenandoah River, and one site near the mouth of the Monocacy River.

At each sampling location, two discrete water samples were collected from a near-shore location using one chemically cleaned 1L amber glass bottles (labelled filtered; I-Chem, Rockwood, Tennessee) and one sterile 500 mL HDPE bottle (labelled unfiltered; Thermo Fisher Scientific, Waltham, Massachusetts). Filtered samples were analyzed to identify cyanotoxins present free in the water column while unfiltered samples account for cellular and acellular toxins. Periphyton was collected by scraping organic benthic material from the surface of submerged rocks and woody debris into a sterile 50 mL conical tube (Becton Dickenson, Franklin Lakes, New Jersey). We targeted benthic mats of periphyton that were dark green, of leathery texture and emitted the strong odor of 2-methylisoborneol. Samples from similar mats collected by West Virginia Division of Natural Resources before and within our sampling period contained the toxin-producing cyanobacteria genera *Microcoleus*, *Oscillatoria*, and *Phormidium* (Table 1). Water temperature (°C), pH, and conductivity (μS/cm) were recorded at each site

using an Oakton® CON 10 Meter (Oakton Instruments, Vernon Hills, Illinois). Total dissolved oxygen (mg/L and percent) was recorded at each site using an Accumet AP74 Meter (Fisher Scientific, Pittsburgh, Pennsylvania).

Following the 12-month reconnaissance sample period, two sites in the South Branch Potomac and one site in the North Branch Potomac were sampled every other week from June 30, 2014 through December 14, 2015 (Figure 1; Biweekly Sites). This was done to increase the temporal resolution given the stability of MCs in the environment (<50% reduction in 16 days simulating wild streams and <14% reduction in 26 days under ideal laboratory conditions as described by Tsuji et al. (1994)). These three sites were sampled on the same day and each sampling day was analyzed within its calendar month. Along with the above-mentioned water quality measurements, we also sampled five nutrient parameters. These were total nitrogen (N; mg/L), total phosphorous (P; mg/L), and total dissolved phosphorous (TDP; mg/L) in the water column, as well as total N (mg/kg) and total P (mg/kg) in the sediment. These samples were collected using one sterile 500 mL HDPE bottles (Thermo Fisher Scientific, Waltham, Massachusetts) for the aqueous metrics and one 250 mL pre-cleaned to EPA standards glass amber bottles (Quality Environmental Containers, Beaver, West Virginia) for the sediment metrics. All nutrient samples were analyzed and reported by Research Environmental Industrial Consultants Inc. (Beaver, West Virginia). Neither medium was filtered prior to shipment.

Smallmouth bass and golden redbreast suckers (GRH; *Moxostoma erythrurum*) were collected by the West Virginia Division of Natural Resources using boat electrofishing. These species were selected to represent two potentially different cyanotoxin exposure routes based on their respective life histories (based on diet and habitat usage as defined in Jenkins and Burkhead (1983)). A necropsy-based fish health analysis was performed on all individuals recording total

length (mm), body weight (g), sex, and noting any grossly observable external abnormalities. Livers were collected from 256 SMB and 291 GRH for total MCs during 19 sampling events between March 29, 2013 and August 12, 2015 (Table 2). Livers of SMB were removed and weighed (nearest g) for calculation of hepatosomatic indices. Weight of GRH livers was not obtained due to the more diffuse organization. The modified Fulton's body condition factor (K) was calculated for both SMB and GRH as follows: $100000 \times ((\text{body weight} - \text{gonad weight}) / \text{total length}^3)$. The SMB hepatosomatic index (HSI) was calculated using the following formula: $(\text{liver weight} / (\text{body weight} - \text{gonad weight})) \times 100$. Liver tissue was placed in plastic bags, held on wet ice until returned to the laboratory (generally less than four hours, vacuum-sealed and stored at -80°C until extraction.

Laboratory Preparation

Filtered water samples were acidified with hydrochloric acid to pH 3.0 and stored at 4°C for no greater than 14 days prior to filtration and extraction. Periphyton samples were centrifuged at 913g for ten minutes. After centrifugation, the supernatant was removed retaining the periphyton pellet which was stored at -20°C until filtration and extraction. Unfiltered water samples were stored at -20°C until filtration and extraction.

Prior to extraction, unfiltered water samples were prepared by three freeze-thaw cycles as described by Loftin et al. (2008). Subsequently, all water samples were extracted using methods modified from Ciparis et al. (2012). Each sample was brought to room temperature and pre-filtered using a 0.7 µm AP40 GF/F filter (Millipore Corporation, Billerica, Massachusetts). We prepared water samples using solid phase extraction via OASIS® HLB (200 mg) glass cartridges (Waters Corporation, Milford, Massachusetts). Cartridges were pre-cleaned using five mL each of ethyl acetate, 50:50 methanol:dichloromethane, methanol, and deionized water (pH adjusted

to 3.0). 800 mL of the filtered or 400 mL of the unfiltered samples were run through the cartridge via continuous vacuum. We then used two consecutive 6 mL solvents containing methanol then 50:50 methanol:dichloromethane and reduced to dryness. Eluates were solubilized in methanol such that the final extraction ratio was 1600:1. Extracted samples were stored at -20°C until analysis.

Periphyton samples were extracted using methods modified from Mohamed et al. (2006). Prior to extraction, periphyton samples were lysed by three freeze-thaw cycles as described by Loftin et al. (2008). Samples were weighed and 6 mL of absolute methanol was added. Samples were vortexed for three minutes, followed by centrifugation at 913g for ten minutes. Supernatant was retained and the process was repeated a second time with 50:50 methanol:dichloromethane. Each supernatant sample was filtered using a 0.7 µm Whatman® Purdisc 13 syringe filter (Whatman, Maidstone, United Kingdom). Once filtered, both samples were reduced to dryness, then solubilized with methanol and pooled such that the final sample volume was 500 µL. Samples were stored at -20°C until analysis.

Liver samples were extracted twice using methods described by Deblois et al. (2008). Whole livers were thawed, weighed, and minced into small pieces in a Falcon ® 15 mL conical tube (Corning Inc., Corning, New York). Six mL of absolute methanol was added, sonicated for one minute at low intensity and then placed on a rotary mixer at 4°C for three hours. The samples were then centrifuged for 20 minutes at 913g and supernatant removed through a 0.7 µm Whatman® Purdisc 13 syringe filter (Whatman, Maidstone, United Kingdom). Filtered supernatants were reduced to dryness, re-solubilized with 500 µL absolute methanol and stored at -20°C until analysis.

Direct competitive ELISA protocol

ELISA methods were adapted from Campas and Marty (2007) to quantify total microcystins/nodularins. These ELISA results will be referred to as total MC/NOD. Wells were coated with 100 μ L of ADDA-specific mAb (0.1 μ g/mL (diluted in carbonate buffer; pH 9.6); AD4G2; Enzo Life Sciences, Farmingdale, New York) and incubated in a 96-well ELISA plate (Costar® 3396, Corning Inc., Corning, New York) for 18 hours at 4°C. Plates were washed 5 times with tris-buffered saline with 0.0005% Tween-20 (TBST; pH 7.4), and blocked with TBST with 1% bovine serum albumin (block buffer; pH 7.4) for two hours at 37°C. Each plate was washed 5 times with TBST and 50 μ L of standards (11 standards ranging from 0.078 – 500.0 ng/mL as well as a solvent control) or sample was added at a 5% dilution in block buffer. 50 μ L of biotin-labelled MC-LR was added to each well (4 μ g/mL) in block buffer and incubated for 1.5 hours at 37°C. Plates were washed five times with TBST and 100 μ L of streptavidin-horseradish peroxidase conjugate (1:5000 in block buffer; ThermoFisher Scientific, Waltham, Massachusetts) was added and incubated for 30 minutes at 37°C. Plates were washed five times with TBST and 100 μ L of SureBlue Reserve™ TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland) was added and incubated at 25°C for 1.5 hours. 50 μ L of TMB stop solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland) was added and optical density was measured at 450 nm using a SpectraMax M4 plate reader (Molecular Devices LLC, Sunnyvale, California). Sample concentration was determined by interpolation to the standard curve and adjusted for sample concentration. Values are reported as ng/mL for filtered and unfiltered water and ng/g for periphyton and liver samples.

To validate the direct competitive ELISA for water and liver extracts a subset of samples was analyzed with a commercially available indirect competitive ELISA for total microcystin/nodularin kit (Abraxis, Inc., Warminster, Pennsylvania). We compared our standards as well as filtered and unfiltered water samples using Abraxis kit product number 520011. To compare the sensitivity and dynamic range of our ELISA to the Abraxis kit, we calculated the average from 20 standard curves and compared this to the Abraxis standard curve. Periphyton, SMB, and GRH liver sample extracts were compared using Abraxis kit product number 520011SAES. Both kits use the same polyclonal antibody and have a dynamic range from 0.05 – 5.0 ng/mL.

Statistical Analysis

All data were analyzed using the R statistical software package (R Core Development Team, 2017) and considered significantly different at $\alpha = 0.05$. Validation of the ELISA method and comparison of the standard curves used a 4-parameter logistic regression in the R package *nplr*. Correlation among the two curves used the R package *drc*. Finally, we compared the interpreted results from the Abraxis and our assays among each of the mediums (filtered water, unfiltered water, periphyton, SMB liver, and GRH liver). Comparison of filtered and unfiltered water samples was performed using the Pearson's correlation. Differences within season/year among these water samples were assessed using a Student's t-test. Variability among sampling sites of water and periphyton data was analyzed separately for the initial 12 months and subsequent 16 month sampling efforts using an analysis of variance (ANOVA) followed by the Tukey's honest significance difference test if a significant difference was detected. We defined season by grouping three consecutive months as follows: spring was between March, April, and May; summer was between June, July, and August; fall was between September, October, and

November; winter was between December, the following January, and February (the year associated with winter corresponds to January and February). If there was no statistical difference among sites, differences among season/year were quantified using an ANOVA followed by the Tukey's honest significance difference test. If there was a significant difference among sites, season/year differences were quantified in the linear mixed effects model (LMM) framework treating "site" as a fixed effect. This was performed with the R package *nlme* (Pinheiro et al. 2013) using a ANOVA (type III sums of squares) followed the Tukey's honest significance difference test if a significant difference was detected.

The linear mixed effects models package *nlme* was also used to define and quantify the explanatory variables responsible for variation in the MC/NOD dataset. The initial model selection framework analyzed the total MC/NOD among all 15 sites during the initial 12 months sampling period. First, we analyzed the global effects of stream discharge (ft^3/s), temperature ($^{\circ}\text{C}$), conductivity ($\mu\text{S}/\text{cm}$), and pH accounting for the seasonal and site variation from the initial models (if applicable). To analyze the subsequent 16 months, we built two separate models due to the limited number of sites (three) given the total amount to variables investigated (six). The first model explored the effects of four physical variables described above on total MC/NOD in the water column and periphyton. The second model incorporated the top candidate model(s) along with the N and P variables. We discarded TDP from analysis due to the high frequency of data below the reporting limit (104 of 117 total samples).

Effects of sex, age, or tissue weight on total MCs in liver was performed with a one-way ANOVA. Seasonal and site differences were determined using a one-way ANOVA. Pairwise comparisons were assessed using the Tukey's honest significance difference test. Species differences within sampling site and sampling effort were quantified using either the Student's or

Welch's t-test depending on equal or unequal variances as reported with an F-test. When applicable, we compared K and HSI to total MCs in the liver using the Pearson's correlation.

Results

ELISA Comparison/Validation

Standard curve from both assays are presented in Figure 2. Our standards fall within the error of the commercial kit's standard curve. The inflection points are -1.43, 1.44 and -0.904, 1.12 for the Abraxis and our curves, respectively (Figure 3). There was no significant difference among these curves ($p = 0.062$). The comparison from the Abraxis kit and our assay among the five sample extract medium's (filtered water, unfiltered water, periphyton, SMB liver, and GRH liver) are presented in Table 4. Among water samples, the data were within the reported range $\pm$ three standard deviations between the two assays or our assay overestimated as compared to the Abraxis kit. However, 36% of the unfiltered water samples quantified were above the reporting limit of the Abraxis kit. These samples were quantifiable without dilution using our assay. 100 % of the GRH livers were within the reporting range among the two assays. There was no discernable pattern for periphyton and SMB livers between the two assays.

Quantification of Total Microcystins in Water Samples

A total of 288 filtered and 288 unfiltered water samples were analyzed. MC/NOD were detected in 40.5% of all water samples. The concentrations of MC/NOD in water ranged from 1.30 to 88.7 ng/L (Table 5). The overall trends among filtered and unfiltered water samples were significantly correlated ($p < 0.001$, $r^2 = 0.342$). However, our initial analysis was designed to quantify differences within season/year between these two mediums. These data are reported in Table 6. As predicted, the unfiltered water samples had an MC/NOD concentration greater than

or equal to the concentration reported in filtered water once adjusted for standard error within each sampling season/year. During the summer and fall 2013 and fall 2015, unfiltered water had a higher MC/NOD concentration than filtered water ($p \leq 0.026$). The source of these differences is unknown.

Site and Seasonal Differences among Water and Periphyton Samples

There were no significant temporal differences among all sites among season/year when we compared total MC/NOD in the water column during the initial 12 months using filtered ($p = 0.144$) or unfiltered ($p = 0.856$). We then examined site differences among all sites within season/year. Although we were unable to statistically compare site differences at this temporal scale based on minimal sampling points within each season/year ($n \leq 3$), we do note differences. For example, unfiltered samples from the three South Branch mainstem sites were consistently higher than the remaining 12 sites during both summer months. However these are only higher in the summer 2014 filtered water samples. The highest value during the summer 2013 was the North Fork of the South Branch while the highest value in the summer 2014 was the South Branch tributary at Mill creek near Romney, WV. We also note that the ten North and South Branch Potomac sites were generally higher for both filtered and unfiltered as compared to the three Shenandoah River sites, one mainstem Potomac River site, and one Monocacy River site. During the subsequent 16 months, we observed a significant difference among sites using both filtered and unfiltered water ($p = 0.003$ and $p < 0.001$, respectively). The two South Branch sites were both significantly higher ($p \leq 0.021$) than the one North Branch Potomac site but were not different from each another ($p = 0.480$).

The summer and fall 2013 filtered samples were higher than the 2014 spring samples ($p < 0.001$ for both). The spring 2014 filtered water samples were lower than the winter and summer

2014 ($p = 0.001$ and $p < 0.001$, respectively). The 2013 summer unfiltered water samples were higher than the other four seasons ($p \leq 0.002$). The 2013 fall unfiltered water samples were higher than the 2014 winter and spring unfiltered water samples ($p < 0.001$ for both). During the biweekly sampling period, seasonal differences in both water sample types were again largely observed in the summer months. When we examined filtered water, 2014 summer was higher than all other season/year except for 2016 winter ($p < 0.001$). Summer 2015 was higher than 2015 autumn ($p = 0.040$). When we evaluated unfiltered samples, 2014 summer was higher than other season/year except for 2016 winter ($p < 0.001$). There were no additional statistical differences.

A total of 288 periphyton samples were analyzed. MC/NOD was reported in 76% of these samples. The range was between 0.280 and 222.620 ng/g (Table 5). We analyzed the periphyton in the initial 12 month and subsequent 16 month groups as described above. During both time frames, there was no significant difference among sites ($p = 0.444$ and 0.908 , respectively). We did observe a significant difference among season/year ($p < 0.001$ and 0.006 , respectively). Samples collected in summer 2013 were lower than winter and spring 2014 ($p < 0.001$). During the biweekly sampling period, spring 2015 samples were lower than fall of 2014 ($p = 0.039$) and 2015 (0.018) samples. The summer 2014 was lower than the fall of 2015 ($p = 0.041$).

Explanatory Variable Data Analysis for Water and Periphyton Samples

All model exploration results are summarized in Table 7. We quantified total MC/NOD in the water column during the initial 12 months as a function of our four physical explanatory variables (water temperature, discharge, pH, and conductivity) given the difference among season and year. Based on the model results, temperature and pH were identified as yielding high

importance to the model (0.94 and 0.88, respectively). When analyzing unfiltered samples, conductivity was identified to be the most important variable (0.69).

During the subsequent 16 months of sampling, we included sample site as a fixed effect to account for the known site differences described above for both methods. When we examined the model results for our physical parameters, similar to the initial time range, only temperature was supported by the model for filtered samples (1.00) and by our unfiltered sample model (0.96). We then built a final model for each sample type which included total N and total P in the water column, as well as water temperature (the top candidate models from above) within the biweekly date range. Again, only temperature was identified to be the most important for filtered and unfiltered samples (1.00 and 0.99, respectively).

We attempted to correlate the total MC/NOD in the periphyton using either our four physical explanatory variables or the two nutrient variables (total N and total P in the sediment) during both of our time periods given the known differences in season/year as described above. Based on the model results, there were no variables that were supported by any model to explain the MC/NOD variance among the periphyton.

Total Microcystins in Liver Samples

A summary of average fish length, weight, age, and total MC/NOD for each site and sampling effort is provided in Table 8. We detected MC/NOD in 81% of SMB and 80% of GRH samples (Table 8). The range among SMB was 0.130 to 29.144 ng/g and the range among GRH was 0.182 to 22.738 ng/g. There was no significant effect of age, sex, or total liver weight among either species analyzed. We observed seasonal variability within sites and within species. Values during the summer sampling season were significantly higher as compared to the spring

sampling season at the two South Branch Potomac sites (Petersburg and Moorefield, WV). We were able to compare seasonal differences among GRH at three sites (North Branch at Cumberland, MD and the South Branch at Petersburg and Moorefield, WV). There was a similar trend for higher total MC/NOD concentrations in the summer compared to the spring. However we note high annual variability in GRH. During nine sampling events, we collected both SMB and GRH. We observed higher MC/NOD in SMB as compared to GRH at the mainstem of the North Branch at Cumberland, MD on 4/8/2013 ($p = 0.039$) and during both the 4/5/2013 and 8/27/2014 samplings at the mainstem of the South Branch near Moorefield, WV ($p = 0.030$ and $p < 0.001$, respectively). There were no other significant differences among species.

Among SMB, there was a general decreasing trend in HSI when we compared spring and summer samples regardless of year (Table 8). There was a similar trend with K. Both HSI and K are inversely correlated to total MC/NOD ($p < 0.001$ and $p = 0.009$, respectively). The K of GRH followed a similarly decreasing trend. However, K was not correlated to total MC/NOD ($p = 0.232$).

Discussion

The MC/NOD concentrations we observed in the environment were much lower than the World Health Organization guideline for MC-LR in drinking water of 1 ng/mL (WHO 2011) and MC/NOD concentrations reported in other systems (the highest MC/NOD reported in individual water samples from the present study were 0.051 ng/mL in filtered water and 0.090 ng/mL in unfiltered water). Wood et al. (2006) documented benthic-oriented cyanobacteria in New Zealand and those taxa are similar to the cyanobacteria reported in the middle and upper Potomac River watershed (Table 1). Six of the 10 streams Wood et al. (2006) sampled contained detectable concentrations of MCs. They report a concentration in the water column of one

system to be 12.70 µg/L which is four orders of magnitude higher than those we observed. They also reported detectable concentrations of MCs in the benthos in five of their streams. The concentrations we detected among periphyton (ranging from 0.28 to 222.62 ng/g) overlap the range of MCs they report (2.1 to 200 ng/g) among the benthos. A variety of hypotheses have been proposed to explain why cyanobacteria release toxins into the environment (e.g. defense mechanism and/or actively reducing competition (Sivonen 2009)). This may (in-part) explain the relatively elevated concentrations of MC/NOD we observed in the summer months. These are generally characterized by warmer water with lower discharge which likely leads to increased growth, competition for available habitat, and immigration of cyanobacterial grazers. All of these factors (which would need to be independently investigated) may influence the release of toxins into the water column.

We attempted to identify the likely source population of MC-producing cyanobacteria in either the pelagic or benthic community by temporally sampling both populations. As highlighted in Table 6, the benthic concentration peaks in the autumn while the pelagic concentration peaks in the summer. Interestingly, the trend of the unfiltered samples correlated to the trend of the filtered samples which would suggest that it is the pelagic community which is driving total MCs in the water. We cannot however rule out the possibility of benthic taxa becoming dislodged and contributing to water concentrations; however we note that the seasonal peak MC/NOD concentrations in water was generally earlier than periphyton. Identification of the specific taxa present in each population would be required to confirm or reject this hypothesis. The concentrations of MC/NOD observed in the water are well below the concentration that has induced a biological effect experimentally. In an aqueous challenge using female zebrafish (*Danio rerio*), Zhao et al. (2015) observed a change in reproductive endpoints

(molecular and histological) at concentrations of MC >10 ng/mL. In a different challenge using medaka (*Oryzias latipes*), Mezhoud et al. (2008) observed a change in hepatic molecular markers at a concentration of MC > 1000 ng/mL. However, it is unclear how a prolonged exposure to particularly sub-lethal concentrations of MCs (such that we observed here) influence transcript abundance or histopathological markers in wild fishes.

Concentration of MC/NOD in SMB and GRH in hepatic tissue was generally below those reported to cause biological effects in wild fishes from other watersheds. Mitsoura et al. (2013) evaluated common carp (*Cyprinus carpio*) from a lacustrine system experiencing cyanobacterial blooms of MC-producing taxa and reported MC concentrations in the water between 2.03 and 3.01 ng/mL and in the liver between 346 and 732 ng/g. At these concentrations, they observed hepatocyte degradation and necrosis. Deblois et al. (2008) analyzed MC concentrations in the water and two tilapia species (*Oreochromis niloticus* and *Tilapia rendalli*) from impoundments in Brazil. They observed concentrations of MCs in the water column between 2710 and 9860 ng/mL and in hepatic tissue between 800 and 2400 ng/g. They suggested this resulted in a decreased HSI and K. As we also observed an inverse correlation among MC/NOD and HSI and K (in SMB), total MCs may be considered as a contributing factor as well as other possible external factors (e.g. temperature, DO, etc.). We sampled SMB and GRH to focus on two separate feeding strategies (higher-order piscivore and lower order omnivore). We generally did not observe differences in MC/NOD in the liver samples between these species. Further investigation is required to determine where MC/NOD is entering the food web.

We observed a correlation of MC/NOD in the water column to stream temperature but not other physical and nutrient variables. This may suggest that primary production is not limited by the availability of nutrients (specifically total N, total P, and TDP). These conclusions are

similar to those reported by Munn et al. (1989) who used chlorophyll *a* as a proxy for cyanobacterial abundance and correlations to temperature and turbidity were observed. They did not observe correlations between N or P in the substrate and it was concluded that primary production among streams within the sample areas was strictly limited by temperature and turbidity. We cannot, however, rule out nutrient loading as a variable of concern in these systems based on the results from a recent study of water quality metrics (including N and P) at sites in the Shenandoah and South Branch Potomac Rivers (Buchanan and Mandel 2015). In addition, we have documented the presence of MC-producing taxa in the watersheds we sampled. *Oscillatoria spp.* is a known MC producer (Brittain et al. 2000) and has been identified in the Potomac River drainage (Table 1). The species *O. limosa* is capable of fixing atmospheric nitrogen and thus, would likely be limited by P (Fay 1992). A detailed time series analysis of taxa richness, as well as total MC concentrations and nutrient analysis would be required to fully understand the contribution of specific species rather than our broadly studied periphyton mat. These data would also allow ecosystem managers to fully understand the interaction of stream nutrients and the MC-producing taxa present in these streams.

Harmful algal blooms are of significant interest with regards to public health (Carmichael and Boyer 2016; Grattan et al. 2016; Wituszynski 2014; Chorus and Bartram 1999; Backer 2002). Overall, the concentrations present in water, periphyton, and fish tissue were low compared to data reported in literature and the guidelines set forth by the WHO. This is the first documentation of MC/NOD in these fish species at these sites. Although we observed variability in individual samples, the overall trend of higher concentrations during the warmer summer months was consistent across all sites. The specific source of MC/NOD reported in the water column remains unclear. Along with richness and biomass, further investigation into the

successional stages in the periphyton as described by Momo (1995) and Brasell (2014) may yield insights into the variables influencing growth and release of MCs. These variables must include both physical and nutrient parameters as recommended by Biggs and Close (1989). Additionally, many cyanobacterial taxa are known to produce multiple toxins (Quiblier et al. 2013). An extensive investigation into specific species present, as well as the toxins they may produce (beyond MCs), would provide a fuller description of influence of cyanotoxins within these systems.

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Figures

Figure 1. The location of all 15 sites included in this study. All sites were located in the states of West Virginia, Maryland, and Virginia within the Potomac River watershed (inset map).

Figure 2. The gray line and circles represents the standard curve from the Abraxis Inc. kit No. 520011 $\pm$ one standard deviation about the mean optical density. The black squares represent the mean Leetown MC-LR standards $\pm$ one standard deviation about the mean optical density on the same plate.

Figure 3. The gray line and circles represents the standard curve from the Abraxis Inc. kit No. 520011 as a function of the optical density. The black circles and line represent the mean Leetown MC-LR standards as a function of the mean optical density from 20 randomly selected assay plates.

Tables

Table 1. A summary of the cyanobacteria richness at the Shenandoah River and South Branch Potomac watersheds. Samples were collected opportunistically between April 5, 2010 and June 7, 2013. Identification was performed by GreenWater Laboratories, Palatka, Florida.

Watershed	Community	Classification	Species	Identification
Shenandoah	Periphyton	<i>Merismopedia</i>	<i>glauca</i>	Pre-2013
Shenandoah	Periphyton	<i>Merismopedia</i>	<i>punctata</i>	Pre-2013
Shenandoah	Periphyton	<i>Oscillatoria</i>	spp.	Pre-2013
Shenandoah	Periphyton	<i>Phormidium</i>	<i>cf. retzii</i>	Pre-2013
Shenandoah	Periphyton	<i>Phormidium</i>	spp.	Pre-2013
South Branch	Pelagic	<i>Aphanocapsa</i>	spp.	Pre-2013
South Branch	Pelagic	<i>cf. Aphanocapsa</i>	spp.	Pre-2013
South Branch	Pelagic	<i>Coelomorion</i>	<i>pusillum</i>	Pre-2013
South Branch	Pelagic	<i>Cyanogranis</i>	<i>ferruginea</i>	Pre-2013
South Branch	Pelagic	cyanophyte cell pair	spp.	Pre-2013
South Branch	Pelagic	cyanophyte filament	spp.	Pre-2013
South Branch	Pelagic	cyanophyte unicell,	spp.	Pre-2013
South Branch	Pelagic	cyanophyte unicell,	spp.	Pre-2013
South Branch	Pelagic	<i>Geitlerinema/Jaaginema</i>	spp.	Pre-2013
South Branch	Pelagic	<i>Komvophoron</i>	spp.	Pre-2013
South Branch	Pelagic	<i>Lyngbya/Oscillatoria</i>	spp.	Pre-2013
South Branch	Pelagic	<i>Lyngbya/Phormidium</i>	spp.	Pre-2013
South Branch	Pelagic	<i>Merismopedia</i>	<i>punctata</i>	Pre-2013
South Branch	Pelagic	<i>Oscillatoria</i>	spp.	Pre-2013
South Branch	Pelagic	<i>Phormidium</i>	spp.	Pre-2013
South Branch	Pelagic	<i>Pseudanabaena</i>	spp.	Pre-2013
South Branch	Pelagic	<i>Synechococcus</i>	spp.	Pre-2013
South Branch	Periphyton	cyanophyte filament	spp.	Pre-2013
South Branch	Periphyton	<i>Heteroleibleinia</i>	spp.	Pre-2013
South Branch	Periphyton	<i>Leptolyngbya</i>	spp.	Pre-2013 and
South Branch	Periphyton	<i>Merismopedia</i>	<i>glauca</i>	Pre-2013
South Branch	Periphyton	<i>Merismopedia</i>	<i>punctata</i>	Pre-2013
South Branch	Periphyton	<i>Microcoleus</i>	<i>cf. autumnale</i>	Pre-2013 and
South Branch	Periphyton	<i>Oscillatoria</i>	<i>princeps</i>	Pre-2013
South Branch	Periphyton	<i>Oscillatoria/Phormidium</i>	spp.	Pre-2013

Watershed	Community	Genus	Species	Identification
South Branch	Periphyton	<i>Phormidium</i>	<i>autumnale/favosum</i>	Pre-2013
South Branch	Periphyton	<i>Phormidium</i>	<i>cf. amoenum</i>	Pre-2013
South Branch	Periphyton	<i>Phormidium</i>	<i>formosum</i>	Pre-2013
South Branch	Periphyton	<i>Phormidium</i>	spp.	Pre-2013 and
South Branch	Periphyton	<i>Pseudanabaena</i>	spp.	Pre-2013 and
South Branch	Periphyton	<i>Snowella</i>	<i>litoralis</i>	Pre-2013

Table 2. Sampling site locations throughout Maryland, West Virginia, and Virginia. Sites are identified by the representation as a reconnaissance and/or biweekly water/periphyton, as well as fish sampling location.

Basin	Site Name	Site Abbreviation	Latitude	Longitude	Reconnaissance Site	Biweekly Site	Fish Sampling Site
Monocacy River	Monocacy River near Mouth near Frederick, MD	Monocacy	39.38778841	-77.37984832	X		X
North Branch of the Potomac River	Mainstem of the North Branch at Cumberland, MD	North Branch Cumberland	39.58690227	-78.73752192	X	X	X
Potomac River	Mainstem of the Potomac River near Sharpsburg, MD	Potomac Sharpsburg	39.41430527	-77.74631932	X		X
South Branch of the Potomac River	North Fork of the South Branch at Seneca Rocks, WV	North Fork South Branch	38.83389380	-79.37185151	X		
	South Fork of the South Branch near Moorefield, WV	South Fork South Branch	38.97944841	-78.97721032	X		
	Mainstem of the South Branch at Petersburg, WV	South Branch Petersburg	38.99917201	-79.08622612	X		X
	Mainstem of the South Branch near Moorefield, WV	South Branch Moorefield	39.10406921	-78.95770572	X	X	X
	Upper Mainstem of the South Branch	Upper South Branch	38.85888361	-79.27907259	X		

Basin	Site Name	Site Abbreviation	Latitude	Longitude	Reconnaissance Site	Biweekly Site	Fish Sampling Site
South Branch of the Potomac River	Mainstem of the South Branch near Mouth near Springfield, WV	South Branch Springfield	39.50934621	-78.60014072	X	X	
	Mill Creek near Dorcas, WV	Mill Creek Dorcas	38.96181921	-79.11440952	X		
	Mill Creek near Romney, WV	Mill Creek Romney	39.32716127	-78.79627972	X		
	Seneca Creek near Seneca Rocks, WV	Seneca Creek	38.83423291	-79.37175894	X		
North Fork of the Shenandoah River	North Fork of the Shenandoah River at Strasburg, VA	North Fork Shenandoah	38.97431587	-78.35507539	X		
South Fork of the Shenandoah River	South Fork of the Shenandoah River at Front Royal, VA	South Fork Shenandoah	38.92916661	-78.21976819	X		
Shenandoah River	Shenandoah River at Millville, WV	Shenandoah Millville	39.27474127	-77.78561892	X		

Table 3. Total MC/NOD concentration (µg/g) based on ELISA or Liquid chromatography/mass spectrometry. All samples were collected in the South Branch Potomac watershed. Analysis was performed by GreenWater Laboratories, Palatka, Florida. Data below the assay detection limit are presented as BD.

		ELISA (µg/g)	Liquid chromatography/mass spectrometry (µg/g)							
Sample Date	Sample Medium	Total MC/NOD Concentration	Total MC-	Total MC-	Total MC-	Total MC-	Total MC-	Total MC-	Total MC-	Total NOD
1/19/2013	Periphyton	0.03	BD	BD	BD	BD	BD	BD	BD	BD
4/8/2013	Periphyton	0.16	BD	BD	BD	BD	BD	BD	BD	BD
7/19/2013	SMB Liver	0.02	-	-	-	-	-	-	-	-
7/19/2013	SMB Liver	0.03	-	-	-	-	-	-	-	-

Table 4. Comparison of the ELISA results between the current study and Abraxis kit. Standard deviation for each sample are presented as SD.

	Current Study		Abraxis	
	Total MC/NOD (ng/L)	SD	Total MC/NOD (ng/L)	SD
Filtered Water Sample 1	3.023	1.039	4.467	0.579
Filtered Water Sample 2	75.189	0.311	35.971	0.147
Filtered Water Sample 3	164.773	19.645	95.033	18.201
Filtered Water Sample 4	114.572	4.206	56.920	2.533
Filtered Water Sample 5	50.868	5.466	25.079	2.337
Filtered Water Sample 6	238.368	2.457	203.792	5.999
Filtered Water Sample 7	175.766	23.896	106.460	24.895
Filtered Water Sample 8	165.294	13.486	95.046	12.525
Filtered Water Sample 9	116.609	22.679	58.708	13.918
Filtered Water Sample 10	238.975	12.545	206.968	31.168
Filtered Water Sample 11	78.195	10.965	37.480	5.274
Unfiltered Water Sample 1	478.321	22.304	AQ	-
Unfiltered Water Sample 2	146.865	1.389	78.980	1.082
Unfiltered Water Sample 3	331.051	25.260	AQ	-
Unfiltered Water Sample 4	526.330	33.445	AQ	-

	Current Study		Abraxis	
	Total MC/NOD (ng/L)	SD	Total MC/NOD (ng/L)	SD
Unfiltered Water Sample 5	67.048	11.170	32.258	5.089
Unfiltered Water Sample 6	167.158	2.844	96.387	2.685
Unfiltered Water Sample 7	249.947	0.832	235.274	2.529
Unfiltered Water Sample 8	252.505	10.346	245.029	33.331
Unfiltered Water Sample 9	230.439	9.677	186.467	20.695
Unfiltered Water Sample 10	365.086	17.122	AQ	-
Unfiltered Water Sample 11	268.937	64.638	502.192	468.182
	Total MC/NOD (ng/g)	SD	Total MC/NOD (ng/g)	SD
Periphyton Sample 1	BD	0.000	14.417	0.005
Periphyton Sample 2	1.135	0.584	15.352	1.104
Periphyton Sample 3	10.392	4.710	51.156	0.961
Periphyton Sample 4	222.618	4.448	69.765	14.476
Periphyton Sample 5	125.070	75.553	747.048	541.871
SMB Liver Sample 1	BD	0.000	3.240	0.045
SMB Liver Sample 2	1.750	0.515	0.277	0.014
SMB Liver Sample 3	4.858	0.177	1.325	0.040
SMB Liver Sample 4	34.940	2.085	104.753	12.005

	Total MC/NOD (ng/g)	SD	Total MC/NOD (ng/g)	SD
SMB Liver Sample 5	18.263	3.632	38.604	2.391
GRH Liver Sample 1	BD	0.000	27.239	4.501
GRH Liver Sample 2	0.955	0.402	4.635	0.062
GRH Liver Sample 3	1.237	0.083	1.478	0.064
GRH Liver Sample 4	6.624	1.808	1.427	0.052
GRH Liver Sample 5	14.181	3.756	20.519	5.515

Table 5. Summary of total microcystins/nodularins in each sample type grouped by season and year. Sample size is presented as *n* and standard error is presented as SE.

Site Name	Season and Year	<i>n</i>	Filtered Water		Unfiltered Water		Periphyton	
			Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/g)	SE
Monocacy River near Mouth near Frederick, MD	2013 Summer	2	5.39	2.26	16.66	0.29	0.05	0.02
	2013 Autumn	2	6.19	1.19	10.76	5.34	1.56	1.49
	2014 Winter	3	4.47	0.86	5.32	1.06	2.70	1.40
	2014 Spring	3	3.68	0.94	3.48	0.18	2.63	1.21
	2014 Summer	1	2.90	NA	4.72	NA	2.54	NA
Mainstem of the North Branch at Cumberland, MD	2013 Summer	3	9.18	2.86	27.33	16.83	1.60	0.61
	2013 Autumn	2	8.97	1.75	11.03	0.13	1.40	0.35
	2014 Winter	3	5.04	1.17	3.33	0.33	2.01	0.84
	2014 Spring	3	3.72	0.92	3.34	0.06	4.18	2.77
	2014 Summer	7	6.43	1.95	7.70	1.48	1.06	0.18
	2014 Autumn	6	6.25	1.59	7.91	3.30	10.37	4.06
	2015 Winter	7	3.76	0.25	3.41	0.53	2.78	0.89
	2015 Spring	6	2.93	0.47	3.10	0.90	1.63	0.78
	2015 Summer	7	6.22	1.36	7.47	1.92	1.81	0.80
	2015 Autumn	7	3.15	1.33	8.45	1.86	5.46	1.50
	2016 Winter	1	2.05	NA	6.47	NA	2.38	NA

			Filtered Water		Unfiltered Water		Periphyton	
Site Name	Season and Year	<i>n</i>	Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/g)	SE
Mainstem of the Potomac River near Sharpsburg, MD	2013 Summer	2	3.15	0.02	4.52	2.09	0.56	0.52
	2013 Autumn	2	7.89	2.89	4.86	1.54	1.26	0.96
	2014 Winter	3	5.09	0.24	3.29	0.74	1.99	1.31
	2014 Spring	3	4.32	0.85	6.00	1.75	3.50	1.22
	2014 Summer	1	4.73	NA	4.62	NA	0.07	NA
North Fork of the South Branch at Seneca Rocks, WV	2013 Summer	2	18.60	2.09	25.75	18.89	5.52	5.33
	2013 Autumn	2	9.17	3.53	9.88	4.99	0.46	0.41
	2014 Winter	3	5.41	0.68	4.35	0.27	2.43	1.93
	2014 Spring	3	4.47	0.71	4.30	0.53	5.68	2.64
	2014 Summer	1	11.21	NA	7.51	NA	3.76	NA
South Fork of the South Branch near Moorefield, WV	2013 Summer	2	7.15	4.02	22.85	12.44	4.23	4.18
	2013 Autumn	2	7.17	2.17	4.48	0.57	0.47	0.07
	2014 Winter	3	4.96	1.25	4.78	1.33	12.01	4.51
	2014 Spring	3	2.53	0.22	3.30	0.87	1.09	0.45
	2014 Summer	1	5.69	NA	9.12	NA	5.18	NA

			Filtered Water		Unfiltered Water		Periphyton	
Site Name	Season and Year	<i>n</i>	Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/g)	SE
Upper Mainstem of the South Branch	2013 Summer	2	8.74	2.60	15.26	5.83	0.61	0.55
	2013 Autumn	2	4.70	0.31	15.97	5.43	1.93	0.31
	2014 Winter	3	5.68	0.59	5.33	2.02	5.48	2.53
	2014 Spring	3	4.28	0.82	4.33	1.65	2.10	1.31
	2014 Summer	1	3.93	NA	11.61	NA	1.78	NA
Mainstem of the South Branch at Petersburg, WV	2013 Summer	2	8.42	5.38	29.32	3.72	5.97	1.84
	2013 Autumn	2	4.87	0.14	6.50	3.18	6.72	5.10
	2014 Winter	3	5.68	0.59	3.99	0.63	2.51	0.95
	2014 Spring	3	4.98	1.12	3.75	0.44	9.19	0.81
	2014 Summer	2	16.28	3.27	24.98	9.68	3.71	1.59
Mainstem of the South Branch near Moorefield, WV	2013 Summer	2	9.24	6.20	30.17	1.84	5.17	2.92
	2013 Autumn	2	11.45	4.69	11.10	6.98	111.62	111.00
	2014 Winter	3	5.21	1.01	3.65	0.22	2.98	1.30
	2014 Spring	4	3.94	0.48	4.81	0.19	0.97	0.79
	2014 Summer	8	23.15	5.05	39.35	11.09	2.25	0.73
	2014 Autumn	6	9.86	3.66	11.75	3.44	23.49	20.37
	2015 Winter	7	4.56	0.56	5.67	1.33	2.81	1.01
	2015 Spring	6	2.66	0.32	4.32	1.32	1.25	0.91
	2015 Summer	7	9.67	2.11	13.93	2.37	3.11	1.15

			Filtered Water		Unfiltered Water		Periphyton	
Site Name	Season and Year	<i>n</i>	Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/g)	SE
Mainstem of the South Branch near Moorefield, WV	2015 Autumn	7	4.05	1.28	9.32	2.74	3.61	1.23
	2016 Winter	1	8.06	NA	23.24	NA	1.64	NA
Mainstem of the South Branch near Mouth near Springfield, WV	2013 Summer	2	12.34	7.92	26.92	0.24	0.04	0.00
	2013 Autumn	2	10.45	5.27	19.80	4.12	1.29	0.69
	2014 Winter	3	5.97	0.63	4.30	1.63	6.56	2.61
	2014 Spring	3	4.65	1.32	6.42	3.20	4.42	2.18
	2014 Summer	6	28.04	5.52	39.70	7.38	2.63	1.43
	2014 Autumn	6	11.43	4.06	16.45	5.22	2.25	0.81
	2015 Winter	7	3.61	0.17	6.16	2.48	3.99	2.30
	2015 Spring	6	6.60	2.91	7.57	2.37	0.97	0.39
	2015 Summer	7	14.65	3.31	22.37	2.51	16.68	9.92
	2015 Autumn	7	4.30	0.70	8.00	1.57	5.48	3.43
	2016 Winter	1	12.19	NA	24.01	NA	1.97	NA
Mill Creek near Dorcas, WV	2013 Summer	2	5.62	2.49	10.95	8.52	0.34	0.27
	2013 Autumn	2	5.67	2.87	8.44	5.04	3.97	3.09
	2014 Winter	3	5.77	0.52	5.46	1.93	2.84	2.56
	2014 Spring	3	4.67	0.70	5.36	2.06	7.54	3.51
	2014 Summer	1	4.16	NA	4.48	NA	0.97	NA

			Filtered Water		Unfiltered Water		Periphyton	
Site Name	Season and Year	<i>n</i>	Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/g)	SE
Mill Creek near Romney, WV	2013 Summer	2	12.21	5.52	30.27	13.24	0.44	0.29
	2013 Autumn	2	3.62	0.58	8.89	3.84	1.78	1.13
	2014 Winter	3	5.77	0.52	5.87	2.34	2.89	1.03
	2014 Spring	3	3.72	0.92	3.34	0.06	1.03	0.48
	2014 Summer	1	39.46	NA	2.59	NA	6.51	NA
Seneca Creek near Seneca Rocks, WV	2013 Summer	2	10.77	1.30	26.52	0.19	0.57	0.49
	2013 Autumn	2	4.53	1.49	3.31	0.47	0.91	0.25
	2014 Winter	3	5.57	1.52	5.28	0.72	6.38	2.78
	2014 Spring	3	2.69	0.10	3.76	1.23	11.38	6.29
	2014 Summer	1	5.26	NA	9.88	NA	2.36	NA
North Fork of the Shenandoah River at Strasburg, VA	2013 Summer	2	6.06	2.93	3.96	1.53	0.17	0.11
	2013 Autumn	2	8.14	3.14	16.65	0.32	0.87	0.84
	2014 Winter	3	6.36	0.40	8.97	5.10	3.16	1.84
	2014 Spring	3	3.11	0.32	4.29	0.24	2.02	1.42
	2014 Summer	1	3.90	NA	4.39	NA	1.08	NA

			Filtered Water		Unfiltered Water		Periphyton	
Site Name	Season and Year	<i>n</i>	Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/L)	SE	Total MC/NOD (ng/g)	SE
South Fork of the Shenandoah River at Front Royal, VA	2013 Summer	2	3.28	1.75	7.32	1.98	0.03	0.00
	2013 Autumn	2	5.28	0.76	9.47	2.97	2.88	0.57
	2014 Winter	3	5.36	1.40	3.51	0.36	10.72	6.71
	2014 Spring	3	2.72	0.10	3.80	0.56	3.61	3.35
	2014 Summer	1	2.56	NA	2.37	NA	0.02	NA
Shenandoah River at Millville, WV	2013 Summer	2	7.74	0.94	13.10	3.72	0.04	0.02
	2013 Autumn	2	4.79	0.21	5.88	3.08	53.79	52.30
	2014 Winter	3	5.95	0.21	4.97	0.56	3.86	0.71
	2014 Spring	3	2.55	0.08	3.00	0.20	8.45	3.73
	2014 Summer	1	6.76	NA	2.81	NA	1.52	NA

Table 6. Seasonal summary of total microcystins/nodularins in all three mediums pooled among all sampling sites. Sample size is presented as *n* and standard error is presented as SE.

Season and Year	<i>n</i>	Filtered Water (ng/L)	Filtered Water SE	Unfiltered Water (ng/L)	Unfiltered Water SE	Significant Difference among Water Fractions		Periphyton (ng/g)	Periphyton SE
2013 Summer	31	8.54	0.99	19.65	2.54	<0.001		1.69	0.53
2013 Autumn	30	6.86	0.64	9.80	1.11	0.026		12.73	8.04
2014 Winter	45	5.49	0.20	4.83	0.43	0.170		4.57	0.73
2014 Spring	46	3.74	0.20	4.23	0.30	0.176		4.44	0.73
2014 Summer	34	15.34	2.36	21.20	3.95	0.208		2.19	0.37
2014 Autumn	18	9.18	1.86	12.04	2.37	0.349		12.04	6.85
2015 Winter	21	3.98	0.22	5.08	0.94	0.267		3.19	0.85
2015 Spring	18	4.06	1.02	5.00	1.01	0.520		1.28	0.40
2015 Summer	21	10.18	1.52	14.59	1.85	0.073		7.20	3.51
2015 Autumn	21	3.83	0.63	8.59	1.17	0.001		4.85	1.26
2016 Winter	3	7.43	2.94	17.91	5.72	NA		1.99	0.21

Table 7. Linear mixed effect model results for total microcystins/nodularins as a function of physical and nutrient explanatory variables among each sample medium and time points.

Filtered Water				
		Estimate	Adjusted SE	Importance:
Initial 12 Months	(Intercept)	-1.51E-02	1.20E-02	
	pH	2.49E-03	1.45E-03	0.88
	Temperature	1.38E-04	6.07E-05	0.94
	Conductivity	-1.80E-06	2.61E-06	0.46
	Discharge	-4.83E-08	1.18E-07	0.35
Subsequent 16 Months		Estimate	Adjusted SE	Importance:
	(Intercept)	3.34E-03	1.24E-02	
	pH	-6.00E-06	5.95E-06	0.25
	Temperature	5.64E-04	1.14E-04	1.00
	Conductivity	-4.23E-08	2.72E-07	0.63
	Discharge	-1.75E-04	1.47E-03	0.26
		Estimate	Adjusted SE	Importance:
	(Intercept)	-4.64E-04	2.37E-03	
	Temperature.C	6.30E-04	1.18E-04	1.00
	Water Column Total N	-9.84E-04	1.17E-03	0.57
	Water Column Total P	-9.84E-04	3.65E-03	0.29
Unfiltered Water				
		Estimate	Adjusted SE	Importance:
Initial 12 Months	(Intercept)	1.23E-02	1.14E-02	
	Conductivity	-7.10E-06	6.40E-06	0.69
	Temperature	5.60E-05	1.13E-04	0.38
	pH	-2.20E-04	1.30E-03	0.29
	Discharge	-5.27E-08	2.03E-07	0.27

Unfiltered Water				
Subsequent 16 Months		Estimate	Adjusted SE	Importance:
	(Intercept)	1.94E-03	2.44E-02	
	Conductivity	-1.03E-05	1.13E-05	0.59
	Temperature	5.42E-04	2.17E-04	0.96
	pH	9.15E-04	2.95E-03	0.29
	Discharge	-2.51E-09	4.23E-07	0.26
		Estimate	Adjusted SE	Importance:
	(Intercept)	3.25E-03	4.67E-03	
	Temperature	7.79E-04	2.29E-04	0.99
	Water Column Total N	-2.96E-03	2.03E-03	0.57
	Water Column Total P	-8.66E-03	7.42E-03	0.34

Periphyton				
Initial 12 Months		Estimate	Adjusted SE	Importance:
	(Intercept)	-8.30E+00	3.91E+01	
	pH	1.86E+00	4.78E+00	0.34
	Temperature	-9.58E-05	5.15E-04	0.25
	Conductivity	-1.81E-03	8.24E-03	0.26
Subsequent 16 Months	Discharge	-2.83E-02	1.36E-01	0.27
		Estimate	Adjusted SE	Importance:
	(Intercept)	-6.05E+00	2.79E+01	
	pH	2.05E-01	2.31E-01	0.30
	Temperature	1.04E+00	3.42E+00	0.57
	Conductivity	-1.92E-04	6.40E-04	0.25
	Discharge	-5.77E-04	4.16E-03	0.30
		Estimate	Adjusted SE	Importance:
	(Intercept)	5.18E+00	3.34E+00	
	Sediment Total N	-1.53E-03	1.23E-02	0.25
	Sediment Total P	1.26E-04	2.16E-03	0.26

Table 8. A summary of average morphometric and liver total MC/NOD data for smallmouth bass (a) and golden redhorse sucker (b). Sample size is presented as *n*. K represents the modified Fulton's condition factor. HSI represents hepatosomatic index. *Significance values for total MCs were determined among sampling dates within a sampling location. Sites in which no seasonal comparison can be made is presented as "--". HSI was not calculated for golden redhorse sucker.

^a Smallmouth Bass											
Site Name	Sample Date	<i>n</i>	Length (mm)	Weight (g)	Age	Total MC/NOD (ng/g)	MC/NOD Sig*	K	K Sig	HSI	HSI Sig
Monocacy River near Mouth near Frederick, MD	8-Apr-2013	18	363.33	686.44	5.06	1.50	-	1.32	e	2.57	a
Mainstem of the North Branch at Cumberland, MD	8-Apr-2013	18	341.00	538.89	6.11	0.92	-	1.28	ace	2.22	ad
Mainstem of the Potomac River near Sharpsburg, MD	29-Mar-2013	17	354.41	629.71	6.41	1.60	-	1.36	ae	2.61	a
	5-May-2015	19	317.32	429.63	5.11	4.02	-	1.28	ace	1.01	bc
Mainstem of the South Branch at Petersburg, WV	4-Apr-2013	18	333.83	520.67	5.67	1.07	a	1.22	bce	1.78	ac
	7-Aug-2013	17	301.59	358.65	4.06	4.86	b	1.20	bce	0.81	b
Mainstem of the South Branch near Moorefield, WV	5-Apr-2013	19	362.05	683.00	6.00	0.78	a	1.29	ace	2.34	a
	8-Aug-2013	20	313.10	393.30	4.37	7.05	b	NA	NA	NA	NA
	7-May-2014	20	312.05	445.10	5.25	1.90	a	1.19	bc	1.09	bc

Site Name	Sample Date	<i>n</i>	Length (mm)	Weight (g)	Age	Total MC/NOD (ng/g)	MC/NOD Sig*	K	K Sig	HSI	HSI Sig
Mainstem of the South Branch near Moorefield, WV	27-Aug-2014	20	258.60	251.45	4.00	13.43	b	1.22	bce	0.72	b
	7-Apr-2015	19	361.42	675.53	6.53	0.74	a	1.26	ace	2.19	ae
	4-May-2015	19	309.05	431.32	4.89	1.85	a	1.29	ace	1.39	bcde
	11-Aug-2015	18	292.28	343.11	3.72	7.74	b	1.26	ace	0.65	b
^bGolden Redhorse Sucker											
Site Name	Sample Date	<i>n</i>	Length (mm)	Weight (g)	Age	Total MC/NOD (ng/g)	MC/NOD Sig*	K	K Sig		
Mainstem of the North Branch at Cumberland, MD	8-Apr-2013	20	442.35	1208.40	8.45	0.42	a	1.30	a		
	22-Aug-2013	11	388.18	687.55	5.27	2.82	b	1.09	ab		
	3-Jun-2014	20	396.20	764.30	5.50	0.65	ac	1.19	ab		
	26-Aug-2014	19	430.16	903.26	6.37	3.52	bc	1.10	ab		
	6-Apr-2015	20	431.45	920.30	7.05	1.45	bc	1.07	ab		
	12-Aug-2015	19	447.37	891.63	6.42	2.37	b	1.02	b		
Mainstem of the South Branch at Petersburg, WV	4-Apr-2013	18	477.61	1312.78	7.33	1.03	a	1.12	ab		
	7-Aug-2013	18	367.56	556.50	3.11	5.72	b	1.10	ab		
Mainstem of the South Branch near Moorefield, WV	5-Apr-2013	20	478.05	1374.75	8.45	0.13	a	1.17	ab		
	8-Aug-2013	20	439.10	964.47	4.65	6.27	cd	NA	NA		
	7-May-2014	20	426.50	988.75	4.95	1.13	b	1.20	ab		
	2-Jun-2014	20	435.95	1022.45	5.95	1.89	cd	1.20	ab		
	27-Aug-2014	20	434.05	996.75	4.30	6.14	cd	1.17	ab		
	7-Apr-2015	20	444.55	1029.00	5.05	2.12	bc	1.10	ab		
	11-Aug-2015	19	454.58	1087.00	5.92	8.77	d	NA	NA		

Chapter Three: Hepatocyte and Leukocyte Transcript Abundance Profiles of Smallmouth Bass (*Micropterus dolomieu*) Challenged with Microcystin-LR

Abstract: Cyanobacterial blooms are of increasing concern worldwide as both a nuisance and due to their production of toxins in select species. The cyanobacterial hepatotoxin microcystin-LR (MCLR) has been shown to disrupt a variety of genes in the liver and anterior kidneys of fishes. These toxins have been shown to occur within the Potomac River watershed in which the smallmouth bass (SMB; *Micropterus dolomieu*) is a popular sport fish. The temporal transcriptional response of SMB hepatocytes to environmentally relevant concentrations of MCLR (0.05, 0.5, and 5.0 ng/mL), as well as the effect of these concentrations of MCLR on peptidoglycan (PGN) stimulated SMB leukocytes was assessed. A reference leukocyte transcriptome was first developed using next-generation sequencing. Independent libraries of leukocyte and hepatic genes-of-interest were subsequently generated. Hepatic transcripts included those which are shown to be affected by MCLR, as well as those indicative of endocrine disruption. Leukocyte transcripts focused on the toll-like receptor 1/2 pathway (which is activated by PGN); as well as transcripts shown to be affected by MCLR. These were included in a customized CodeSet and analyzed using the NanoString® nCounter technologies. No significant differences among hepatocyte treatments or leukocyte treatments treated only with MCLR were reported. However differences in PGN-stimulated and PGN-MCLR coexposed leukocytes were observed when compared to unexposed. Although these data do not show statistically or biologically relevant transcriptional disruption by MCLR, it does show that MCLR acts concomitantly with peptidoglycan to up- or down-regulate select leukocyte transcripts.

1. Introduction

Chronic mortality of fish has been reported in a variety of species within the Chesapeake Bay drainage. Some of these events have been linked to specific pathogens such as *Aphanomyces invadans* which infects Atlantic menhaden *Brevoortia tyrannus* (Blazer et al. 2002) and *Mycobacterium* spp. which infect striped bass *Morone saxatilis* (Gauthier et al. 2008) and other species. There are however, reported fish kill events which have no clear correlation to any one specific pathogen or contaminant. Although a variety of pathogens were identified (such as trematodes, myxozoans, *Aeromonas hydrophila*, *A. salmonicida*, *Flavobacterium columnare*, and largemouth bass virus) in recent kills of smallmouth bass *Micropterus dolomieu*, no clear cause could be derived. This is likely due to a variety of contributing factors which lead to diminished overall fish health (Blazer et al. 2010).

Cyanobacteria are a large phylum which inhabits marine and freshwater systems. Cyanotoxins are a secondary metabolite of a subset of these cyanobacteria. Cyanotoxins are generally grouped by the target tissue. Hepatotoxins are a group which includes the 89 known variants of microcystin (MC), as well as nodularin. Much of the research specific to microcystins has focused on the variant –LR (MCLR). Microcystins act as a potent inhibitor of the serine/threonine-specific protein phosphatases 1 and 2A (Runnegar et al. 1995). The inhibition of these enzymes leads to the dysregulation of intracellular glycogen, as well as the collapse of sinusoidal capillaries (Dawson 1998; Dittmann and Wiegand 2006).

One cellular process to detoxify compounds is the generation of reactive oxygen species (ROS; Lushchak (2014)). Oxidative stress is the condition within cells when the production of ROS exceeds the cell's ability to neutralize their effects and may be induced by a variety of compounds (Lushchak 2016). The MCLR exposure has been shown to induce various enzymes

associated with oxidative stress. Pflugmacher et al. (1998) identified increased activity of glutathione (GSH), as well as the catalyst enzyme glutathione-S-transferase (GST), which is believed to be the first step in the detoxification of MC. Bouaicha and Maatouk (2004) evaluated rat hepatocytes *in vitro* and observed an acute increase in GSH three hours post-exposure, as well as an increase in ROS 0-60 minutes post-exposure. Glutathione was not different than controls 24 or 48 hours post-exposure. Jayaraj et al. (2006) showed a decrease in the activity of glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT) 120 minutes post-exposure while only CAT was significantly decreased one, three, and seven days post-exposure. They theorized this resulted from a depletion of intracellular stores of glutathione resulting from the effects of oxidative stress. This is supported by the increase in the genes that encode heat shock protein-70 (HSP70) and glutathione reductase (GSR) within the first two hours post-exposure, both of which protect against the effects of oxidative stress.

Microcystin-producing cyanobacteria have been shown to modulate the expression of a suite of genes *in vivo*. Rogers et al. (2011) report an up-regulation in the gene that encodes vitellogenin (*vtg*, *vtg1*, *vtg2*, and *vtg3*) in zebrafish (*Danio rerio*) when exposed to MC-producing cyanobacterial lysate. They also observed a significant down-regulation among genes associated with a variety of other pathways such as cell signaling and development. Marie et al. (2012) showed similar results using medaka (*Oryzias latipes*). Wei et al. (2008) identified genes associated with mitogen-activated protein kinases (MAPK) and immune pathways in zebrafish which were up-regulated when exposed to purified MCLR. Zhao et al. (2015) observed the up-regulation in a suite of genes associated with the hypothalamic-pituitary-gonadal axis, ovarian maturation, and vitellogenin and exposure to MCLR 2 µg/L and 10 µg/L. Interestingly, these associations were not consistently observed at 50 µg/L (likely due to cytotoxicity).

Microcystins have been shown to up-regulate immune-relevant genes as well. Rymuszka and Adaszek (2012) quantified the pro-inflammatory cytokines interleukin-1 β (IL1B) and tumor necrosis factor- α (TNF- α), as well as the anti-inflammatory interleukin-10 (IL-10) and transforming growth factor- β (TGFB) in common carp *Cyprinus carpio* leukocytes isolated both from blood and anterior kidney. The authors observed an up-regulation at the low MCLR dose (0.01 μ g/mL). Conversely, they reported a down-regulation at the high MCLR dose (0.1 μ g/mL). Their results began to shed light on the observed immunomodulation as previously mentioned. They document an upregulation of certain pro-inflammatory cytokines at low doses of MC and a nominal upregulation of anti-inflammatory cytokines at these concentrations. However, at higher concentrations the anti-inflammatory transcripts are significantly higher and may be overwhelming the pro-inflammatory cytokines. It should be noted that their treatment concentrations were 10x and 100x higher than the WHO guideline of 1.0 μ g/L for the MC variant -LR in drinking water (WHO 1998).

Chen et al. (2017) quantified a variety of endpoints of oxidative stress (the enzymes superoxide dismutase, catalase, and glutathione peroxidase), as well as pro-inflammatory cytokines in common carp following intraperitoneal injection of 150 μ g/kg MCLR. The specific transcriptional endpoints were the pro-inflammatory IL1B, TNF- α , interferon type I, as well as the chaperonins heat shock protein 70 and 90. They observed an upregulation in all of the oxidative stress endpoints 12 hours post-injection. Most of these enzymes returned to pre-challenge levels after 96 hours. Similarly, both chaperonins were upregulated 12 hours post-injection following MCLR exposure but returned to baseline levels after 96 hours. All three pro-inflammatory cytokines were upregulated at all time periods (12 – 96 hours post injection).

Overall, the response of oxidative stress and chaperonin endpoints suggest detoxification and clearing of the toxin.

The first objective of this chapter is to quantify select transcript abundance of smallmouth bass (SMB) hepatocytes to *in vitro* exposure of three concentrations of purified MCLR. The second objective was to investigate the effects of MC toxin on the transcriptional response of leukocytes to a bacterial antigen.

2. Methods

2.1. Hepatocyte assay

Yearling smallmouth bass were acclimated at the U.S. Geological Survey in Leetown, WV. Eight bass (6 males and 2 females) were randomly selected and euthanized using a lethal dose of tricaine methanesulfonate (MS-222) in each of two independent trials. Hepatocytes were isolated using methods modified from Mommsen et al. (1994). Briefly, fish were bled via a puncture of the caudal vein to minimize the contamination of tissue by erythrocytes. Following exsanguination, the liver was aseptically removed and separated from the gall bladder. Livers were transported in Leibovitz's L-15 growth medium containing 5% (v/v) of charcoal/dextran stripped fetal bovine serum (FBS), supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 292 µg/ml L-glutamine (L-15 5% PSG; Sigma-Aldrich, St. Louis, MO) on wet ice. Tissues were punctured and perfused using a 3 mL syringe with a 25 3/8-gauge needle containing 5mL Dulbecco's Phosphate-Buffered Saline (DPBS; Sigma-Aldrich, St. Louis, MO), 5 mM (Ethylenedinitrilo)tetraacetic acid calcium disodium salt (EDTA; Sigma-Aldrich, St. Louis, MO) and 10 KU of sodium heparin (Sigma-Aldrich, St. Louis, MO) per mL. All media was removed and repeated with a 5mL second rinse of the DPBS-EDTA-heparin solution. Once

all erythrocytes were flushed (identified by a color change in tissue), the tissue was perfused with 10 mL of DPBS with type-2 collagenase (1.0 mg/mL; Sigma-Aldrich, St. Louis, MO) and massaged for 10 minutes. The media was removed and 10mL of DPBS-EDTA-heparin was added. The liver was then cut into small pieces using a sterile surgical scalpel and disassociated for 20 minutes by gently expressing them with a 3 mL syringe. The resulting suspension was pipetted through a cell strainer (213 μ m sterile steel mesh pre-filter in series with a 70 μ m cell strainer). The resulting substrate was strained into a 50 mL polypropylene conical tube. Following 10 minutes of centrifugation at 70 x g average at 4°C, the solution was resuspended in 10 mL DPBS-EDTA-heparin and centrifuged again for 10 minutes at 70 x g average at 4°C. Cells were resuspended in 10 mL L-15 5% PSG and 10 μ L of this suspension was added to 10 μ L of 4% trypan blue and the total number of cells and viability was determined using the Countess® II automated cell counter (Life Technologies, Carlsbad, CA). Hepatocytes were adjusted to a concentration of 4×10^5 cells/100 μ L in L-15 5% PSG. A total of 105 μ L of the cell suspension was transferred to each cell of a 96-well plate (Costar 3396, Corning, NY).

The concentrations of MCLR used are based on those presented for cultured cells (X. Li et al. 2003; Takumi et al. 2010) and preliminary data for concentrations present in the environment. The concentrations are 0.05, 0.5, and 5.0 ng/mL. Additionally, the concentration of 50.0 ng/mL was included in the second challenge. Stock concentrations of MCLR were solubilized in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). Microcystin-LR was diluted to the desired working concentrations of 0.1% DMSO in L-15 5% PSG and 105 μ L of MCLR at the desired concentration to each well. The final hepatocyte concentration was 2×10^5 / 100 μ L in L-15 5% PSG + 0.05% DMSO. Plates were placed within a humidified chamber in a 20 °C incubator. Hepatocytes were challenged for 6, 12, and 24 hours. Following each

incubation, 10 μ L of the cell suspension was reserved for viability determined using the Countess® II automated cell counter as described above. The remaining 200 μ L suspension was centrifuged for 10 minutes at 70 x g average (noting that 200 μ L yielded the optimized cellular concentration for downstream transcript abundance analysis). Supernatant was removed and 100 μ L of TRK lysis buffer (Omega Biotek, Norcross, GA) containing 0.02% 2-Mercaptoethanol (Sigma-Aldrich, St. Louis, MO) was then added to each sample well. The resulting lysate was transferred to a 0.2 mL 8-strip PCR tube with cap (USA Scientific, Ocala, FL) and stored at -80°C until analysis.

2.2. Anterior kidney stimulation

The first step was to map the anterior kidney tissue transcriptome for SMB. This was performed using tissue from adult SMB collected in 2015 from the Susquehanna River basin. Anterior kidney cells were isolated and stimulated using methods described by Sharp et al. (1991). Briefly, whole anterior kidney was aseptically removed and placed into a Falcon® 15 mL conical tube (Corning Inc., Corning, NY) containing sterile Leibovitz's L-15 media (Sigma-Aldrich, St. Louis, MO) supplemented with 10 KU/ml sodium heparin, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2% (v/v) fetal bovine serum (L-15 2%) and stored on wet ice.

A single-cell suspension was then created by pouring the contents of the conical tube into a sterile 100 mm x 15 mm petri dish and expressed with a sterile three mL BD Luer-Lok™ syringe (Becton Dickinson, Franklin Lakes, NJ). The suspension was filtered using a 70 μ m Falcon™ cell strainer (Becton Dickinson, Franklin Lakes, NJ) into a 50 mL conical tube (Corning Inc., Corning, NY) and then transferred back into the original 15mL conical tube. The suspension was centrifuged for 10 minutes at 4°C at 500 g. The supernatant was removed, and the resulting pellet was suspended in 10 mL of fresh L-15 2%. The cells were centrifuged for 10

minutes at 4°C at 500 g and the cells washed two more times with L-15 2%. We then combined 10 µL of the resulting suspension with 90 µL of Trypan blue (Sigma Aldrich, St. Louis, MO) and counted using one square in a hemocytometer. The calculation is as follows: Total cells per mL = total calls counted * 100000. Cells were diluted to a final concentration of 5×10^5 cells per mL in Leibovitz's L-15 media supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 5% (v/v) fetal bovine serum (L-15 5%) and 1 mL was transferred to a 24-well Costar® 3527 TC-Treated multiple well plate (Corning Inc., Corning, NY). Cells were challenged for 18 hours at 15°C with 0.05 ng/mL MCLR, 20 µg/mL peptidoglycan (PGN; Sigma Aldrich, St. Louis, MO), 10 µg/mL polyinosinic-polycytidylic acid sodium salt (Poly I:C; Sigma Aldrich, St. Louis, MO), 5 mg/mL zymosan A from *Saccharomyces cerevisiae* (Sigma Aldrich, St. Louis, MO), or lectin from *Phaseolus vulgaris* (PHA-P; Sigma Aldrich, St. Louis, MO).

2.3. RNA isolation and sequencing

Each sample was lysed, and total RNA extracted (including DNase) using protocols specific to Omega Bio-tek (Norcross, GA). Total RNA was quantified among individual samples using the Quant-iT™ RNA assay kit (Invitrogen Corporation, Carlsbad, CA) and normalized accordingly. Sample aliquots were then pooled among immune-stimulant treatment group. The RNA integrity number (RIN) for each pooled treatment group was quantified using an Agilent RNA 6000 Nano Chip (Agilent Technologies, Santa Clara, CA) on the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) and verified to be ≥ 8 . Samples were shipped to the University of Maryland School of Medicine Institute for Genome Sciences (Baltimore, MD) for sequencing on the Illumina HiSeq 4000 (Illumina, San Diego, CA). Barcoded strand specific RNA-seq was performed.

2.4. Bioinformatics

Paired-end (PE) sequencing reads were imported into CLC Genomics Workbench v9.3 (Quiagen, Hilden, Germany) for quality trimming and *de novo* assembly. These PE reads were imported from control, Poly I:C, PHA-P and zymosan A stimulated cells to establish a *de novo* reference transcriptome (the PGN treatment was omitted as the other immunostimulants provided a more comprehensive contig list). Assembly utilized the default parameters and contigs less than 200 base pairs (bp) were culled from the reference. A reference transcriptome was assembled and annotated by pooling all four select treatment groups. This set of reference sequences was annotated using Blast2Go. An RNAseq analysis was conducted in CLC Genomics Workbench v9.3 (Qiagen, Hilden, Germany) to coarsely identify genes differentially regulated by immune stimulation.

2.5. Leukocyte isolation and PGN/MCLR challenge

Nine SMB (5 females and 4 males) were collected from ponds located at the United States Geological Survey Leetown Science Center (Kearneysville, WV). Fish were acclimated indoors for three weeks at 13.7°C prior to exposure and fed twice weekly to satiation with live fathead minnows *Pimephales promelas* and bluegill *Lepomis macrochirus*. Fish were euthanized using a lethal dose of MS-222. Leukocytes were isolated as described above with the following addendum. Prior to counting cells, cells were suspended in 6 mL L-15 2% and carefully layered on top of a 32% Percoll in Hanks Balanced Salt Solution (HBSS; Sigma Aldrich, St. Louis, MO) in a 15 mL conical tube. This was centrifuged at 500 g for 45 minutes at 4°C. Leukocytes were removed and washed three times in L-15 2%. Following the final wash, cells were suspended in 10 mL L-15 2% and combined 1:1 by volume with Trypan blue. Cells were counted, and viability was determined using the Countess® II automated cell counter (Life Technologies,

Carlsbad, CA). Cells were diluted to a final concentration of 1×10^7 cells/mL in L-15 5%. Duplicate 96-well Costar® 3598 Polystyrene TC-Treated Microplates (Corning Inc., Corning, NY) were used containing 5×10^5 cells per each treatment well. Treatment groups were: carrier control (methanol), 20 µg/mL PGN, 0.05 ng/mL MCLR, 0.50 ng/mL MCLR, 5.00 ng/mL MCLR, 0.05 ng/mL MCLR + 20 µg/mL PGN, 0.50 ng/mL + 20 µg/mL PGN MCLR, or 5.00 ng/mL MCLR + 20 µg/mL PGN. Cells were challenged at 17.5°C for 18 hours. Following the challenge, cells from one plate were lysed using TRK lysis buffer (Omega Bio-tek, Norcross, GA) supplemented with 2-mercaptoethanol (Sigma Aldrich, St. Louis, MO) and stored at -80°C prior to analysis using the NanoString nCounter SPRINT™ (NanoString Technologies, Seattle, WA). Viability was quantified using the second plate using the automated cell counter method described above.

2.6. Transcript list development

Genes were selected for the hepatocyte assay primarily based on the known oxidative stress and endocrine disrupting effects of MC on fishes in the literature (Table 1). In addition to these, a suite of genes associated with endocrine disruption, stress, and contaminants were also included; as well as the six housekeeper genes: 40S ribosomal protein S12 (RPS12), elongation factor 1α (EF1α), eukaryotic translation initiation factor 3D (ETIF3D), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L8 (RPL8), and tata box binding protein (TBP). Specific sequences adapted to SMB were based on Hahn et al. (2016). A full summary of the genes selected are included in Table 2.

A SMB leukocyte custom CodeSet was developed using target and housekeeper genes identified both in the literature and from our HiSeq 4000 runs for MC and PGN (Table 3). Specifically, TNF-α, TGFB, IL1B, and suppressor of cytokine signaling 3 (SOCS3) have been

shown to be differentially expressed by exposure to microcystins (Rymuszka and Adaszek 2012, 2013; LiLi Wei et al. 2018). Additionally, genes were included from the TLR-1/2 pathway (Figure 1) which responds to a PGN stimulus (Rebl et al. 2010). Additional genes were included which are associated with general immune function.

2.7. Transcript abundance normalization and quantification

Transcriptome databases for SMB were utilized in the creation of each custom CodeSet designed by NanoString Technologies (Seattle, WA). Transcript abundance was analyzed at the United States Geological Survey, Leetown Science Center (Kearneysville, WV) using the NanoString nCounter SPRINT™ (NanoString Technologies, Seattle, WA). Abundance counts were normalized using nSolver Analysis Software (v3.0). Each study was analyzed independently.

2.8. Quantitative analysis

All statistical analysis was performed within the R statistical package (R Core Development Team, 2017). Statistical significance was determined at $\alpha=0.05$. Housekeeping genes were assessed using the *Normfinder* algorithm as presented in Andersen et al. (2004). Transcript abundance was normalized using the nSolver™ analysis software v3.0 (NanoString Technologies, Seattle, WA). Quality control was performed as defined by Hahn et al. (2016). Transcript abundance was initially quantified using parametric methods which incorporate Bayesian methodologies to reduce the likelihood of false discovery. We examined both the Bioconductor *edgeR* package defined by Robinson et al. (2010) and the *NanoStringDiff* package as defined by Wang et al. (2016). These analyses were rejected based on inconsistent differences observed between these analyses techniques among treatment groups. These inconsistencies

likely result from the low sample size within each treatment group. Therefore, the Kruskal-Wallis test was used with the Multiple Experiment Viewer software defined in Howe et al. (2011). The Wilcoxon rank-sum test was incorporated if significant differences were identified.

3. Results

3.1. Transcript abundance using hepatocyte assay

A low decrease in viability was observed in each of the hepatocyte challenges ($\leq 1.27\%$ and $\leq 1.94\%$ for the challenges on January 16, 2018 and February 26, 2018, respectively). These were not associated with any specific treatment, individual, or time point. Both challenges were normalized together as one large experiment. Based on the stability analysis, only the housekeeping genes 40S12, EF1a and RPL8 were used.

A summary of the hepatocyte transcript abundance results is provided in Tables 4 and 5. The controls for each time period were significantly different between the two challenges; therefore, each challenge was analyzed independently. Vitellogenin counts were only observed in two individuals (one female in each challenge) and thus were removed from further analysis. No transcripts were significantly different either among treatment or among exposure time during either challenge.

3.2. Leukocyte Sequencing and RNAseq

Metrics of treatment library specific sequencing are listed in Table 6. A total of 189,359 contigs were identified greater than 200bp. Sequences mapped to 33,950 annotated contigs representing complete or partial gene sequences during RNAseq analysis. A total of 2431, 1667, or 1366 genes were upregulated greater than 2.5-fold relative to controls following zymosan A, Poly I:C or PHA-P stimulation, respectively.

3.3. MCLR-PGN leukocyte challenge

A minimal decrease in cell viability was observed ($\leq 0.7\%$) which was not associated with any treatment group or individual. The housekeeping genes ETIF3D, RPL8, and 40S S12 were rejected due to poor stability among sex and among treatment. Only EF1a was retained as a housekeeping gene based on high stability among sex and among treatment group. A total of 12 genes were removed from analysis due to minimal expression among treatment groups.

Table 7 provides a summary of all statistically significant differentially expressed transcript abundance results. An additional 33 transcripts were removed as there were no significant differences among treatment groups. There were also no significant differences among sex. A significant upregulation of the pro-inflammatory cytokine IL1B, cyclooxygenase-2 (PTGS2); the cytokine CCL-CUa; the two chemokines interleukin 8 (CXCL8) and RANTES (CCL5); and suppressor of cytokine signaling-3 (SOCS3) were reported in the PGN and PGN co-exposure treatments as compared to the MeOH control. The only transcripts that were significantly different among MCLR treatments were MAP3K8 (high-dose only) and NCF1 (medium and high dose). All other differences were only reported in PGN-associated treatments.

Log₂ fold changes relative to the MeOH control are presented in Figure 2. All treatments except for B-cell receptor CD22 resulted in a relative upregulation. Appreciable fold changes (>2.0) were observed in all of our significantly differentially expressed genes except for NCF1. Generally, these differences were only the PGN and PGN co-stimulated treatment groups.

4. Discussion

No significant transcriptional activation or suppression was observed in hepatocytes in any of the treatment groups. Only weak temporal and/or dose trends were observed. The minimal

effects of CYP1A are consistent with Wang et al. (2006) which suggest that CYPs do not play a pivotal role in the metabolism of MCLR. We did not find significant dysregulation of GST θ or GST ρ . As discussed earlier, GSH (catalyzed by GST) has been shown to be the first step in the detoxification of MCLR (Pflugmacher et al. 1998). However, an upward trend in rho-class transcripts is noted as compared to theta-class GST. It is unclear whether the minimal effect of theta-class represents a downregulation as compared to rho-class or is rho-class play a greater role in detoxification in SMB. This may result from a temporal and dose-response in response to ROS as indicated by GPX transcripts. Glutathione peroxidase, SOD, and CAT would all be expected to be involved in protecting cells from the effects from ROS as discussed in Li et al. (2003). Moderate changes in GPX were observed at the 12- and 24-hour time points in the second and first challenge, respectively. This may suggest variable response time and intensity to intracellular hydrogen peroxide as noted by the increased abundance in GSR in the 50 ng/mL dose. However, the lack of significant results cannot be discounted meaning these concentrations of MCLR simply may not result in a hepatic transcriptional response under these conditions.

Activation of genes associated with most components of the TLR pathway by PGN was observed (Figure 1). Most of the statistically significant activation was observed at (but not restricted to) end nodes (cytokines and chemokines). There is also observed evidence of both NF- κ B and MAPK pathway activation. One omission was TLR2. This may have resulted from an inadequate probe or the experiment's kinetics. Leukocytes were challenged for 18 hours and the possibility that many of the transcripts had already been "turned off" when cells were sampled cannot be ruled out. We observed an activation of SOCS3. This may be compensatory as SOCS3 is activated by cytokines and acts to inhibit their effects (Wang et al. 2011). Reduced

activation of CD22 was also observed. As CD22 acts to inhibit B-cell signaling (Li et al. 2017), a reduction of transcripts may suggest an increase in B-cell signaling.

Several dose-dependent trends are noted in the leukocyte challenge although there were no statistically significant differences among MCLR treatments. For example, an increased abundance of IL1B was observed similar to Rymuszka and Adaszek (2012). However, unlike their results, a reduction of IL1B at higher concentrations was not observed. Similar non-significant dose-response in the chemokines CCL5 and CXCL8, and the RELA subunit of the transcription factor NF- κ B were reported. Overall, this may suggest that increased concentration of MCLR may be driving increased transcription of pro-inflammatory cytokines and chemokines. However, given the minimal log₂ fold changes and statistical insignificance in most transcripts, it is more likely that MCLR at these concentrations does not influence these transcripts at a biologically meaningful level.

Differential transcript abundance was reported in 14 genes. In all of these except for NCF1, the coexposure with PGN yielded greater up- or downregulation as compared to MCLR alone (noting no significant differences in most MCLR treatment as compared to controls). A slight concomitant effect was observed among PGN and MCLR in ten transcripts (CCL5, CD22, CXCL8, IL1B, JUN, PTGS2, SOCS3, CHUK, NFKB2, and PIK3R5). While the possible additive effect is noteworthy, perhaps of more importance is coexposure with PGN elevated (or lowered) these transcript counts to a statistically significant level as compared to the control. As shown in Chapter Two, the concentrations of microcystin/nodularins in the environment are $\leq$ 0.05 ng/mL and persisted throughout much of the calendar year. This study has shown that acute exposure to MCLR does not negate the effects of PGN and may additively influence the expression of cytokines and chemokines transcript abundance. It is unclear whether such chronic

exposure to MCLR causes associated stress on the immune system. A further investigation would be needed to test this hypothesis both *in vitro* and in the environment.

To our knowledge, this is the first MCLR challenge using SMB hepatocytes, as well as the first PRN-MCLR coexposure challenge using SMB leukocytes. This exposure taken with the literature would suggest that these concentrations of MCLR alone are unlikely to elicit a strong immune response. However, given the known occurrence of microcystins and bacteria in the environment, the biological significance of these results in the context of concomitant exposure cannot be ruled out. Hepatocytes were not coexposed to MCLR and an additional compound. However given the leukocyte results, we also cannot rule out the possibility of an effect (synergistic, antagonistic, or concomitant) given the suite of other endocrine disrupting compounds, bacteria, viruses, and parasites which have been previously shown to be present within the Potomac River watershed (Alvarez et al. 2009; Blazer et al. 2010; Kolpin et al. 2013). Experiments addressing these mixture effects should also incorporate temporal effects. In leukocytes for example, Kono et al. (2004) noted maximum expression of bactericidal permeability-increasing protein/LPS binding protein after four hours while granulocyte colony stimulating factor receptor activity peaked after 12 hours. Challenging SMB leukocytes at various temporal points may yield further insights into not only the response to PGN stimulation, but also the temporal response to MCLR. Further research would be required to determine the hepatic transcriptional response of hepatocytes to this mixture.

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Figures

Figure 1. Toll-like receptor 1/2 pathway modified from full *Danio rerio* KEGG TLR pathway (http://www.genome.jp/kegg-bin/show_pathway?dre04620). Gene abbreviations represent HUGO equivalent. Color codes represent transcript activation across all treatment groups.

Figure 2. Log₂ fold changes for each significantly differentially expressed leukocyte transcripts as compared to the MeOH control.

Tables

Table 1. Description of genes associated with microcystin-LR treatment and expected change in regulation.

Gene Name	Abbreviation	Category	Citation	Expected Association with Microcystin toxins
Glutathione Peroxidase	<i>GPX</i>	Oxidative stress	Clark et al. 2007	Positive
Glutathione Reductase	<i>GSR</i>	Oxidative stress	Clark et al. 2007	Positive
Glutathione-S-Transferase p-class	<i>GSTp</i>	Oxidative stress	Liang et al. 2007	Positive
Glutathione-S-Transferase θ-class	<i>GSTθ</i>	Oxidative stress	Fu and Xie 2006, X. Li et al. 2003	No effect
Superoxide Dismutase	<i>SOD</i>	Oxidative stress	Clark et al. 2007	Negative
Proliferating Cell Nuclear Antigen	<i>PCNA</i>	Neoplasia	Wei et al. 2008	Positive
Cytochrome P450, family 1, member A1	<i>CYP1A</i>	Endocrine distruption	Zikova et al. 2013	No effect
Vitellogenin	<i>vtg</i>	Endocrine distruption	Rogers et al. 2011	Positive
Heat Shock Protein 70	<i>hsp70</i>	Oxidative stress	He et al. 2010	Positive
Heat Shock Protein 90	<i>hsp90</i>	Oxidative stress	Y. Li et al. 2009	Positive
Interleukin-1β	<i>IL-1β</i>	Pro-inflammatory Cytokine	Rymuszka and Adaszek 2012	Negative
Catalase	<i>CAT</i>	Oxidative stress	Clark et al. 2007	Negative
Transforming growth factor β	TGFβ	Neoplasia	Rymuszka and Adaszek 2012	Positive

Table 2. Specific primers used in NanoString nCounter liver custom CodeSet. Tm CP and Tm RP represents the melting temperatures of the capture and reporter probe, respectively.

Transcript Name	Classification	HUGO Gene Abbreviation	Abbreviation	Tm CP	Tm RP	Primer
17B Hydroxysteroid Dehydrogenase	Endocrine Disruption	HSD17B3	17BHD	83	84	CATCCTCAACATCTCGTCTGCC AGTGGGATGTACCCTGTTCTT CTCCTCACTGTCTACTCTGCCT CCAAGGCGTTTGTGGACTTCTT CTCCCGAGGACTG
40S ribosomal protein S12	Housekeeper	Micropterus_0108	RPS12	86	85	CTCCCTGATCCACGATGGCCTT GCCCCGTGGTATCCGTGAGGCC ACAAAGGCCCTGGACAAGCGT CAGGCTCATCTCTGCGCTCTG GCTGCCAACTGCGAT
Androgen Receptor α	Endocrine Disruption	ARA	AR α	85	86	GACTGCACCATCGACAACTG AGGCGGAAAACTGCGCCTCG TGCCGTTTAAAGAGATGCTTC ATGTCGGGAATGAGCCTTAAA GGTCGCAGGCTAAAGG
Androgen Receptor β	Endocrine Disruption	ARB	AR β	83	84	ATTAACATCGAATGACCACC AACTGCTCTCAGAGGTTCTAC CAACTACCCGAGTCCTGGAC TCTCTCCAGATGACGGTCAAG AAGCTCCATCAGTTA
Apolipoprotein A1	Endocrine Disruption	APOA1	APA1	79	84	TTTAGCCGCTGAAATCAAGGA TAAGTTCCAAGCTATCTTCGA GGCCATCGCTGCTACCGTCAC CAAGAGCTAAATCCTCTCCAC CCTGTCCCTGTAATTA
Aryl Hydrocarbon Receptor	Contaminants	AHR	AHR	85	83	CGCCGCCTCAGCTCGCCCTGTT CGCCATCGCCACACCCCTCCA GCCTCCAGCCATCCTTGAAAT CAGGACGAGGAACATGATTTT CAGGACCAACACAA
B-cell lymphoma 2	Neoplasia	BCL2	BCL2	85	82	GATGACAGAGTACTTAAACGG ACCTCTGAACAGCTGGATACA AGATAACGGGGGATGGGTAG GTCTGATTCCGTGTAGTTGAG CGCTTTGCAGTTGTGTG
Complement Component 3	Immune Response	GDQU01000040	C3	79	84	CAATCTTGATATTGGCTTGCT AACTGGCTTTACTGCTAACAC AAATGACCTGGACTTACTGTC CAAAGGACGTGCCCGCACTAT TGCAAAATATGAGAT

Transcript Name	Classification	HUGO Gene Abbreviation	Abbreviation	Tm CP	Tm RP	Primer
Catalase	Endocrine Disruption	CAT	CAT	84	82	GCCCAGCTCTTCATCCAGAAA CGCATGGTCGAGAATTTGAAG GCTGTCCATCCACACTATGGA AACCGAGTTCAGGCTCTTCTT GACAAGTACAATGTGG
Choriogenin	Endocrine Disruption	HSD3B	CHG	84	80	GAAAGCAAAAATGTGAGCAG CCTTCCTCTTGACCCTCTATGG GTCCCATTTTCTGCGGTTAAGG TGGCTGAGGAATTCTTATACTT CACCTGAAACTCA
C-reactive protein-like	Stress Response	GDQU01027741	CRP	81	86	CAGCTGTGGTTTGATGGACAA CCATCAATAAGGAAATATATC AGCTCTGGATCAAGCATCAAA GGGCCCACTATTATCACCTTA GGACAGGAGCAGGATT
Cytochrome P450, family 1, member A1	Endocrine Disruption	CYP1A	CYP1A	83	78	CAGCTCAACACTGTTATGAAG GCTGAAGGCAGTTTCGACCCA TTCCGCTACATCGTTGTCTCTG TTGCTAATGTGATCTGTGCCAT GTGCTTTGGCCGAC
Cytochrome P450, family 3, subfamily A	Endocrine Disruption	CYP3A	CYP3A	79	82	TTCGGCACTATGCTGGCATAT AAAAAGGGATTTCATGCACTTT GATTCGGAGTGCTTCAAGAAA TATGGGAAAACATGGGGCATT TTTGATGGCCGTCAGC
Elongation Factor 1 α	Housekeeper	EF1A	EF1 α	85	85	AGATCAACGCAGGTTACGCAC CTGTGCTGGATTGCCACACAG CTCACATTGCCTGCAAGTTCA AGGAGCTCATCGAGAAGATCG ACCGTCGTTCTGGCAA
Epidermal Growth Factor Receptor	Neoplasia	EGFR	EGFR	86	84	GGTCTGTGGGTTCCAGAAGGA GAGGATGTGAAGATCCAGTG GCCATCAAAGTTTTGAGAGAG GCCACATCACAAAAGCAAAC AAAGAGATCTTGGACG
Epoxide Hydrolase 1	Contaminants	EH	EH1	83	84	GATAGACCAGACTCGCCCTGT CCCTTCACTAGAGGATAGCCA GTTTAATTATGGCTTCAATTCC CAGTACCTGCAGAAGGTGGTC TCTTACTGGAGAAAT
Estrogen Receptors α	Endocrine Disruption	ESRA1	ER α	85	85	CTCTCCACCATCAGGCACAT GAGCGAACAAAGGTAATGGA GCATCTCTACAGCATGAAGTG CAAGAACAAAGTGCCTCTGTA CGACCTTCTGCTGGAGA

Transcript Name	Classification	HUGO Gene Abbreviation	Abbreviation	Tm CP	Tm RP	Primer
Estrogen Receptors β 1	Endocrine Disruption	ESRB1	ER β 1	85	85	GCCTGAGCAGAGAAGAGGGG AGCTGTGTCCAGGGCTTCGCA GAGATCTTTGATATGCTGATA GCTGCCACGTCCAGGGTGAGA GAGCTCAAGCTCCAGAG
Estrogen Receptors β 2	Endocrine Disruption	ESRB2	ER β 2	85	86	CTGCAGCTCCATCCTCGGTTGT AACAAAGCCGCTGGGGAAGAG ATTAGAGGAAGGAGTGGAAG GCGTGAACCTCTCCTTGTGTTT TTCTGCGGTAGGGAAA
Eukaryotic Translation Initiation Factor 3D	Housekeeper	EIF3D	ETIF3D	85	84	ACATGACTCAGTTCAACATGC AGACGCTACCTAAGAGCGCCA AGCAAAAGGAGAGGGATCGT ATGCGCCTGCAGAAGAAGTTC CAAAAGCAGTTTGGTGT
Glucocorticoid Receptor	Stress Response	GR	GR	82	84	GCAGATGCTGAAGATCTGTAA TGAGTTTGTGCACTGCAGGT GTCCTATGATGAGTACCTGTG CATGAAGGTCTGTACTGCT CAGTACAGTACCGAAA
Glucokinase	Neoplasia	GCK	GLK	83	81	TACTCTATTCCTGAAGATGCC ATGACAGGCACTGCCGAAATG CTATTTGATTACATAGCAGAG TGCATGTCAGACTTCTTGGAC AAACATCATCTCAAGC
Glutathione Peroxidase 1	Stress Response	GPX	GPX	83	83	GAACGAGCTTCACAGTCGTTA CACTGCCAAGGGACTCGTTAT TCTGGGTGTGCCCTGCAATCA GTTTGGACATCAGGAGAACTG CAAGAATGATGAAATT
Glutathione Reductase	Stress Response	GSR	GSR	83	83	TCTGCCAAAGCGCAGTGTGAT TGTCGGTGCGGGTTATATTGC AGTGGAGATGGCAGGCATCCT TTCCACCCTGGGGTCCAAAC ATCCCTCATTATTCTGA
Glutathione S-Transferase theta-1	Stress Response	GST	GST θ	84	83	CCTGCTGGAGGCGAAATTCCT GCAGAACAAACCGTTTATCAT CGGCGACAAAATCTCTCTGGC TGATCTGGTGGCTATAGTTGA GATCATGCAGCCTGTT
GST A-like (rho-class)	Stress Response	GDQU01079 959	GST ρ	79	82	TCGTGTCTGTCAGATATTTAAC AAACACAGGCAATGTTGGTCT GTGCAGCATGTGATGTTTGCA GGGTAACAGGCATAGGTTGCA GGCCAAATGTAATGT

Transcript Name	Classification	HUGO Gene Abbreviation	Abbreviation	Tm CP	Tm RP	Primer
haptoglobin	Stress Response	GDQU01003 865	HP	83	79	TCTACAGCGACCTCTTTGGAG GCATCAGCTTCCGCCC GTTGT GAAATTTCCAGGTACACTTTA GGAATGACGGGATCTTTTCCC TGAATATCCTGTCTCGAC
Heat Shock Proteins 71	Stress Response	HSP71	HSP71	80	84	TCATTGCATTGTTTGCACACA ACTGAGGAGCAACTTGTTGGC ACAGTTGAGCTGTCAGTTCAC ATGTAAAGTGTCTGGTATACT GAGAGGTCACCTGCCT
Heat Shock Proteins 90B	Stress Response	HSP90A	HSP90A	81	84	ACATCAAGCTGTATGTCAGGA GGGTCTTTATTATGGACAACT GTGAAGAGCTCATTCCAGAGT ACCTGAACCTTGTCCGTGGTGT GGTGGACTCTGAGGA
Hepcidin 1	Endocrine Disruption	HAMP1	HEP1	82	80	TCTCATGGGTGTCAGGCCACC AAAAGAGCACCACAATATCTT CACTTTATTCTTCAGCTCTAAC GATGGATGGCGATTGTCTGGG CAAGTGGATTCTCTC
Hepcidin 2	Endocrine Disruption	HAMP2	HEP2	85	81	ACTCAGAGGAGCTGACAAGAG TCACCAAAGAGTCAAAGGATT GAACAACTTAAACCTCTCAAA CCCTTCTAAGATGAAGACACT CAGTGTTGCAGTTGCA
Hypoxanthine Phosphoribosyltransferase 1	Housekeeper	HPRT1	HPRT1	83	83	ACTTCATTTCGCTCAAGAGCT ACTGTAACGACCAGTCGACAG GTGAAATCAAAGTAATCGGTG GGGATGACCTGTGTACGTTGA CAGGCAAGAATGTCTT
Interleukin-1 β	Immune Response	GDQU01054 274	IL1b	83	81	ATCGTCGCCATAGAGAGGTTT AAGGGCAGCAGATGCATTGAC TCTGAAAACCTGCTCAACTTC ATGCTGGAGAGCTTAGTGGAA GAGCATATAGTGTGTTG
Insulin-like Growth Factor 1	Endocrine Disruption	IGF1	IGF1	83	84	CGGAGACCCGTGGGGATGTCT AGCGCTCTCTCCTTTCAGTGGC ATTTATGTGATGTCTTCAAGA GTGCGATGTGCTGTATCTCCTG TAGCCACACCCTCT
Largemouth Bass Virus MCP	Virus	LMBV MCP	LMBV MCP	85	86	AGCAGTTTAACTGCTTTTCCT GGATGCCTGGAACGAGTACAC CATGCCCAGGCCAAGCGCAT CGGGTATTACAACATGATTGG CAACTAGCGATCT

Transcript Name	Classification	HUGO Gene Abbreviation	Abbreviation	Tm CP	Tm RP	Primer
Metallothionein	Contaminants	MT1	MT	85	75	CAGCGCTCTACTGCCCTTGTG ATGGCGCCTGTGTGAACAAC AATGACTAACTGCACATGTCT ACATAAAATGTATTTGTACTC GTCTCAGCGTTGCAG
Proliferating Cell Nuclear Antigen	Neoplasia	PCNA	PCNA	84	84	CATCAGCTCAGAGCAGAAGA CAATGCAGACACACTCGCACT TGTGTTTGAGACGATCAACCA GGAGAAGGTCTCAGATTATGA GATGAAATTGATGGAC
Ribosomal Protein L8	Housekeeper	RPL18	RPL8	85	85	TTCTCCTCTGCAAACAGAGCT GTTGTTGGTGTGGTAGCTGGA GGTGGTCGTATTGACAAGCCC ATCCTGAAGGCTGGTCGTGCC TACCATAAGTACAAGG
Serum amyloid A-5	Immune Response	GDQU01041317	SAA-5	83	81	TCAGAGTATGCTCGCCACATA TCACGAGTTCCTTGAGCAGCT TCAACAGGAAACCAGTACCAC TGGGCATTGGTTTCCACAATT AGAACCAGAACAATTC
Serum amyloid P	Immune Response	GDQU01051042	SAP	80	83	TTCCGTGTGCTCGGGTTTCCAT TGACCCACAGCTGAGCGACTC CTGTTTTCCCTTCCCACGTAGC GCACACTGAATTGAAGACATT GGGATTATCCGGCA
Superoxide Dismutase	Stress Response	SOD	SOD	79	83	ATGGTGTTTAAAGCTGTTTGTG TTTTAAAAGGAGCCGGAGAGA CCACCGGGACCGTTTATTTTG AGCAGGAGGGCGATTTCAGACC CTGTGAAGGTGACAG
Tata Box Binding Protein	Housekeeper	TBP	TBP	82	84	GTTAGCTGCCAGAAAATACGC TCGTGTGGTGCAGAAGCTCGG TTTTCTTGCAAAGTTCCTGGAC TTCAAGATTTCAGAACATGGTG GGAAGCTGCGACGTG
Thyroid Hormone Receptor β	Endocrine Disruption	THRB	THR β	83	84	TGCATTACCTGTGAAGGTTGC AAGGGTTTCTTCAGGCGGACG ATCCAGAAGAATCTCAACCCCT ACCTACGCCTGTAAGTATGAG GCGAAATGCGTCATCG
Transferrin	Immune Response	GDQU01000776	TF	83	83	ATCAGAGCAAGAATATCGGAA ATGTTTGGATCTGGTAGCCAA AGCGCCCGCGTTCTCCTGCGT GAAGAAGGAAAACACCATCG ACTGCATCACTGCTATT

Transcript Name	Classification	HUGO Gene Abbreviation	Abbreviation	Tm CP	Tm RP	Primer
Transforming Growth Factor β	Neoplasia	TGFB	TGF β	83	84	AATAACACAGAGAACACCAA GACCTCCAAAAGCATCCCGAT GTTCTTCAGCATCTCTAACATA CGGGAAAGTGTGGGGGATTCC AGTTTGCTGACCAGTG
Type I Deiodinase	Endocrine Disruption	DIO1	DIO1	86	85	ACTGTCTGGAGCAGTCCACAG TGCAGAATACAACTATGAGT AGGACGACTGAGACCGACGAT GCAGCGTTGTGCGTAATGAAG GGTAGTCTGGAATAAA
Type II Deiodinase	Endocrine Disruption	DIO2	DIO2	79	75	GCTGTGAAATTGTGAGTTCAC CACCAAGGAGCAAGCATGTTT TTGCAAAGACTGATGGTCTAT TTATCGACAGTATGCCTGTTTT GCTATATGATAGGAC
Vitellogenin	Endocrine Disruption	VTG	VTG	80	84	CATTGGTAATCATGTTGCGCT GAGGTTTCATCAAGGAGCACTT CCTCGCTGGTGAGCTATCTTTT GCTGAAACTGCTCAAGCCCTG CTGGCATCTGTTCAC
Warm Temperature Acclimation Protein 65	Stress Response	WAP65	WAP65	82	83	TGGATGCTGTTGCAGTGAACG AAGAGGGAATCCCGTACTTTT TCAAGGGTGACCATCTGTTCA AGGGCTTCCATGGCAAAGCAG AGCTGTCCAATAATTC
Zona Pellucida 3	Endocrine Disruption	ZP3	ZP3	84	82	CTGTGTGGCCACTGCTGTCCCT GATATAAATGCAGTCCCCAGA TATTCCTTCATTGAGAACCATG GGTGTTTGATTGATGCCAAGC TAACCCAGTCCAGT

Table 3. Specific primers used in NanoString nCounter leukocyte custom CodeSet. Tm CP and Tm RP represents the melting temperatures of the capture and reporter probe, respectively.

Transcript Name	Classification /Association	HUGO Gene Abbreviation	Tm CP	Tm RP	Primer
RAC-alpha serine threonine- kinase	TLR1/2	AKT1	84	82	AGGTCTACCTGACCAAAATCAGACACAAA GTGACTATGCACGACTTTGAATACCTCAA ACTCCTGGGAAAAGGCACCTTTTGGCAAAG TTATCCTGGTAAA
caspase 8	TLR1/2	CASP8	80	84	TAAAAGACCTCAGCGGAAGGGATTTTTTT GCTGATGATGCCTTGTTGGTATGCGTGCT TTCCCACGGAGAAAAGGGTTGTGTCTTTG GGACGGATGAGAA
MIP1alpha-like	TLR1/2	CCL3	70	78	TTCTGGTTACCCAAAAGTCTCGTCATATCT GTGTGGATCCAAATCTCTCCTGGGTTGAG GACATCATGAAAATGGTGGATGAACGCTC CTTGTAAGC
C-C motif chemokine 4-like	TLR1/2	CCL4	83	79	GCAATGGAATTGGTCCTGATGACTGCTGC TTCAAATTCTACCCAAGAAGATTGAACAA AAACCTCATCACTTCATATTACATGACTG ATTACCGGTGCCC
monocyte chemotactic 1B-like	TLR1/2	CCL5	82	85	ATCATAATTTTCGGACATGGCGTGTGCCGA TCCAGAATTGGACGTGGCGAAGATTCTCTC TGATTGCGCTGAATGTGAGCGTGCGGAAA AAGTTGGGGCTGT
chemokine CCL-CUa precursor	RNASeq	CCL-CUa	86	85	AGGCTGGACCAGCTGAGAAGTTGGCAGA ATGCTGCAAATCAGTCAACAAACAGGAG ATAACCGAACCAATCATAGGATACTTGAT CCAGAAACGTGCGCC
B-cell receptor CD22-like	Addition	CD22	82	86	AGGAGACATTTTCAAAGGAAGTTTCGATGA CTCTCACCTGCAGCAGTGATGCAAACCCA CCTGTGACACAAAGCGGATACAGCCTGTA TAAAGACGGACAC
inhibitor of nuclear factor kappa-B kinase subunit alpha	TLR1/2	CHUK	80	79	CCTGGCACCTGAGCTTTTTGAGAATAAGC CATACACAGTTACTGTGGATTACTGGAGC TTTGGCACGATGGTATTTGAATGCAGTTG TGGTTTCCGTCCA
cytokine-inducible SH2-containing protein	Addition	CISH	85	86	ACTTCTGCTATCTAGAAAATTCAGGATGG TACTGGGGAGCTGTAAGTGC GGCTCAGGC CCATGCTGCACTTCAAGAGGCATCTGAAG GAGCTTTTCTGGT
C-X-C motif chemokine 11-like	TLR1/2	CXCL1	78	85	CTCTCATCACTGATGTCAAGGAGATTGAT CCTCTCCATATTGCAACAAGAAAGAAGT CATTGTCACACTGAGAGATAACAGCCAAA GGTGTCTTGACCC

Transcript Name	Classification /Association	HUGO Gene Abbreviation	Tm CP	Tm RP	Primer
Interleukin 8	TLR1/2	CXCL8	85	86	ACATGAAGAGCAGCAAAGTCATTGTCACC TCTATCGTGGTGCTCCTGGCCTTCCTGGCC ATCAGTGAAGGGATGAGTTTGAGGAGCCT GGGTGTAGAGCT
Elongation Factor 1A	Housekeeper	EF1A	85	85	AGATCAACGCAGGTTACGCACCTGTGCTG GATTGCCACACAGCTCACATTGCCTGCAA GTTCAAGGAGCTCATCGAGAAGATCGACC GTCGTTCTGGCAA
Eukaryotic Translation Initiation Factor 3D	Housekeeper	EIF3D	85	84	ACATGACTCAGTTCAACATGCAGACGCTA CCTAAGAGCGCCAAGCAAAAGGAGAGGG ATCGTATGCGCCTGCAGAAGAAGTTCCAA AAGCAGTTTGGTGT
Interferon gamma	PGN	IFNG	83	81	CTTCACCAGGGAACCACTGACTGACAAAA TTGAGGCAAAGATGGTGTATTATGGGTGGC GTTTTGGACACGTATGAAAAGCTGATTAG CCACATGTTGAAG
BCR IgM mu	Addition	IGHM	82	84	CTGCAGGGGAAGTGGATTTGATTTCAGTC AGTATGGAATGCACTGGATCAGACAGCCT TCAGGAAAAGCACTGGAATGGATGGGGC TTATTTACTATGAT
inhibitor of nuclear factor kappa-B kinase subunit beta	TLR1/2	IKBKB	82	84	TGGGCGACGGACAGACTTGCCTCTGGTCT TCCTGTTTGATCGTTCTTCCTGCACTTATG AACCTCAGTTTGCTCCTCGCACCTTCCAG AGAACATCCGC
NF-kappa-B essential modulator	TLR1/2	IKBKG	85	82	CAGACCAGCAATATTACTGAGGAGACTGT TCAACATCGCTCTGAAGCACAGCACTCTT CTGACTGGTATCGTGACTTGGAGGAGAAG CTTGATTACCTTC
Interleukin 10	MCLR	IL10	85	85	TGGAGAGTAAAGGTCTATATAAGGCCATG GGTGAGCTGGACCTGCTGTTTAACTACAT TGAGACATATCTGGCTTCAAACCGGCACA GAAAACCCAGAGC
Interleukin 12a	TLR1/2	IL12A	81	82	GGAATTCATCCTTTAGTGAGAGTGAATGT CTGAGGAACATCATGAAGGACTTGACCCA CTACACCGCTGTTATTCAGTCTTACCGCAG CTCCACACTCAT
IL1b	TLR1/2 and MCLR	IL1B	83	81	ATCGTCGCCATAGAGAGGTTTAAGGGCAG CAGATGCATTGACTCTGAAAACCTGCTCA ACTTCATGCTGGAGAGCTTAGTGGAAGAG CATATAGTGTTTG
Interleukin-6	TLR1/2	IL6	81	84	GAATTCAGAGACGTGCAATATCTCTTTCT GGACAACTACAAACCCTCCTCACTTCCAG AAAAGTGCCTAAATCCAACCTCAGCAAG GAGGCATGTCTCC

Transcript Name	Classification /Association	HUGO Gene Abbreviation	Tm CP	Tm RP	Primer
interleukin-1 receptor-associated kinase 1	TLR1/2	IRAK1	83	85	CCTCCTCTTCTCCTCCTCGTTTCGCTGGTC CGTGTGAAACCGATGAGAGTCGAGGTTTC TCCCAGTATGACCCTCGCGCCAAGTCAG ATCTAACGGGAC
interleukin-1 receptor-associated kinase 4	TLR1/2	IRAK4	80	84	ACATGAGAACTTGGTTGACATGGTTGGAT TTTGTGTGATGGACAGCACCCATGTTTG GTGTATGCCCTTATGGCCGCAGGTGACAC CTCAGGTGAGGAG
interferon regulatory factor 5	TLR1/2	IRF5	85	85	GGGAGGACTGGCCCGACAAGAAACCCAA AGAGAAAAAACTCATCATCGTGCAGGTGG TTCCTGTGGTGGCTCGAATCCTGACGGAG ATGTTCTCTGGAGA
c-Jun N-terminal kinase 1	TLR1/2	JNK	83	84	TCATCTTCCAGGGCACCACCACATTGAC CAATGGAATAAGGTAATTGAGGTTCTGGG CACGCCAGTCTTGAGTTTATGAACCGAC TGATGGAAACCGT
transcription factor AP-1	TLR1/2	JUN	83	84	ACCGTTTACAACAACAACGCCACCATGCG CTCCGACTCGCCGGTTTATGAGGACTTGA ACACTTTCAACCCAGCCATCAGCACCGTC TCGGCTCCTAATT
lymphotoxin alpha	Addition	LTA	83	85	AGAACTTACCCAAATGTCACTCCTGCTCC CTTGTCCTACATAACAACCTCTATCCACTGC AAGGAGAACAGCTTCTACTTCATCTACGC CCAGGTCAACTT
dual specificity mitogen-activated protein kinase kinase 2	TLR1/2	MAP2K 2	81	82	TCTTGAAATTAAACCTGCTATCAGAAACC AGATCATCCGAGAGCTTCAGGTGCTGCAT GAGTGTAACCTCTCCCTACATTGTGGGCTTC TATGGAGCATT
dual specificity mitogen-activated kinase kinase 4	TLR1/2	MAP2K 4	83	84	AGAATGGCTCCTAAAAAGAGACCAGTTCA TTTAACCATTGGGCCTACTGGCGATGGGA TGTCTACCTCCACCAACACTGATGTTACAT CTGAGGCCAATT
dual specificity mitogen-activated protein kinase kinase 6	TLR1/2	MAP2K 6	82	84	ACATCTTGGGCAAGATCACAGTAGCGATT GTCAAGGCATTAGAGCATCTGCACAGTAA CCTGTCAGTGATACACAGAGATGTGAAGC CCTCCAATGTTCT
P38 mitogen-activated protein kinase kinase 13	TLR1/2	MAP3K 13	83	84	GTTGTGTTGTGGGAACCTTCTGACCGGAGA GATTCCCTACAAAGACGTAGACTCCTCGG CCATCATTTGGGGTGTGGCAGCAACAGT CTCCACCTTCCTG
mitogen activated protein kinase kinase 7	TLR1/2	MAP3K 7	91	92	CCTGCTGATCCAGAGAAAGAAGGAGCTCA TCGCTGAACTGGACCAAGATGAGAAGGA CCAGCAGAACACGTCCCGTCTGGTGCAGG AGCACAAAAAGCTC

Transcript Name	Classification /Association	HUGO Gene Abbreviation	Tm CP	Tm RP	Primer
mitogen-activated protein kinase kinase 8-like	TLR1/2	MAP3K8	86	85	CAAACCCAGTAACATTGTCCTGATGTCAG ACAAGGCAGTGCTGGTGGACTTTGGCCTG ACAGTTCAGATGACAGAGGACATATACAT TCCTAGAGACCTG
mitogen-activated kinase 1	TLR1/2	MAPK1	84	84	TCAGTGGGTTCATCCTGGCTGAGATGTT GTCCAACAGGCCAATCTTTCCTGGGAAGC ACTACTTGATCAGCTTAACCACATTTTG GGGATCCTGGGTT
myeloid differentiation factor 88	TLR1/2	MYD88	85	85	CTGGAGGAGGTGGAGAGAAAAGACATTG TGGAGGATCTTCGTCCTTAATAGATGAG GATGTCAGGAAGTATTGTGAAAGTCTGAA GAAGAAGGCTGAAC
neutrophil cytosol factor 1	Addition	NCF1	85	81	CACACCGGAGACCTGGTGGAAATTGTAGA GAAAAGTCAGAACGGTTGGTGGTTCTGCC AGTATGAGAATAAACACGGCTGGGTTCCT GCATCCTATTG
nuclear factor NF-kappa-B p105	TLR1/2	NFKB1	85	85	AAGCAGGAAGTGTGTCGTGCTCTGGAGAG TGAAGGATGCTGGGAGAATCTGGCTCACG GTCTGGGTCTCGGGATCCTCAACACGGCC TTCAGACTGAGCC
nuclear factor NF-kappa-B p100 subunit	Addition	NFKB2	85	86	ACGCCTCCAACCTGAAAATCTCTCGGATG GACAAAACGTGCGGCTCGGTGCTCGGGGG AGACGAGATCTTCTGCTCTGTGACAAAG TCCAGAAAGACGA
NF-kappa-B inhibitor alpha	TLR1/2	NFKBIA	85	86	ACACGCCTCTTCACATCGCCTGTCGCCAT GGTAACCTGCTGTGCTTCAGCGTCATCAC GCAGAACTGTCAGCCGGAGTATCTACACA GGGTGATGGCTGC
phosphoinositide 3-kinase regulatory subunit 5	TLR1/2	PIK3R5	80	83	GCTCCATCCCTCTTCTCTGTCACTTCTGGA GCTGACAGTGACTATTTTGAAGATTCTGA TGAGAACATCTGCTCCTCTCCCGTTGCCG AAAAATGCTCGC
cyclooxygenase 2	Addition	PTGS2	82	79	AAGACAATTTCATTCCGGACCCGCAGGGCA CCAGTCTGATGTTTGCATTCTTCGCGCAGC ATTTACCCACCAGTTCTTTAAATCCGATA TGAAGAAAGGG
ras-related C3 botulinum toxin substrate 1	TLR1/2	RAC1	79	83	TACCCACAGACGGATGTGTTTCTCATCTG CTTTTCGCTTGTGAGTCCCGCCTCGTTTGA AAACGTCCGTGCCAAGTGGTACCCTGAGG TGAGACACCATT
transcription factor p65	TLR1/2	RELA	86	85	GACGTCCATAGGCAGGTGGCCATTGTGTT CCGCACACCGCCGTACCGTGACACTAACC TCTCTGAGCCTATTAGAGTGAAAATGCAG CTCCGCCGGCCCT

Transcript Name	Classification /Association	HUGO Gene Abbreviation	Tm CP	Tm RP	Primer
Ribosomal Protein L8	Housekeeper	RPL18	85	85	TTCTCCTCTGCAAACAGAGCTGTTGTTGGT GTGGTAGCTGGAGGTGGTCGTATTGACAA GCCCATCCTGAAGGCTGGTCGTGCCTACC ATAAGTACAAGG
40S ribosomal protein S12	Housekeeper	RPS12	86	85	CTCCCTGATCCACGATGGCCTTGCCCGTG GTATCCGTGAGGCCACAAAGGCCCTGGAC AAGCGTCAGGCTCATCTCTGCGCTCTGGC TGCCAACCTGCGAT
natural resistance-associated macrophage 2	Addition	SLC11 A1	85	85	GGGTAAGCTATCCACCTGCTGTGTTTCATA GAGGACGCCGTCTGAGTACGACTCCTAGA GCTGAACAGGAGCACTAACCACCAGCATT CCTCTTTCCTCTT
suppressor of cytokine signalling 1	Addition	SOCS1	87	92	GTATCTGACCCACTTCCCCACCTTCTCCTG CAAGGAGGACTGTGTGATCATCACAGACA CAGCGTCCAAGCTCGAGCGCAGCTCCTTC TACTGGGGCCCT
suppressor of cytokine signalling 2	Addition	SOCS2	85	86	CCACCAACCTGCGGATCGAGTACAAACAC GGCAAATTCAAGCTGGACTCGGTGGTTCT GGTGAAGCCCAAGCTGAAGCAGTTCGACA GCGTGGTCCACCT
suppressor of cytokine signalling 3	MCLR	SOCS3	82	85	CCCACTTTGACTGTGTCTCAAGCTGGTCC ATTACTACATGTCTCAGAACAAAGGGAAC ACTCGCAGTGGGAATATCTACTACATTTA CTCTGGCGGGGA
suppressor of cytokine signalling 4	MCLR	SOCS4	84	85	TGGCCACACTGACTACATCTTGGTCCCAG ATCTCCTCCAGATCAACAACAGCTCTTGTT ACTGGGGTGTGCTTAACCGCTTTGAGGCA GAGGAGCTACTG
suppressor of cytokine signalling 5	Addition	SOCS5	86	84	GTGGGAACCGTAGGGACAGACGTTATGG AGTGTGCTCCATCCAGGACATGAGCGACT CTGTATCTGGAGGACGCAGTCTGAATGCT CGGTCTTTGCGCCA
suppressor of cytokine signalling 6	Addition	SOCS6	82	84	CGGAAAGAATTTACGATTTCCAGATGGA AGGGCTCTTTCAGGACCAAGCAGAATCCT TAAAGAATCTCCAGCAGCCTCAAAACGGT GAGCTGCATCTAA
suppressor of cytokine signalling 7	Addition	SOCS7	83	82	CACTACAGAGGTACGTTACGCCTCTGGTG TCACCCTAAGTTTGAGGATCGCTGTCACT CTGTGGTGGAGTTCATTGAACGAGCTATC ATGCACTCCAAGA
TGF-beta-activated kinase 1 and MAP3K7-binding 1	TLR1/2	TAB1	92	89	GCTAGCTGACGGAGCGGTACGCCCACAC AGGGTGGACGTATCTACCCTGTATCAGTG CCCTACTCCAACAGCCAGAGCACTAGTAA AACCAGTGTACC

Transcript Name	Classification /Association	HUGO Gene Abbreviation	Tm CP	Tm RP	Primer
TGF-beta-activated kinase 1 and MAP3K7-binding 2	TLR1/2	TAB2	79	86	TCCTCTGGTGTCTCCTCGTGCTCTTCTTCC TCTTCCTCCTCCTCTGTCATGCCCACATCC CTGTCTACCATCAGCCAGTACAACATCCA GAACATCTCCA
TGFB1	MCLR	TGFB	83	84	AATAACACAGAGAACACCAAGACCTCCA AAAGCATCCCGATGTTCTTCAGCATCTCT AACATACGGGAAAGTGTGGGGGATTCCA GTTTGCTGACCAGTG
toll interleukin-1 receptor domain-containing adapter	TLR1/2	TIRAP	83	85	CGCTGTTGCGATGGAGCCGAAAGTATGAC GTGTTTGTGTGCCACAGCTCTGTGGACAG CGACATTGAAGAGGCTGGACGCCTGGTTT CTTCCTGGAGGC
Toll-like receptor 1	TLR1/2	TLR1	79	82	ACTCACTTTGTAAACGTGGTTCTGTGTACA TCTCTTACCAGTCTGAGCGCCTCTGATGTG GCCCTCAACAAGTTGCCTTATATAGACAC TAATGTGACCA
Toll-like receptor 2	TLR1/2	TLR2	83	84	ACCCTGACTTTATGAATAAACTACTCAAG GTGATCTGCACGTCCAACCAGCAGCTTCA AGGGTCCACTGTGATGCCCTTCCAGCCTC CGTCTATATACAA
tumor necrosis factor-alpha	TLR1/2 and MCLR	TNF	82	85	TCAGGGAGGTTTCCAAGTAGTGAAGAACC AGATTGTTATCCCACAACTGGCCTCTAC TTCGTCTACAGCCAGGCATCGTTCAGAGT CTCCTGCAGCAAT
toll-interacting isoform 2	TLR1/2	TOLLIP	83	84	GTTGAAGTGGAGTTGATGAATTTGACTGG AGGCTACAAAGACCTAAGTGAGCAGTTGA GAGAGAGAGCCAAAATCCACACATCTCAT CTTGATCTCAGCA
tumor necrosis factor receptor type 1-associated DEATH domain	TLR1/2	TRADD	92	93	GCTGTCGGGCTCTGAAGGGTCCGGCTATA GACAACCTGGCCTACGAGTACGAGAGAG AAGGGCTCTATGAGCAAGCCTACCAGCTG CTCAGCCGCTTCAT
TNF Receptor Associated Factor 6	TLR1/2	TRAF6	83	85	CCGCGCTGCTCCAAGTATATCTCCCTCTTT GTTACACCATGCAAGGAGTTTTTGATGG GCAGCTGACATGGCCCTTCCAGGGCACCA TTCGCCTTGCCA

Table 4. Median, maximum, and minimum transcript abundance counts for specific genes of interest in the liver at each time point (6, 12, and 24 hr.) during the first hepatocyte challenge on Jan-16-2018. Data below the detection limit are reported as BD.

	6 Hour											
	Control			Low			Medium			High		
	Median	Max	Min	Median	Max	Min	Median	Max	Min	Median	Max	Min
CAT	315	481	258	389	545	277	402	528	296	386	449	340
CYP1A	220	343	106	275	473	143	266	442	163	281	429	129
CYP3A	622	941	540	766	1306	619	741	1266	664	723	1258	657
GSTrho	BD	8	BD	BD	BD	BD	BD	4	BD	BD	BD	BD
GLK	3	50	BD	BD	33	BD	BD	38	BD	BD	30	BD
GPX	4	25	BD	BD	6	BD	2	8	BD	BD	33	BD
GSR	2	33	BD	BD	4	BD	BD	10	BD	BD	BD	BD
GSTtheta	619	1040	366	726	1025	459	622	983	335	668	1178	415
HSP71	2329	2774	1155	2649	4168	2193	2840	4316	2363	2775	3897	2271
HSP90B	1226	1637	875	1437	2151	1239	1427	2023	1258	1427	1969	1163
IL1b	BD	48	BD	BD	BD	BD	BD	BD	BD	BD	23	BD
PCNA	BD	5	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD
SOD	50	87	BD	51	80	BD	54	88	BD	41	73	BD
TGFB	BD	23	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD

	12 Hour											
	Control			Low			Medium			High		
	Median	Max	Min	Median	Max	Min	Median	Max	Min	Median	Max	Min
CAT	333	438	272	332	393	295	353	489	240	352	460	231
CYP1A	206	398	99	212	387	147	218	450	102	233	395	155
CYP3A	953	1486	807	1069	1596	876	1077	1466	919	1162	1310	936
GSTrho	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	2	BD
GLK	37	133	BD	53	135	BD	54	118	BD	29	128	BD
GPX	BD	2	BD	BD	6	BD	BD	16	BD	BD	15	BD
GSR	1	29	BD	BD	31	BD	7	28	BD	BD	46	BD
GSTtheta	565	890	218	572	861	366	521	1139	361	487	864	270
HSP71	2863	4431	2255	2668	3908	2417	2521	4233	2033	2887	5708	2101
HSP90B	1510	2021	1202	1477	1904	1319	1422	2432	1272	1473	2492	1250
IL1b	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD
PCNA	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	12	BD
SOD	45	79	BD	55	91	BD	59	94	BD	36	96	BD
TGFB	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD

	24 Hour											
	Control			Low			Medium			High		
	Median	Max	Min	Median	Max	Min	Median	Max	Min	Median	Max	Min
CAT	315	400	204	312	354	237	306	332	235	272	322	205
CYP1A	105	157	1	118	147	37	105	203	50	93	129	38
CYP3A	1389	1951	1229	1370	1656	1241	1465	1610	1122	1373	1698	1273
GSTrho	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD
GLK	8	76	BD	7	93	BD	14	77	BD	BD	66	BD
GPX	BD	BD	BD	BD	BD	BD	BD	15	BD	BD	BD	BD
GSR	BD	29	BD	BD	32	BD	BD	36	BD	BD	35	BD
GSTtheta	408	572	86	394	574	204	410	624	167	417	541	219
HSP71	3066	6264	2356	3221	4236	2964	2951	4524	2519	2927	3850	2240
HSP90B	1619	2811	1364	1637	2062	1480	1542	2264	1425	1535	2178	1336
IL1b	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD
PCNA	BD	19	BD	BD	BD	BD	BD	13	BD	BD	8	BD
SOD	57	87	BD	48	82	10	56	116	BD	34	59	BD
TGFB	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD

Table 5. Median, maximum, and minimum transcript abundance counts for specific genes of interest in the liver at each time point (6, 12, and 24 hr.) during the first hepatocyte challenge on Feb-26-2018. Data below the detection limit of the assay are represented as BD.

	6 Hour														
	Control			Low			Med			High			Severe		
	Median	Max	Min	Median	Max	Min	Median	Max	Min	Median	Max	Min	Median	Max	Min
CAT	163	439	135	168	285	110	155	298	104	166	231	136	170	207	142
CYP1A	121	446	53	131	390	92	115	421	82	120	431	95	94	323	61
CYP3A	1324	1909	486	1204	1742	552	1197	1833	501	1127	1835	517	1231	1866	501
GSTrho	BD	BD	BD	BD	9	BD	BD	BD	BD	BD	5	BD	BD	BD	BD
GLK	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD
GPX	BD	9	BD	BD	18	BD	BD	3	BD	BD	9	BD	BD	BD	BD
GSR	BD	BD	BD	BD	9	BD	BD	BD	BD	BD	16	BD	BD	4	BD
GSTtheta	502	1012	460	463	533	367	358	458	304	435	629	348	407	623	335
HSP71	1691	4497	1437	1753	2023	1404	1706	2441	1627	1953	2330	1552	1867	2614	1388
HSP90B	1429	3441	1135	1258	1480	1102	1283	1500	1121	1333	1637	1095	1325	1480	1126
IL1b	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD
PCNA	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	7	BD	BD	4	BD
SOD	9	24	BD	10	33	BD	7	45	BD	13	52	BD	3	32	BD
TGFB	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD

	12 Hour														
	Control			Low			Med			High			Severe		
	Median	Max	Min	Median	Max	Min	Median	Max	Min	Median	Max	Min	Median	Max	Min
CAT	141	237	105	138	248	118	140	275	101	126	260	95	118	231	92
CYP1A	187	568	105	186	566	106	153	566	95	163	535	92	102	321	75
CYP3A	2115	2936	764	2122	2950	791	2163	2850	868	2065	3136	794	1744	2641	796
GSTrho	BD	BD	BD	BD	12	BD	BD	3	BD	BD	1	BD	BD	3	BD
GLK	3	144	BD	BD	91	BD	BD	87	BD	5	78	BD	BD	46	BD
GPX	BD	1	BD	BD	5	BD	BD	4	BD	BD	11	BD	BD	29	BD
GSR	11	39	BD	23	47	BD	13	27	BD	21	73	BD	54	95	12
GSTtheta	335	350	213	355	431	313	339	439	217	336	384	295	296	366	216
HSP71	2191	2629	1976	2069	2462	1877	2176	2534	1799	1999	2740	1723	1992	2368	1554
HSP90B	1500	1616	1296	1538	1691	1327	1521	1684	1282	1473	1686	1274	1535	1766	1333
IL1b	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD
PCNA	BD	BD	BD	BD	6	BD	BD	7	BD	BD	5	BD	BD	5	BD
SOD	9	33	BD	7	30	BD	BD	29	BD	7	20	BD	10	30	BD
TGFB	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	15	BD

	24 Hour														
	Control			Low			Med			High			Severe		
	Median	Max	Min	Median	Max	Min	Median	Max	Min	Median	Max	Min	Median	Max	Min
CAT	121	239	89	137	235	72	103	259	79	123	317	83	101	149	67
CYP1A	151	399	105	149	373	109	135	425	65	150	401	102	102	154	27
CYP3A	2085	3236	1236	2215	3359	1280	2137	3644	1279	2163	3673	1277	1500	2525	749
GSTrho	BD	2	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD
GLK	41	230	9	27	225	BD	25	197	BD	26	196	BD	BD	54	BD
GPX	BD	6	BD	BD	2	BD	BD	BD	BD	BD	8	BD	BD	BD	BD
GSR	35	74	9	36	72	4	23	49	BD	47	64	BD	77	119	BD
GSTtheta	202	265	124	220	264	144	208	264	163	192	215	131	189	209	135
HSP71	2465	2860	1814	2432	2705	1744	2315	3148	1765	2680	3563	1940	2490	3090	1863
HSP90B	1641	1998	1330	1719	1851	1327	1697	1883	1410	1745	2006	1344	1938	2324	1503
IL1b	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD
PCNA	BD	8	BD	BD	BD	BD	BD	BD	BD	BD	7	BD	BD	1	BD
SOD	24	60	BD	23	41	BD	10	40	BD	22	41	BD	7	26	BD
TGFB	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD

Table 6. Contig summary (by immunostimulant) for the generation of reference transcriptome based on leukocyte stimulation.

Sample	Insert Size	Total Reads	Bases
Control	394	226,613,464	34,218,633,064
Poly I:C	388	207,461,274	31,326,652,374
PHA-P	412	186,103,334	28,101,603,434
Zymosan	410	216,455,450	32,684,772,950

Table 7. Median, maximum, and minimum transcript abundance counts for specific genes of interest in the leukocyte challenge. Significance was determined among treatment and within each transcript. Significance codes represent differences among treatment groups within each treatment. Data below the detection limit of the assay are presented as BD.

Treatment									
		Control	PGN	MCLR-L	MCLR-M	MCLR-H	PGN-MCLR-L	PGN-MCLR-M	PGN-MCLR-H
CCL5	Median	BD	24	BD	BD	12	49	27	20
	Significance	a	ac	a	a	ac	c	ac	ac
	Max	19	46	33	36	44	61	62	47
	Min	BD	BD	BD	BD	BD	13	BD	BD
CCL-CUa	Median	991	3693	832	880	773	3927	4316	4515
	Significance	a	b	a	a	a	b	b	b
	Max	1406	4890	1991	1539	1463	5346	5024	6476
	Min	428	2435	310	279	338	2918	2693	3049
CD22	Median	40	BD	BD	31	44	8	BD	BD
	Significance	a	ab	ab	ab	a	b	b	a
	Max	131	63	112	49	70	14	26	56
	Min	12	BD	BD	BD	20	BD	BD	BD
CXCL8	Median	BD	130	2	14	28	175	141	174
	Significance	a	b	a	a	a	b	b	b
	Max	40	196	38	46	43	230	215	311
	Min	BD	80	BD	BD	10	77	83	69
IL-1 β	Median	77	594	213	243	224	779	747	978
	Significance	a	bc	ac	ac	ac	b	b	b
	Max	276	817	412	352	406	873	995	1149
	Min	BD	185	91	125	155	360	343	162

Treatment									
		Control	PGN	MCLR-L	MCLR-M	MCLR-H	PGN-MCLR-L	PGN-MCLR-M	PGN-MCLR-H
JUN	Median	6	29	BD	8	29	58	25	24
	Significance	a	b	ab	ab	ab	ab	ab	ab
	Max	27	40	69	35	57	68	50	50
	Min	BD	BD	BD	BD	4	BD	BD	BD
MAP3K8	Median	BD	36	14	28	43	80	47	36
	Significance	a	ab	ab	ab	b	ab	b	ab
	Max	30	62	50	55	63	104	85	83
	Min	BD	BD	BD	BD	14	1	16	BD
NCF1	Median	69	60	97	136	119	95	73	64
	Significance	a	a	ab	b	b	ab	ab	a
	Max	110	76	141	204	176	123	119	103
	Min	BD	35	44	94	106	29	26	4
PTGS2	Median	BD	40	BD	BD	15	94	73	63
	Significance	ac	bc	ac	a	c	b	b	b
	Max	11	79	72	42	46	157	145	161
	Min	BD	22	BD	BD	2	26	28	34
RAC1	Median	BD	13	BD	BD	17	32	19	BD
	Significance	a	ab	ab	ab	ab	b	ab	ab
	Max	15	33	31	32	26	57	33	27
	Min	BD	BD	BD	BD	BD	BD	BD	BD
SOCS3	Median	BD	53	BD	6	16	92	61	40
	Significance	a	cd	ac	ac	ac	bde	bc	ace
	Max	45	64	63	66	63	119	86	83
	Min	BD	9	BD	BD	BD	45	BD	BD
CHUK	Median	BD	BD	BD	BD	2	16	BD	BD
	Significance	b	ab	ab	ab	ab	a	ab	b
	Max	2	11	28	20	8	36	25	5
	Min	BD	BD	BD	BD	BD	BD	BD	BD

Treatment									
		Control	PGN	MCLR-L	MCLR-M	MCLR-H	PGN-MCLR-L	PGN-MCLR-M	PGN-MCLR-H
NFKB2	Median	BD	BD	BD	BD	BD	20	3	BD
	Significance	a	ab	ab	ab	ab	b	ab	ab
	Max	7	20	36	20	27	34	23	27
	Min	BD	BD	BD	BD	BD	BD	BD	BD
PIK3R5	Median	BD	BD	BD	BD	BD	12	1	BD
	Significance	a	ab	ab	ab	ab	b	ab	ab
	Max	BD	5	11	20	16	36	12	8
	Min	BD	BD	BD	BD	BD	BD	BD	BD

Chapter Four. Associations among estrogenic activity, select chemical compounds and microcystin toxins in the Potomac River drainage, USA

Abstract

A suite of compounds identified to interfere with hormone pathways are termed endocrine disrupting compounds (EDCs). Considerable attention has been directed towards estrogenic chemicals in the environment within the Chesapeake Bay watershed. We quantified the net cumulative effects of these compounds in mixtures using a bioluminescent yeast estrogenicity screen (BLYES). Samples were collected at 15 sites through the middle and upper Potomac River basin and quantified the estrogenic equivalency potential in the water column. Data are presented as the estrogenic equivalency of strain *Saccharomyces cerevisiae* BLYES as compared to a 17 β -estradiol standard curve (EEQ_{BLYES}) and ranged from BD – 5.75 ng/L. We observed considerable seasonal variability reporting data higher in the summer months. Estrogenicity was positively correlated with temperature; however, there were no significant correlations among discharge, pH, conductivity, total nitrogen or total phosphorous in the water column or benthos. The EEQ_{BLYES} was positively correlated with equol. Additionally, EEQ_{BLYES} was positively correlated with total microcystins/nodularins and we verified that purified microcystin variant –LR was not estrogenic by this assay. We assessed EEQ_{BLYES} in the context of land cover/land use and did not observe any significant correlations in any of our sampling watersheds. These data highlight temporal changes in estrogenicity at these sites. They also show a contribution from phytoestrogens to the instream estrogenicity. Both of these should be taken into account when quantifying environmental estrogenicity.

1. Introduction

Endocrine disrupting compounds (EDCs) have been identified in surface waters throughout the United States (Kolpin et al. 2002, Snow et al. 2010). A subset of these compounds termed estrogenic endocrine disrupting compounds (EEDCs) has been shown to modulate estrogen receptor-mediated pathways in aquatic organisms (Campbell et al. 2006). Examples of EEDC include both steroidal and non-steroidal compounds. Many of these compounds have been correlated to adverse biological effects in fishes (Segner et al. 2013, Senthilkumaran 2015). The specific sources and fate of these EEDCs in surface waters is important in understanding their environmental impact and potential management (Adeel et al. 2017). Biological assays (specifically yeast-based assays) are incorporated to monitor EEDCs (Murk et al. 2002, Petrovic et al. 2004, Vigano et al. 2008, Di Paolo et al. 2016).

Extensive research has been conducted in the Chesapeake Bay watershed focusing on the associations of EEDCs with biological effects in aquatic organisms. Blazer et al. (2007) report widespread testicular oocyte (TO) occurrence and severity in smallmouth bass (SMB; *Micropterus dolomieu*) within the Potomac River watershed. They note that this system is dominated by agricultural input. Iwanowicz et al. (2009) further studied TO and noted a high occurrence of TO regardless of proximity to wastewater treatment facilities (WWTP). This would suggest that other inputs may be driving endocrine disruption. Recent research has highlighted the association of agricultural operations (such as confined animal feedlot operations) with estrogenicity (Blazer et al. 2012, Ciparis et al. 2012, Young et al. 2014). A variety of compounds are associated with agricultural land use (Zhang et al. 2015). One extensively studied pesticide that has been shown to induce TO is atrazine (Hayes et al. 2011). Kolpin et al. (2013) documented the occurrence of atrazine in the Potomac River watershed and

identified a significant positive correlation with TO in male smallmouth bass. Other EEDCs were identified as well. Some of this sampling occurred proximate to SMB nesting sites. The presence and concentration of these bioactive compounds are an important component for associating the biological effects potential in the environment.

Agricultural inputs are not limited to high density animal excretions and land applied pesticides. Phytoestrogens are also present in agricultural settings. These are a group of non-steroidal compounds produced by plants and other photosynthetic organisms. Chemically they are structurally similar to estrogens and some are known to interact with estrogen receptors and modulate ER-mediated signaling pathways (Verdeal and Ryan 1979, Morito et al. 2001, Cappelletti et al. 2006, Lecomte et al. 2017). Legumes and agricultural crops have been principally identified as producing these compounds (Franke et al. 1994, Jarosova et al. 2015, Kang and Price 2009, Kolpin et al. 2010, Kuhnle et al. 2009, Liu et al. 2010). However, the presence of phytoestrogens is not strictly limited to agricultural operations. For example, equol is a bacterial metabolite of the soy isoflavones daidzin and daidzein (Setchell and Clerici 2010). It is released in the waste of animals containing these bacteria and can occur in humans, livestock, and in the wild. Thus, occurrence in the environment may be linked to point- and non-point source contamination. The specific timing and magnitude of release of these compounds into surface waters is poorly understood. For example, Rearick et al. (2014) were unable to report a discernible temporal or spatial trend in select phytoestrogens; however, they did note that phytoestrogens could be detected in anthropologically impacted streams and absent from reference streams.

Other possible, but lesser investigated sources of EEDCs are the instream algal communities. The frequency of algal blooms in aquatic environments has increased over time as

a result of elevated temperatures and available eutrophication (O'Neil et al. 2012); and harmful algal blooms are a significant matter of public health (Carmichael and Boyer 2016, Grattan et al. 2016). One critical component to the public health aspect of harmful algal blooms is the production of toxins by select species (particularly cyanobacteria). Microcystin (MC) is a secondary metabolite of select cyanobacteria species and best recognized as a potent hepatotoxin (Sierosławska 2010). Oziol and Bouaicha (2010) showed the microcystin (MC) variant -LR (MC-LR) to cause an increase in estrogenic activity in culture via classical estrogen receptor mediated signaling. Interestingly, lysates from both cyanobacteria and true algae have been shown to be estrogenic *in vitro* (Štěpánková et al. 2011, Sychrová et al. 2012). The contribution of algae should be considered when assessing the total instream estrogenicity.

The objective of this research was to monitor the temporal and spatial distribution of estrogenic compounds in the middle and upper Potomac River watershed, and identify possible associations with land cover/land use. This estrogenic signal was compared to the occurrence of common phytoestrogens and steroidal estrogens, as well as total microcystin/nodularin toxins. A secondary objective was to compare the estrogenic signal to both physical and nutrient parameters.

2. Methods

2.1. Field collection

Fifteen sites along streams located within the Potomac River watershed in West Virginia, Maryland, and Virginia were selected for surface water sampling based on the historic presence of EEDCs or sites of interest by fisheries biologist staff from the West Virginia Division of Natural Resources. Here we define these as the reconnaissance sites. The 15 reconnaissance sites

were sampled monthly from June 26, 2013 through June 18, 2014 (Figure 1). Two grab water samples were collected from a near-shore location using one chemically clean 1 L amber glass bottle (I-Chem, Rockwood, Tennessee). These will be referred to as filtered water samples. Water temperature (°C), pH, conductivity ($\mu\text{S}/\text{cm}$), total dissolved oxygen (mg/L and percent), and discharge (ft^3/s) were recorded at each site. Additional water samples were collected in chemically clean 1 L amber glass bottles for analytical chemistry. This was performed at 2 sites on the South Branch of the Potomac River monthly between July 9, 2013 and August 11, 2014 (Figure 1). These water samples were filtered using a 0.7 μm AP40 GF/F filter (Millipore Corporation, Billerica, Massachusetts) to remove coarse and fine particulates and stored at -20°C . Frozen samples were then shipped to the USGS Kansas Water Science Center (Lawrence, Kansas) for analysis of steroidal estrogens (17- β estradiol (E2), and estrone (E1)), as well as the isoflavones genestein, daidzein, formonentin, equol, and biochain A.

A subset of three reconnaissance sites was selected to increase the temporal resolution and sampled biweekly for 16 months (Figure 1). Locations sampled during this phase of the study are defined as biweekly sites. Biweekly sampling occurred between June 30, 2014 and December 14, 2015. We collected an additional discrete water sample in one clean sterile 500 mL HDPE bottle (Thermo Fisher Scientific, Waltham, Massachusetts) in addition to the physical parameters described above, beginning with the first sampling date in 2015 (January 13th) through the final collection date (December 14th). These samples were defined as unfiltered. Filtered samples contain acellular EEDCs present in the water column while unfiltered samples contain cellular and acellular analytes.

We also measured total nitrogen (N; mg/L), total phosphorous (P; mg/L), and total dissolved phosphorous (TDP; mg/L) in the water column from the biweekly collections. In

addition, sediment N (mg/kg) and P (mg/kg) was also measured. Water was collected in sterile 500 mL HDPE bottles (Thermo Fisher Scientific, Waltham, Massachusetts) while sediment was collected in 250 mL pre-cleaned to EPA standards glass amber bottles (Quality Environmental Containers, Beaver, West Virginia). Both samples were stored at 4°C for no more than 14 days prior shipment to Research Environmental Industrial Consultants Inc. (Beaver, West Virginia) for analysis.

2.2. Laboratory preparation

Filtered water samples were adjusted to pH 3.0 with 6N hydrochloric acid and stored at 4°C. All samples were extracted within two weeks of collection (Havens et al. 2010). Unfiltered water samples were subjected to three freeze-thaw cycles to room temperature to disrupt the cells (Graham et al. 2010, Loftin et al. 2008).

Filtered and unfiltered water samples were extracted using methods modified from Ciparis et al. (2012). Briefly, all water samples were filtered with a 0.7 µm AP40 GF/F filter (Millipore Corporation, Billerica, Massachusetts) prior to extraction to remove coarse particulates (unfiltered water samples were adjusted to pH 3.0 with 6N hydrochloric acid prior to filtration). We then prepared water samples by solid phase extraction (SPE) using OASIS® HLB (200mg) glass cartridges (Waters Corporation, Milford, Massachusetts). Cartridges were conditioned using 5 mL each of ethyl acetate, 50:50 methanol:dichloromethane, methanol, and 18MΩ-H₂O pH 3.0. Then 800 mL filtered or 400mL unfiltered water samples were run through the cartridge by continuous vacuum using methods modified from Laganà et al. (2004). Analytes were eluted with 6 mL absolute methanol and 6 mL 50:50 methanol (absolute):dichloromethane and reduced to dryness. Eluates were solubilized in methanol to a final sample concentration of 1600x. Samples were stored at -20°C until analysis.

2.3. Bioluminescent Yeast Estrogenicity Screen (BLYES)

The *Saccharomyces cerevisiae* Bioluminescent Yeast Estrogenicity Screen (BLYES) was used to quantify total estrogenicity of environmental samples using methods previously described by Ciparis et al. (2012) with modification. Strain BLYES was purchased from 490 Bio Tech (Knoxville, TN). Yeast cultures were grown to early stationary phase in yeast minimal drop-out media (YMM; leu-, ura-) at 30°C for 48 hours on a rotary shaker (Routledge and Sumpter 1996). The 48 hour culture was centrifuged at 2000 x g for 10 minutes, re-suspended in fresh YMM, and normalized to an OD₆₀₀ of 0.40. The assay was performed in sterile, clear bottom 96-well black plates (Costar, Corning Incorporated, Corning, New York). Sample extracts were diluted to 5% in YMM and assayed in triplicate. All plates included a 12-point standard curve of E2 that ranged from 10 ng/mL to 3.4 pg/mL and included a YMM-only blank. The OD₆₀₀ normalized yeast reporter was added to all wells (100 µL/well). The final concentration of methanol in all assay and standard wells was 2.5%. Plates were incubated for 4 hours at 30°C on an orbital shaker. Luminescence was quantified using a SpectraMax M4 (Molecular Devices, Sunnyvale, California) with the integration time at 500 milliseconds and automatic photo multiplier tube gain. Sample concentrations were interpolated from the standard curve in SoftMaxPro v6.2.2 (Molecular Devices, Sunnyvale, California) using a 4-parameter logistic regression. Sample concentrations were then back calculated based on sample dilution.

The detection limit (LoD) and reporting limit (LoQ) were determined based on 20 randomly selected standard curves generated over a 2-year period. The LoD was defined as the minimum detection limit plus one standard deviation. When corrected for sample concentration, the LoD was 0.145 ng/L. The LoQ based on E2 was defined as the minimum detection limit plus

three standard deviations and corrected for starting water volume was 0.230 ng/L. Data were reported as EEQ_{BLYES} . Data below the LoQ are reported as $0.5 * LoD$.

We explored the possible relationship between estrogenicity and total MCs using purified microcystin variant-LR (MC-LR; EMD Millipore, Burlington, Massachusetts) at environmentally relevant concentrations ranging from 5 fg/mL to 500 ng/mL.

2.4. Direct competitive enzyme-linked immunosorbent assay (ELISA) protocol

We developed an ELISA specific for the ADDA side chain present in microcystins and nodularins synthesized by some cyanobacteria. This method was adapted from Campas and Marty (2007). Wells were coated with 100 μ L of ADDA-specific monoclonal antibody (0.1 μ g/mL; AD4G2; Enzo Life Sciences, Farmingdale, New York) and incubated in a 96-well ELISA plate (Costar® 3396, Corning Inc., Corning, New York) for 18 hours at 4°C. Plates were washed five times with tris-buffered saline plus 0.5% v/v Tween-20 (TBST; pH 7.4), and blocked with TBST plus 1% bovine serum albumin (block buffer pH 7.4) for 2 hours at 37°C. Each plate was washed 5 times with TBST and 50 μ L of standards or sample was added at a 5% dilution in block buffer. We then added 50 μ L of biotin-labelled MC-LR to each well at a concentration of 4 μ g/mL in block buffer and incubated for 1.5 hours at 37°C. Plates were washed 5 times with TBST and 100 μ L of streptavidin-horseradish peroxidase conjugate (1:5000 in block buffer; ThermoFisher Scientific, Waltham, Massachusetts) was added and incubated for 30 minutes at 37°C. Plates were washed 5 times with TBST and 100 μ L of SureBlue Reserve™ TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland) was added and incubated at 25°C for 1.5 hours. Finally, 50 μ L of TMB stop solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland) was added and plates were read

at 450 nm with a SpectraMax M4 plate reader (Molecular Devices LLC, Sunnyvale, California). Data are reported as total MC/NOD due to non-specificity of the mAb.

2.5. Spatial analysis

Land cover/land use was determined using the U.S. Department of Agriculture Cropland Data Layers from 2013, 2014, and 2015 (USDA 2013, 2014, 2015). We identified the crop cover for each year among the full watershed upstream of our sampling site. We also quantified the crop cover for all three years within a 30 meter buffer around each stream located within the watershed upstream of our sampling site. Land cover types were then grouped into six categories (Table 1). Streams and watersheds were determined using both the National Hydrography Dataset (<https://nhd.usgs.gov>, accessed 2016), and the National Elevation Dataset (<https://lta.cr.usgs.gov/NED>; accessed 2016). Wastewater treatment facilities were derived from the U.S. EPA Facility Registry Service (FRS): Wastewater Treatment Plants (<https://catalog.data.gov/dataset/epa-facility-registry-service-frs-wastewater-treatment-plants>).

2.6. Statistical analysis

All data were analyzed using the R statistical software v3.4.3 (R Core Development Team 2017). Significance was determined at $\alpha=0.05$. All variables were assessed for normality using the Shapiro-Wilk test of normality. If variables were not normally distributed, they were transformed with either a $\log_{10}$ or square root transformation. We then determined the correlation between filtered and unfiltered water samples using the Pearson correlation. We analyzed the relationship between filtered and unfiltered water within season/year using a Student's t-test accounting for homogeneity.

We quantified the association among EEQ_{BLYES} in both water fractions among sampling locations using a one-way ANOVA test. We did not observe significant differences in EEQ_{BLYES} among our sampling sites in either the reconnaissance or intensive sampling period in filtered water ($p = 0.604$ and 0.160 , respectively) or unfiltered water ($p = 0.077$).

Because no significant differences were observed among sampling sites, the correlation between estrogenicity (in both water fractions) was assessed among sampling season/year using a one-way ANOVA test. Tukey's honest significant difference tests were used to differentiate significant results. We defined season by the following: spring sampling occurred during March, April, and May; summer sampling occurred during June, July, and August; fall sampling occurred during September, October, and November; winter sampling occurred during December, the following January, and February (the year associated with winter corresponds to January and February). Next we quantified the explanatory variables responsible for each water fraction among each respective sampling time period. This was analyzed using multiple linear regression models given that no significant differences were observed across sites. First, we evaluated estrogenicity in filtered water as a function of stream discharge (m^3/s), temperature ($^{\circ}\text{C}$), conductivity ($\mu\text{S}/\text{cm}$), and pH during the initial reconnaissance time period. To analyze the subsequent 16 months, we developed two separate models. The first model explored the effects of four physical variables described above on total estrogenicity in both water fractions (independently). The second model incorporated the top candidate model(s) based on significance along with the N and P variables. We did not incorporate TDP into the models due to the high frequency of data below the reporting limit (104 of 117 total samples).

The two locations in which estrogenic compounds E2, E1, genestein, daidzein, formonentin, equol, and biochain A were quantified were analyzed independently. The

association between EEQ_{BLYES} (in filtered water) and these compounds was quantified using a simple linear regression.

Land cover/land use data are reported annually. Therefore we pooled EEQ_{BLYES} and the percent agricultural, developed, and forested (both for the entire basin and within 30m of a stream) among each calendar year. We then qualitatively summarized both the specific and reclassified land cover classifications among each watershed. We quantified the association between EEQ_{BLYES} and each land cover grouping using Spearman's rank correlation coefficient. The associations between total MC/NOD and EEQ_{BLYES} of field samples were compared using simple linear regression at each of the sites grouped among season/year.

3. Results

3.1. Seasonal/annual variability

We analyzed 291 filtered water samples and 75 unfiltered water samples. First, we analyzed the correlation among filtered and unfiltered grab water samples. We observed a significant weakly positive correlation among filtered and unfiltered grab water samples ($p < 0.001$; $r^2 = 0.29$; range: BD – 2.036 ng/L). There was no difference among filtered or unfiltered water within season/year ($p > 0.05$). We then quantified the estrogenicity of both water samples above LoD both among site and among season/year (Figure 2). While there were not statistically different among sites within season/year, we did observe considerable variability in filtered EEQ_{BLYES} among individual sites; specifically in the summer and autumn 2013. This was driven by the elevated values measured at the three sites: South Branch downstream from Moorefield, West Virginia, South Branch downstream from Franklin, West Virginia, and Mill Creek (Hampshire County, West Virginia) (Table 2). We observed an overall trend for higher values in

the summer and fall compared to the winter and spring. We also note a slight reduction in estrogenicity from 2013 through to 2015 although this was not statistically significant.

3.2. Variability among season/year pooled across sites

We did not observe a significant difference among sampling season/year during the reconnaissance sampling period ($p = 0.296$); however, we did observe a difference among sites in the biweekly sampling period ($p = 0.002$; Table 2). The summer 2014 was significantly higher than the winter and spring 2015. There were no other significant differences. We observed significant difference among sampling season/year in the unfiltered water fraction ($p = 0.010$) during the biweekly period. Spring and summer 2015 were higher than winter 2015 (Table 3).

3.3. Explanatory variable analysis

Next, we quantified our estrogenic response variable in the filtered water as a function of the four predictor variables (discharge, temperature, pH, and conductivity) during the initial reconnaissance time period. Among these variables, only water temperature was significantly positively correlated to EEQ_{BLYES} ($p = 0.002$). Next, we performed the same analysis during the subsequent intensive sampling period. Again, only temperature was significantly correlated to our response variable ($p = 0.004$). Finally, we quantified the association among the sediment N and P, as well as the top variable from above (water temperature) to EEQ_{BLYES} . Water temperature was the only variable significantly correlated in this model ($p < 0.001$) and it was always a positive association.

We then quantified estrogenicity in unfiltered water as a function of the explanatory variables as described for filtered water. Again, water temperature was the only significantly

correlated physical variable ($p = 0.002$) and the only significant variable when included with the two nutrient parameters (0.017).

3.4. Chemical results summary

We analyzed estrogenic compounds among two sites (Figure 1). We did not statistically analyze E2 or biochain A due to the low frequency of data above the LoD. Equol was significantly positively correlated with estrogenicity in the water column at the South Branch Potomac River downstream from Petersburg, West Virginia site ($p = 0.018$; $r^2 = 0.42$; Figure 3). There was no statistical association with any other compound to either of these sites. However, upon examining the data, we observed an apparent additive association between E1 and Equol to estrogenicity. Therefore we compared total E1 and equol to estrogenicity and again, only the South Branch Potomac River downstream from Petersburg, West Virginia site was positively correlated to these two estrogenic compounds ($p = 0.019$; $r^2 = 0.42$). Interestingly, we see a stronger association when we extend our analysis to include all five estrogenic compounds ($p = 0.007$; $r^2 = 0.53$). Water temperature and discharge were inconsistently associated with the five estrogenic compounds we analyzed. We did not observe any significant associations with any compounds at the South Branch Potomac River downstream Moorefield, West Virginia. We observed a weakly inverse correlation among E1 and discharge at the South Branch Potomac River downstream Moorefield, West Virginia site ($p = 0.034$, $r^2 = 0.29$). There were no other associations between discharge and any other estrogenic variables. We did observe a positive association between temperature and E1 ($p = 0.043$, $r^2 = 0.31$), equol ($p = 0.034$, $r^2 = 0.34$), and the sum of E1/equol ($p = 0.027$, $r^2 = 0.38$) at the South Branch Potomac River downstream Petersburg, West Virginia site. Temperature was only significantly associated with estrone at the South Branch Potomac River downstream Moorefield, West Virginia site ($p = 0.002$; $r^2 = 0.55$).

3.5. Land cover analysis

The total basin area among each site ranged between 120.79 km² and 16229.73 km² (Tables 4-6). We omitted the “NA”, “Open Water” and “Wetlands” classification groupings due to minimal representation on the landscape. Overall, the dominant specific land cover type in each watershed was deciduous forest which comprised between 32% – 87% of the overall watershed. Among agricultural land cover types, grass/pasture occurred on the highest percentage of the landscape followed by other hay/non alfalfa, corn, and soybeans. There was no statistical correlation among estrogenicity in the water column and any individual land cover type at either spatial scale ($p > 0.05$).

3.6. Comparison of MC-LR and estrogenicity

MC-LR did not activate the BLYES reporter. However, when we compared environmental samples, we observed a significant positive correlation among estrogenicity and MC/NOD in filtered water at two sites in the water column (Table 2). There was no association between EEQ_{BLYES} and MC/NOD in unfiltered water samples. When independently pooled among all sites, both endpoints were greatest during the summer/fall and approached minimum concentrations during the winter/spring (Figures 2 and 4).

4. Discussion

Overall, the estrogenicity values we observed were within the ranges recently reported around the Potomac River watershed (Ciparis et al. 2012, Young et al. 2014). It is unclear what accounts for the seasonal pattern we observed. It is unlikely the result of a dilution effect reflecting the higher flows in the winter and spring given that there was no correlation (positive or inverse) between estrogenicity and discharge. Despite the uncertainty surrounding the specific

cause(s), this seasonality highlights the dynamic nature of environmental samples. Careful consideration should be given to this variability when designing future studies.

We did not observe any association with estrogenicity and land cover/land use. Although this may seem to contradict earlier studies showing an association with agricultural sources, this is likely simply a result of our site selection. Studies such as Young et al. (2014) and Ciparis et al. (2012) asked specific questions regarding land cover/land use and included a gradient across various land use types. Our site selection included ten minimally impacted agricultural sites (South and North Branch Potomac sites), three moderately impacted agricultural sites (all within the Shenandoah River watershed), and one highly impacted agricultural site (Monocacy River). However, we did not include any specific gradient of land use because the specific question we were addressing was the temporal variability across sampling locations. We did not observe statistically significant differences across any of our sites which would suggest that the seasonal pattern we report is consistent regardless of land use among these 15 sites. Further research is needed to specifically examine the temporal distribution of estrogenicity between a gradient of agricultural, urban, and forested land cover/land use types.

We report positive correlations between estrogenicity and the phytoestrogen equol, the sum of equol and estrone, and the sum of all estrogenic compounds only at the South Branch Petersburg site. This would suggest that phytoestrogens and steroidal estrogens are significantly contributing to the observed total estrogenicity at this site. However, these associations were not reported at the South Branch downstream Moorefield site which is located approximately 20 kilometers downstream. This is likely driven by the reporting of 5.75 ng/L on 11-September-2013 which is well above the predicted no-effects concentration of 0.73 ng E2/L as suggested by Wu et al. (2014). It is unclear what is responsible for this event. Once this point is removed, a

similar positive trend in equol and estrone is observed. This likely results from the similarities between the two sites (noting the inclusion of the South Fork of the South Branch Potomac, as well as two WWTPs at the Moorefield site). These additions constitute a nearly 50% increase in total basin area. We report estrone above the detection limit (0.25 ng/L) at a higher frequency at the Moorfield site (62%) as compared to the Petersburg site (36%). Estrone has been associated with both WWTP effluent (Tan et al. 2007) and animal waste (Raman et al. 2004). Estrone reports a ten-fold weaker response in strain *S. cerevisiae* BLYES as compared to 17 β -estradiol (Sanseverino et al. 2009). Therefore, it is unclear what net effects estrone may be having on our reported estrogenicity when combined with equol and the three other estrogenic compounds. Our analysis only included select steroidal hormones and phytoestrogens which were able to show associations with estrogenicity. However, given the discrete event described above which was not associated with the chemicals we quantified, further research should include a more comprehensive list of estrogenic compounds.

We observed a similar seasonal trend between estrogenicity and total MC/NOD; however, purified MC-LR did not report any estrogenic effect with the BLYES assay. However both report the same seasonal signal and both are positively correlated with stream temperature. Cyanobacteria are found in many aquatic habitats worldwide (Chorus and Bartram 1999). Focus of these species is largely on the toxins they produce (Codd et al. 2005), as well as the reduced dissolved oxygen associated with bloom failure. An emerging caveat of cyanobacterial presence is their possible contribution to the estrogenic potential of a stream. The estrogenic potential of cyanobacteria should be considered when explaining the total EEQ of a stream. For example, Procházková et al. (2017) quantified the phytoestrogens present in water bodies which were dominated by cyanobacteria (which included the five flavonoids we quantified). Although they

identified phytoestrogens in all sites, these were not sufficient to explain the total EEQ they observed. They went on to describe the total phytoestrogen contribution (>10%) and suggested that additional sources were likely from anthropogenic pathways. Although these were observed in lentic systems, it does elucidate the potential for the environmental estrogenic contribution from phytoestrogens.

Both cyanobacteria and green algae have been associated with estrogen receptor signaling (Štěpánková et al. 2011, Sychrová et al. 2012). They have also been shown to exhibit possible synergistic and/or antagonistic effects on ER when challenged in mixtures. Given the temporal variability among algal communities as reviewed by Fogg (1975), this may explain the seasonal trends we observe. The predominant estrogenic signal we observed occurred between late spring and early fall which may indicate optimal growing temperature. Further research into the specific estrogenic potential among the algal species present within our samples is needed to accurately quantify their contribution.

Finally, any discussion involving cyanobacterial growth must include mention of nutrients. Considerable attention has been directed at documenting and curbing nitrogen (N) and phosphorous (P) in the environment. We did not observe any correlation among EEQ_{BLYES} and total N or P in the water column. This may result from the variable N fixation abilities of specific taxa present (Fay 1992). We did not observe any correlation among nutrients and MC concentrations which could mean that there was a mixed population of MC-producing cyanobacteria which drowned out the nutrient signal. A second possibility is that the concentrations of nutrients were consistently at or above the biological threshold for those cyanobacteria. Further research is needed to temporally quantify algal richness and abundance to test these hypotheses.

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Figures

Figure 1. Location of sampling sites throughout the Potomac River watershed.

Figure 2. Temporal variability among total estrogenicity among filtered water during A) the 12 sites specific to the reconnaissance time period and both filtered and unfiltered water fractions at the three sites included in B) the biweekly sampling period. Data are pooled among all sampling locations. Error bars represent standard error.

Figure 3. Temporal variability in EEQ_{BLYES} and select estrogenic compounds at A) the South Branch Potomac below Petersburg, WV and B) the South Branch Potomac below Moorefield, WV. The detection limits of the analysis are as follows: The LoQ for EEQ_{BLYES} was 0.230 ng/L and 0.250 for estrone, genestein, daidzein, formonentin, and equol.

Figure 4. Temporal variability among total microcystins/nodularins among filtered and unfiltered water fractions. Data are pooled among all sampling locations. Error bars represent standard error.

Tables

Table 1. Reclassification scheme for land cover. Original classification derived from the U.S.

Department of Agriculture Cropland Data Layer. Unclassified groups presented as NA.

USDA Classification Name	Reclassification Group
Alfalfa	Agricultural
Almonds	Agricultural
Apples	Agricultural
Apricots	Agricultural
Aquaculture	Agricultural
Asparagus	Agricultural
Barley	Agricultural
Blueberries	Agricultural
Broccoli	Agricultural
Buckwheat	Agricultural
Cabbage	Agricultural
Camelina	Agricultural
Caneberries	Agricultural
Canola	Agricultural
Cantaloupes	Agricultural
Carrots	Agricultural
Cauliflower	Agricultural
Celery	Agricultural
Cherries	Agricultural
Chick Peas	Agricultural
Christmas Trees	Agricultural
Citrus	Agricultural
Clover/Wildflowers	Agricultural
Corn	Agricultural
Cotton	Agricultural
Cranberries	Agricultural
Cucumbers	Agricultural
Dbl Crop Barley/Corn	Agricultural

USDA Classification Name	Reclassification Group
Dbl Crop Barley/Sorghum	Agricultural
Dbl Crop Barley/Soybeans	Agricultural
Dbl Crop Corn/Soybeans	Agricultural
Dbl Crop Durum Wht/Sorghum	Agricultural
Dbl Crop Lettuce/Barley	Agricultural
Dbl Crop Lettuce/Cantaloupe	Agricultural
Dbl Crop Lettuce/Cotton	Agricultural
Dbl Crop Lettuce/Durum Wht	Agricultural
Dbl Crop Oats/Corn	Agricultural
Dbl Crop Soybeans/Cotton	Agricultural
Dbl Crop Soybeans/Oats	Agricultural
Dbl Crop WinWht/Corn	Agricultural
Dbl Crop WinWht/Cotton	Agricultural
Dbl Crop WinWht/Sorghum	Agricultural
Dbl Crop WinWht/Soybeans	Agricultural
Dry Beans	Agricultural
Durum Wheat	Agricultural
Eggplants	Agricultural
Fallow/Idle Cropland	Agricultural
Flaxseed	Agricultural
Garlic	Agricultural
Gourds	Agricultural
Grapes	Agricultural
Grass/Pasture	Agricultural
Greens	Agricultural
Herbs	Agricultural
Honeydew Melons	Agricultural
Hops	Agricultural
Lentils	Agricultural
Lettuce	Agricultural
Millet	Agricultural
Mint	Agricultural
Misc Veggies & Fruits	Agricultural
Mustard	Agricultural

USDA Classification Name	Reclassification Group
Nectarines	Agricultural
Oats	Agricultural
Olives	Agricultural
Onions	Agricultural
Oranges	Agricultural
Other Crops	Agricultural
Other Hay/Non Alfalfa	Agricultural
Other Small Grains	Agricultural
Other Tree Crops	Agricultural
Peaches	Agricultural
Peanuts	Agricultural
Pears	Agricultural
Peas	Agricultural
Pecans	Agricultural
Peppers	Agricultural
Pistachios	Agricultural
Plums	Agricultural
Pomegranates	Agricultural
Pop or Orn Corn	Agricultural
Potatoes	Agricultural
Prunes	Agricultural
Pumpkins	Agricultural
Radishes	Agricultural
Rape Seed	Agricultural
Rice	Agricultural
Rye	Agricultural
Safflower	Agricultural
Sod/Grass Seed	Agricultural
Sorghum	Agricultural
Soybeans	Agricultural
Speltz	Agricultural
Spring Wheat	Agricultural
Squash	Agricultural
Strawberries	Agricultural

USDA Classification Name	Reclassification Group
Sugarbeets	Agricultural
Sugarcane	Agricultural
Sunflower	Agricultural
Sweet Corn	Agricultural
Sweet Potatoes	Agricultural
Switchgrass	Agricultural
Tobacco	Agricultural
Tomatoes	Agricultural
Triticale	Agricultural
Turnips	Agricultural
Vetch	Agricultural
Walnuts	Agricultural
Water	Agricultural
Watermelons	Agricultural
Winter Wheat	Agricultural
Barren	Developed
Developed	Developed
Developed/High Intensity	Developed
Developed/Low Intensity	Developed
Developed/Med Intensity	Developed
Developed/Open Space	Developed
Deciduous Forest	Forested
Evergreen Forest	Forested
Forest	Forested
Mixed Forest	Forested
Shrubland	Forested
Background	NA
Clouds/No Data	NA
NA	NA
Nonag/Undefined	NA
Perennial Ice/Snow	NA
Open Water	Open Water
Herbaceous Wetlands	Wetlands
Wetlands	Wetlands

USDA Classification Name	Reclassification Group
Woody Wetlands	Wetlands

Table 2. Summary of EEQ_{BLYES} and total microcystins/nodularins in filtered water samples during the reconnaissance and biweekly sampling sites. r^2 is only represented in sites with significantly correlated association between MC/NOD and EEQ_{BLYES}. SE represents standard error.

Stream Name	Sampling Season/Year	Filtered EEQ_{BLYES} (ng/L)	Filtered EEQ_{BLYES} SE	Filtered Total Microcystins (ng/mL)	Filtered Total Microcystins SE	Association among Total Filtered MC/NOD and EEQ_{BLYES} (p-value, r^2)
Monocacy River near Mouth near Frederick, Maryland	2013 Summer	0.174	0.124	5.39	2.26	0.904
	2013 Autumn	0.359	0.024	6.19	1.19	
	2014 Winter	0.420	0.187	4.47	0.86	
	2014 Spring	0.479	0.080	3.68	0.94	
	2014 Summer	0.436	NA	2.90	NA	
Mainstem of the North Branch at Cumberland, Maryland	2013 Summer	0.352	0.054	9.18	2.86	>0.001*, 0.232
	2013 Autumn	0.309	0.259	8.97	1.75	
	2014 Winter	0.296	0.133	5.04	1.17	
	2014 Spring	0.318	0.180	3.72	0.92	
	2014 Summer	0.518	0.069	6.43	1.95	
	2014 Autumn	0.224	0.110	6.25	1.59	
	2015 Winter	0.218	0.090	3.76	0.25	
	2015 Spring	0.162	0.071	2.93	0.47	
	2015 Summer	0.501	0.147	6.22	1.36	
	2015 Autumn	0.382	0.072	3.15	1.33	
	2016 Winter	0.274	NA	2.05	NA	
Mainstem of the Potomac River near Sharpsburg, Maryland	2013 Summer	0.289	0.239	3.15	0.02	0.075
	2013 Autumn	0.224	0.174	7.89	2.89	
	2014 Winter	0.440	0.040	5.09	0.24	
	2014 Spring	0.577	0.063	4.32	0.85	
	2014 Summer	0.050	NA	4.73	NA	

Stream Name	Sampling Season/Year	Filtered EEQ_{BLYES} (ng/L)	Filtered EEQ_{BLYES} SE	Filtered Total Microcystins (ng/mL)	Filtered Total Microcystins SE	Association among Total Filtered MC/NOD and EEQ_{BLYES} (p-value, r²)
North Fork of the South Branch at Seneca Rocks, West Virginia	2013 Summer	0.296	0.246	18.60	2.09	0.620
	2013 Autumn	0.437	0.128	9.17	3.53	
	2014 Winter	0.397	0.209	5.41	0.68	
	2014 Spring	0.417	0.041	4.47	0.71	
	2014 Summer	0.448	NA	11.21	NA	
South Fork of the South Branch near Moorefield, West Virginia	2013 Summer	0.331	0.059	7.15	4.02	0.419
	2013 Autumn	0.651	0.601	7.17	2.17	
	2014 Winter	0.312	0.146	4.96	1.25	
	2014 Spring	0.251	0.108	2.53	0.22	
	2014 Summer	0.454	NA	5.69	NA	
Upper Mainstem of the South Branch	2013 Summer	1.003	0.953	8.74	2.60	0.647
	2013 Autumn	0.180	0.130	4.70	0.31	
	2014 Winter	0.163	0.113	5.68	0.59	
	2014 Spring	0.288	0.146	4.28	0.82	
	2014 Summer	0.399	NA	3.93	NA	
Mainstem of the South Branch at Petersburg, West Virginia	2013 Summer	0.474	0.096	8.42	5.38	0.074
	2013 Autumn	0.170	0.120	4.87	0.14	
	2014 Winter	0.342	0.052	5.68	0.59	
	2014 Spring	0.327	0.034	4.98	1.12	
	2014 Summer	0.475	0.037	16.28	3.27	

Stream Name	Sampling Season/Year	Filtered EEQ _{BLYES} (ng/L)	Filtered EEQ _{BLYES} SE	Filtered Total Microcystins (ng/mL)	Filtered Total Microcystins SE	Association among Total Filtered MC/NOD and EEQ _{BLYES} (p-value, r ²)
Mainstem of the South Branch near Moorefield, West Virginia	2013 Summer	0.627	0.063	9.24	6.20	0.161
	2013 Autumn	3.099	2.650	11.45	4.69	
	2014 Winter	0.353	0.077	5.21	1.01	
	2014 Spring	0.155	0.061	3.94	0.48	
	2014 Summer	0.417	0.057	23.15	5.05	
	2014 Autumn	0.319	0.023	9.86	3.66	
	2015 Winter	0.229	0.075	4.56	0.56	
	2015 Spring	0.381	0.331	2.66	0.32	
	2015 Summer	0.330	0.048	9.67	2.11	
	2015 Autumn	0.177	0.047	4.05	1.28	
	2016 Winter	0.050	NA	8.06	NA	
Mainstem of the South Branch near Mouth near Springfield, West Virginia	2013 Summer	0.505	0.082	12.34	7.92	0.035*, 0.070
	2013 Autumn	0.316	0.035	10.45	5.27	
	2014 Winter	0.453	0.046	5.97	0.63	
	2014 Spring	0.549	0.089	4.65	1.32	
	2014 Summer	0.382	0.071	28.04	5.52	
	2014 Autumn	0.199	0.067	11.43	4.06	
	2015 Winter	0.152	0.049	3.61	0.17	
	2015 Spring	0.157	0.068	6.60	2.91	
	2015 Summer	0.274	0.050	14.65	3.31	
	2015 Autumn	0.266	0.102	4.30	0.70	
	2016 Winter	0.050	NA	12.19	NA	
Mill Creek near Dorcas, West Virginia	2013 Summer	0.050	0.000	5.62	2.49	0.440
	2013 Autumn	0.317	0.031	5.67	2.87	
	2014 Winter	0.840	0.610	5.77	0.52	
	2014 Spring	0.532	0.057	4.67	0.70	
	2014 Summer	0.050	NA	4.16	NA	

Stream Name	Sampling Season/Year	Filtered EEQ_{BLYES} (ng/L)	Filtered EEQ_{BLYES} SE	Filtered Total Microcystins (ng/mL)	Filtered Total Microcystins SE	Association among Total Filtered MC/NOD and EEQ_{BLYES} (p-value, r²)
Mill Creek near Romney, West Virginia	2013 Summer	3.088	2.476	12.21	5.52	0.312
	2013 Autumn	1.058	0.752	3.62	0.58	
	2014 Winter	0.359	0.155	5.77	0.52	
	2014 Spring	0.354	0.036	3.72	0.92	
	2014 Summer	0.696	NA	39.46	NA	
Seneca Creek near Seneca Rocks, West Virginia	2013 Summer	0.490	0.036	10.77	1.30	0.770
	2013 Autumn	0.227	0.177	4.53	1.49	
	2014 Winter	0.279	0.121	5.57	1.52	
	2014 Spring	0.423	0.038	2.69	0.10	
	2014 Summer	0.525	NA	5.26	NA	
North Fork of the Shenandoah River at Strasburg, Virginia	2013 Summer	0.398	0.080	6.06	2.93	0.907
	2013 Autumn	0.655	0.176	8.14	3.14	
	2014 Winter	0.265	0.131	6.36	0.40	
	2014 Spring	0.246	0.196	3.11	0.32	
	2014 Summer	0.438	NA	3.90	NA	
South Fork of the Shenandoah River at Front Royal, Virginia	2013 Summer	0.606	0.046	3.28	1.75	0.055
	2013 Autumn	0.736	0.032	5.28	0.76	
	2014 Winter	0.307	0.142	5.36	1.40	
	2014 Spring	0.452	0.093	2.72	0.10	
	2014 Summer	0.551	NA	2.56	NA	
Shenandoah River at Millville, West Virginia	2013 Summer	0.478	0.003	7.74	0.94	0.205
	2013 Autumn	0.696	0.133	4.79	0.21	
	2014 Winter	0.239	0.111	5.95	0.21	
	2014 Spring	0.519	0.038	2.55	0.08	
	2014 Summer	0.522	NA	6.76	NA	

Table 3. Summary of EEQ_{BLYES} and total microcystins/nodularins in unfiltered water samples during the reconnaissance and biweekly sampling sites. SE represents standard error.

Stream Name	Sampling Season/Year	Unfiltered EEQ _{BLYES} (ng/L)	Unfiltered EEQ _{BLYES} SE	Unfiltered Total Microcystins (ng/mL)	Unfiltered Total Microcystins SE	Association among Total Unfiltered MC/NOD and EEQ _{BLYES} (p-value)
Mainstem of the North Branch at Cumberland, Maryland	2015 Winter	0.102	0.052	3.41	0.53	0.086
	2015 Spring	0.494	0.240	3.10	0.90	
	2015 Summer	0.531	0.132	7.47	1.92	
	2015 Autumn	0.353	0.087	8.45	1.86	
	2016 Winter	0.235	NA	6.47	NA	
Mainstem of the South Branch near Moorefield, West Virginia	2015 Winter	0.050	0.000	5.67	1.33	0.306
	2015 Spring	0.345	0.152	4.32	1.32	
	2015 Summer	0.302	0.049	13.93	2.37	
	2015 Autumn	0.161	0.057	9.32	2.74	
	2016 Winter	0.050	NA	23.24	NA	
Mainstem of the South Branch near Mouth near Springfield, West Virginia	2015 Winter	0.231	0.107	6.16	2.48	0.730
	2015 Spring	0.350	0.104	7.57	2.37	
	2015 Summer	0.298	0.060	22.37	2.51	
	2015 Autumn	0.179	0.061	8.00	1.57	
	2016 Winter	0.050	NA	24.01	NA	

Table 4. Spatial summary for each sample site. Wastewater treatment facilities presented as WWTP.

Watershed	Stream Name	Latitude	Longitude	Basin Area (km ²)	Total WWTP's
Monocacy	Monocacy River near Frederick, Maryland	39.387788	-77.379848	2119.41	24
North Branch Potomac	North Branch Potomac at Cumberland, Maryland	39.586902	-78.737522	2544.65	28
South Branch Potomac	North Fork South Branch Potomac near Petersburg, West Virginia	38.833894	-79.371852	443.07	0
	South Fork South Branch Potomac near Moorefield, West Virginia	38.979448	-78.977210	673.08	1
	South Branch Potomac below Petersburg, West Virginia	38.999172	-79.086226	2207.83	4
	South Branch Potomac below Moorefield, West Virginia	39.104069	-78.957706	3115.99	5
	South Branch Potomac below Franklin, West Virginia	38.858884	-79.279073	778.01	2
	South Branch Potomac near Springfield, West Virginia	39.509346	-78.600141	3822.54	7
	Seneca Creek at Seneca Rocks, West Virginia	38.834233	-79.371759	176.77	0
	Mill Creek (Grant County, West Virginia)	38.961819	-79.114410	122.57	0
	Mill Creek (Hampshire County, West Virginia)	39.327161	-78.796280	120.80	0
Mainstem Potomac	Potomac Mainstem Below Antietam Creek near Sharpsburg, Maryland	39.414305	-77.746319	16229.73	87
Shenandoah River	North Fork Shenandoah River at Strasburg, Virginia	38.974316	-78.355075	1981.05	7
	South Fork Shenandoah River at Front Royal, Virginia	38.929167	-78.219768	4230.90	23
	Shenandoah River Mainstem near Millville, West Virginia	39.274741	-77.785619	7852.31	37

Table 5. Land cover summary for each sample site. These percentages represent the full watershed upstream from each sampling site. The percentage of wetland/open water are excluded.

Stream Name	Agricultural			Developed			Forested		
	2013	2014	2015	2013	2014	2015	2013	2014	2015
Monocacy River near Frederick, Maryland	53.05%	52.87%	49.68%	12.26%	13.00%	12.93%	34.09%	33.25%	36.43%
North Branch Potomac at Cumberland, Maryland	10.64%	10.50%	8.18%	7.83%	7.96%	8.45%	80.76%	80.52%	82.41%
North Fork South Branch Potomac near Petersburg, West Virginia	10.19%	11.63%	8.62%	3.88%	3.85%	3.79%	85.85%	84.47%	87.54%
South Fork South Branch Potomac near Moorefield, West Virginia	8.27%	8.77%	7.14%	3.43%	3.42%	3.46%	88.12%	87.59%	89.15%
South Branch Potomac below Petersburg, West Virginia	16.29%	17.54%	14.24%	3.68%	3.68%	3.68%	79.89%	78.61%	81.92%
South Branch Potomac below Moorefield, West Virginia	15.30%	16.42%	13.43%	3.82%	3.82%	3.83%	80.70%	79.54%	82.50%
South Branch Potomac below Franklin, West Virginia	20.89%	21.98%	18.76%	4.23%	4.13%	4.15%	74.81%	73.82%	77.01%
South Branch Potomac near Springfield, West Virginia	15.28%	16.32%	13.39%	3.77%	3.79%	3.82%	80.66%	79.54%	82.42%
Seneca Creek at Seneca Rocks, West Virginia	5.69%	6.59%	4.31%	2.46%	2.55%	2.46%	91.83%	90.82%	93.21%
Mill Creek (Grant County, West Virginia)	16.96%	18.14%	14.09%	3.77%	3.87%	3.81%	79.24%	77.92%	82.07%
Mill Creek (Hampshire County, West Virginia)	12.00%	12.68%	9.44%	4.33%	4.27%	4.33%	83.66%	83.00%	86.16%
Potomac Mainstem Below Antietam Creek near Sharpsburg, Maryland	22.62%	22.68%	20.58%	7.36%	7.67%	7.73%	69.54%	68.99%	71.04%
North Fork Shenandoah River at Strasburg, Virginia	34.90%	35.38%	33.78%	6.29%	6.29%	6.30%	58.60%	57.96%	59.51%
South Fork Shenandoah River at Front Royal, Virginia	33.99%	34.60%	33.13%	10.26%	10.30%	10.32%	55.39%	54.57%	55.99%
Shenandoah River Mainstem near Millville, West Virginia	34.28%	34.71%	33.11%	8.95%	9.00%	9.02%	56.37%	55.71%	57.27%

Table 6. Land cover summary for each sample site. These percentages represent the land cover/land use within a 30 meter buffer on either side of streams within each watershed upstream from each sampling site. The percentage of wetland/open water are excluded.

Stream Name	Agricultural			Developed			Forested		
	2013	2014	2015	2013	2014	2015	2013	2014	2015
Monocacy River near Frederick, Maryland	39.23%	38.90%	33.79%	11.06%	11.49%	11.50%	47.17%	45.68%	50.71%
North Branch Potomac at Cumberland, Maryland	7.91%	7.32%	5.54%	10.90%	11.01%	11.02%	77.96%	76.93%	78.57%
North Fork South Branch Potomac near Petersburg, West Virginia	15.42%	17.42%	12.15%	8.02%	8.02%	7.94%	76.19%	74.38%	79.70%
South Fork South Branch Potomac near Moorefield, West Virginia	10.18%	10.46%	7.71%	6.58%	6.52%	6.60%	82.56%	82.23%	84.83%
South Branch Potomac below Petersburg, West Virginia	24.44%	25.50%	20.07%	8.04%	8.01%	8.02%	66.89%	65.88%	71.19%
South Branch Potomac below Moorefield, West Virginia	21.57%	22.49%	17.60%	7.64%	7.59%	7.63%	69.96%	69.04%	73.72%
South Branch Potomac below Franklin, West Virginia	33.46%	33.40%	28.48%	9.65%	9.49%	9.50%	56.64%	56.82%	61.74%
South Branch Potomac near Springfield, West Virginia	21.09%	21.87%	17.08%	7.38%	7.35%	7.38%	69.93%	69.06%	73.62%
Seneca Creek at Seneca Rocks, West Virginia	3.36%	3.95%	2.51%	8.02%	8.05%	8.08%	88.53%	87.94%	89.38%
Mill Creek (Grant County, West Virginia)	18.95%	19.61%	13.76%	7.30%	7.10%	7.24%	73.73%	73.22%	78.96%
Mill Creek (Hampshire County, West Virginia)	16.66%	15.85%	10.87%	10.41%	10.43%	10.40%	72.91%	73.69%	78.60%
Potomac Mainstem Below Antietam Creek near Sharpsburg, Maryland	19.65%	19.66%	16.69%	8.71%	8.84%	8.86%	69.36%	68.56%	71.42%
North Fork Shenandoah River at Strasburg, Virginia	32.06%	32.56%	30.04%	7.15%	7.12%	7.11%	59.31%	58.05%	60.44%
South Fork Shenandoah River at Front Royal, Virginia	31.58%	32.37%	30.15%	10.70%	10.62%	10.69%	55.27%	53.99%	56.01%
Shenandoah River Mainstem near Millville, West Virginia	30.02%	30.57%	28.24%	9.25%	9.22%	9.25%	58.10%	56.98%	59.16%

Chapter Five. Evaluation of possible interference as reported by the yeast strain *Saccharomyces cerevisiae* BLYR

Abstract

Estrogenic endocrine disrupting compounds (EEDCs) are a class of chemicals which have been shown to dysregulate reproductive pathways. The identification of these compounds is critical for understanding the biological effects potentially caused by EEDCs. The yeast strain *Saccharomyces cerevisiae* BLYR incorporates a constitutively expressed reporter and is designed to be a measure of toxicity; however, we reference output as interference. BLYR output was quantified among various concentrations of two solvents, methanol (MeOH) and dimethyl sulfoxide (DMSO), as well as 17 β -estradiol and extracts from both environmental water and periphyton samples. Challenged *S. cerevisiae* cells were also evaluated using flow cytometry to determine whether a decrease in BLYR output was a result of cell mortality. Temporal and spatial variability in BLYR output was assessed at select sites in the Potomac River watershed. Strain BLYR was run in conjunction with *S. cerevisiae* strain BLYES (bioluminescent yeast estrogen screen) to determine whether interference could be diluted out without a subsequent loss in the estrogenic signal. Low BLYR output was reported only at high concentrations of MeOH (>10%), DMSO (>2.5%) and 17 β -estradiol (>0.25 ng/mL). There was only significant cell mortality at the highest concentrations of MeOH (50%) and DMSO (5%). Despite evaluating a range of BLYR output (3.21 – 98.61%) in environmental and 17 β -estradiol samples, we only observed $\pm$ 5% mortality as compared to the control. Periphyton samples reported significantly higher interference as compared to water samples. There were minimal but site specific interference among water samples. Seasonally, summer and fall water samples have higher interference as compared to winter and spring. The dilution of individual samples resulted in a

reduction of interference; however there was not a linear response in BLYES results suggesting diluting samples may be a viable option on a case-by-case basis. The comparison of strains BLYR and BLYES in diluted samples yielded variable results. Despite the minimal interference in water samples, it is recommended to report the BLYR interference along with estrogenicity values. This would aid in evaluating spatial and temporal trends; specifically outlier analysis. Due to the high reporter interference, it is recommended to only qualitatively assess estrogenicity in periphyton samples.

1. Introduction

Biological assays (bioassays) are designed to quantify an effect in the context of a living organism. Estrogenic endocrine disrupting compounds (EEDCs) are a group of compounds which have been shown to modulate estrogen receptor-mediated pathways in aquatic organisms (Campbell et al. 2006). Many of these compounds (which include both steroidal and non-steroidal chemicals) have been correlated to deleterious biological effects in fishes (Segner et al. 2013; Senthilkumaran 2015). Routledge and Sumpter (1996) described a yeast (*Saccharomyces cerevisiae*) bioassay which incorporates human estrogen receptor (hER) into the yeast genome, as well as the reporter gene *Lac-Z*. This allows for the identification of compounds which activate this specific pathway and is termed the yeast estrogen screen assay (YES). Sanseverino et al. (2005) further developed the strain *S. cerevisiae* and instead of the colorimetric *Lac-z* reporter, they incorporate the bioluminescent *luxA/B* reporter. This strain is termed bioluminescent yeast estrogen screen (BLYES). The new strain *S. cerevisiae* BLYES yielded a similar detection range of 17 β -estradiol standards; however shortened the duration of the assay from 72 hours to 6 hours.

An important component of bioassays is the determination of cellular viability both before and after a challenge experiment. This ensures that the observed effect is not due to cell death (termed toxicity) but from the experimental agent. The strain *S. cerevisiae* BLYR(a) was first published by Eldridge et al. (2007) and was designed to constitutively express the *luxA/B* bioluminescent reporter. This was done to report cytotoxicity among (in this example) potentially androgenic compounds (incorporating human androgen receptor instead of hER and termed the bioluminescent yeast androgen screen (BLYAS)). The authors identified toxicity by a reduction in the bioluminescent reporter as compared to a control. Sanseverino et al. (2009)

analyzed a comprehensive suite of compounds and quantified the estrogenic and androgenic potential using the BLYES and BLYAS assays as well as the toxicity as reported by BLYR.

Strain *S. cerevisiae* BLYES has been used to report estrogenicity in a variety of aquatic systems (Ciparis et al. 2012; Iwanowicz et al. 2016). Variability among EEQ_{BLYES} in the middle and upper Potomac River watershed is reported in Chapter Three; however the possible contribution to this variance because of reporter interference was not discussed. An investigation into these data may yield insights as to the site-specific variability observed.

To our knowledge, there are no data quantifying the toxicity reported in strain *S. cerevisiae* BLYR in environmental samples. Interference is defined as a significant deviation from the control luminescence. The specific objective of this study was to determine whether the interference (specifically the lower output luminescence) determined by the BLYR assay constitutes dead cells as reported by flow cytometry. A subsequent objective was to determine the variability in the BLYR assay to differing concentrations of the solvents absolute methanol (MeOH) and dimethyl sulfoxide (DMSO), as well as 17 β -estradiol (E2) and environmental samples. An additional objective of our study was to examine the potential interference in the estrogenicity assay using the BLYR reporter with environmental samples from the middle and upper Potomac River watershed.

2. Methods

2.1. Field collection

Fifteen sites along streams located within the Potomac River watershed in West Virginia, Maryland, and Virginia were selected for surface water sampling based on the historic presence of EEDCs or sites of interest by fisheries biologist staff from the West Virginia Division of

Natural Resources (Figure 1). These 15 reconnaissance sites were sampled monthly from 26-June-2013 through 18-June-2014 (Table 1). Two grab water samples were collected from a near-shore location using one chemically clean 1 L amber glass bottle (referred to as filtered samples; I-Chem, Rockwood, Tennessee). A subset of three sites from the original 15 was selected and sampled biweekly for 16 months to evaluate fluctuation at a finer time scale (Table 1). Biweekly sampling occurred between 30-June-2014 and 14-December-2015. Along with the physical parameters described above, beginning with the first sampling date in 2015 (13-January) through the final collection date (14-December), an additional discrete water sample was collected in one clean sterile 500 mL HDPE bottle (Thermo Fisher Scientific, Waltham, Massachusetts). These samples were defined as unfiltered. Filtered samples contain soluble and small particulate ($0.7\ \mu\text{m}$) compounds present in the water column while unfiltered samples also contained larger particulates.

A periphyton sample was collected at every sampling event in both the reconnaissance and biweekly sampling periods by scraping organic benthic material from the surface of submerged rocks and woody debris into a sterile 50 mL conical tube (Becton Dickinson, Franklin Lakes, New Jersey). Benthic mats of periphyton were targeted based on dark green coloration, leathery texture and the strong smell from 2-methylisoborneol.

2.2. Laboratory preparation and extraction

Filtered water samples were treated with hydrochloric acid until the pH was at 3.0 and stored at 4°C no longer than 2 weeks until extraction as recommended by Havens et al. (2010). Unfiltered water samples were subjected to three freeze-thaw cycles to room temperature to disrupt the cells (Graham et al. 2010; Loftin et al. 2008). Periphyton samples were centrifuged at 500 x g for 10 minutes. After centrifugation, supernatant was removed retaining the periphyton

pellet. Centrifuged periphyton samples were lysed by three freeze-thaw cycles (brought to room temperature) to ensure that all targeted compounds were released from the cells (Graham et al. 2010; Loftin et al. 2008). Lysed samples were stored at -20°C until extraction.

Filtered and unfiltered water samples were extracted using methods modified from Ciparis et al. (2012). Briefly, samples were pre-filtered using a 0.7 µm AP40 GF/F filter (Millipore Corporation, Billerica, Massachusetts) prior to extraction (unfiltered samples were adjusted to pH 3.0 with 6N hydrochloric acid prior to filtration). Water samples were prepared by solid phase extraction (SPE) using OASIS® HLB (200mg) glass cartridges (Waters Corporation, Milford, Massachusetts). Cartridges were conditioned using 5 mL each of ethyl acetate, 50:50 MeOH:dichloromethane, MeOH, and MiQ-H₂O pH 3.0. Eight hundred mL of each water sample was run through the cartridge by continuous vacuum using methods modified from Laganà et al. (2004). Two subsequent 6 mL eluents of MeOH and 50:50 methanol:dichloromethane were reduced to dryness. Eluates were solubilized in MeOH such that the final extraction ratio was 800:1. Samples were stored at -20°C until analysis.

Periphyton samples were extracted using methods modified from Mohamed et al. (2006). Once the sample weight was recorded, 6 mL of absolute MeOH was added. Samples were vortexed for 3 minutes, followed by centrifugation at 500g for 10 minutes. Supernatant was retained and the process was repeated a second time with 50:50 MeOH:dichloromethane. Each supernatant sample was filtered using a 0.7 µm Whatman® Purdisc 13 syringe filter (Whatman, Maidstone, United Kingdom). Once filtered, both samples were reduced to dryness, then solubilized with MeOH and pooled such that the final sample volume was 500 µL. Samples were stored at -20°C until analysis.

2.3. Determination of toxicity, interference, and viability

In order to evaluate toxicity or interference with the bioreporter, samples were screened at the same assay dilution used for estrogenicity using the constitutive bioluminescence strain *S. cerevisiae* BLYR (Eldridge et al. 2007). Strain BLYR was purchased from 490 Bio Tech (Knoxville, Tennessee). This strain was grown under identical conditions of strain *S. cerevisiae* BLYES and then brought to approximately OD₆₀₀ of 0.40. The assay was performed in sterile 96-well white plates (Costar, Corning Incorporated, Corning, New York). Sample extracts were diluted 5% in 100 μ L yeast minimal media (YMM) and placed in each well. Control samples were added in duplicate to each plate. *S. cerevisiae* BLYR was then added to each well (100 μ L) resulting in a final MeOH concentration of 2.5%. Each plate was incubated for 4 hours at 30°C on an orbital shaker. Luminescence was quantified using a SpectraMax M4 (Molecular Devices, Sunnyvale, California) with the integration time at 500 milliseconds and automatic photo multiplier tube gain. Data were reported as the ratio of sample to a 2.5% MeOH control. The detection limit of the BLYR assay was determined by quantifying the variance among 96 2.5% MeOH control wells. The interference threshold is defined as the mean $\pm$ 3 SD about the mean.

A range of 17 β -estradiol (E2) standards (10.0, 5.0, 1.0, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008, and 0.004 ng/mL), as well as a range of MeOH (20.0, 10.0, 5.0, 2.5, 1.25, 0.625, and 0.313%) and DMSO concentrations (5.0, 2.5, 1.25, 0.625, 0.313, 0.15625, and 0.078%) and a subset of filtered water and periphyton extracts which previously reported a wide range of BLYR output percentages were all evaluated. The output ratios for MeOH and DMSO treatments were compared to media-only wells and all environmental samples were compared to the 2.5% MeOH control wells. Following the measurement of luminescence, samples were centrifuged for 10 minutes at 805 x g and supernatant removed. A 1% solution of propidium iodide (Molecular

Probes, Eugene, Oregon) was prepared in sterile Dulbecco's phosphate buffered saline (pH 7.4; DPBS; Sigma Aldrich, St. Louis, Missouri) and 100 μ L was added to each well and incubated on a rotary shaker for 30 minutes (protected from light). The samples were centrifuged for 10 minutes at 805 x g, supernatant removed and washed using 100 μ L of DPBS. Viability was quantified via flow cytometry on an Amnis[®] FlowSight[®] (MilliporeSigma, Billerica, Massachusetts). Ten thousand events were analyzed using the 488 nm laser, channel four. Analysis was performed with IDEAS 6.2 software (Amnis/MilliporeSigma, Billerica, MA). An example of the gating strategy is shown in Figure 2. First, all single cells which were in focus were targeted (represented by the green box in Figure 2B and expanded as Figure 2B). Next, a second gate was created which identified only those dead cells accounting for auto-florescence in live cells. Dead cells report relatively high propidium iodide intensity and are indicated in Figure 2C by the pink box. The frequency distribution and grouping of these dead cells are presented in Figures 2D. Examples of propidium iodide positive and negative cells are presented in figures 2eE and 2F, respectively. Dead cells are defined as propidium iodide-positive and live cells as propidium iodide-negative. This gating template was used for all samples. Apoptotic-like cells were identified (Figure 3) in which the nucleus is small and compact when compared to the normal cell morphology (George et al. 2004; Henery et al. 2008; Vorobjev and Barteneva 2017). This gating strategy was used on all samples. Outputs are recorded as the percent of dead or apoptotic-like cells. Cell densities are recorded for all samples (noting that cells were optimized according to optical density, not specific cell density prior to plating).

2.4. Statistical Analysis

All analysis was performed using the R statistical software. Statistical significance was determined at $\alpha = 0.05$. Analysis of Variance (ANOVA) test with a Tukey's Honest Significance

test (if statistically significant differences were reported) was used to compare site differences pooled across month and year, as well as the monthly differences pooled across all sites and years. An ANOVA accompanied with the Tukey's Honest Significance test was also used to identify differences among sites within season/year. Seasons were defined as spring occurring between March, April, and May; summer occurring between June, July, and August; fall occurring between September, October, and November; winter occurring between December, the following January, and February (the year associated with winter corresponds to January and February). Filtered and unfiltered water fractions were compared using a Student's t-test.

3. Results

3.1. Viability and interference among Solvent, E2, and water samples

All samples were screened for reporter interference/toxicity/viability. The interference threshold for the BLYR assay was determined to be 11.0%. BLYR and viability results are summarized in Table 2. In general, an increase of interference as reported by BLYR was associated with higher E2 and MeOH concentrations. The range of BLYR output among MeOH was 47.9 to 117.6% and the range of BLYR output for E2 was 61.5% to 98.6%. The background mortality in our unchallenged cells was 10.7%. To better represent the dead cells resulting from each respective treatment, this value was subtracted from the absolute mortality for each treatment and presented this as the adjusted percent dead cells. The highest adjusted mortality in MeOH and E2 challenged cells was extremely minimal (0.8%). There was no dose-dependent pattern for either compound.

A kinetic difference in BLYR output was noted when testing the solvents. At the highest three MeOH concentrations (20, 10, and 5%) output was suppressed during the first two hours of

the experiment (Figure 4). Following this time, the cells exposed to 5% MeOH were within the range of the lower four concentrations. The cells exposed to 20 and 10% MeOH concentrations remained below all other MeOH concentrations. The highest two DMSO concentrations (5.0 and 2.5%) suppressed output as compared to the other five concentrations throughout the experiment (Figure 5). There were no differences among the lower concentrations.

There was a relatively wide range of interference among water and periphyton samples. The range of BLYR output among water samples was 29.9 to 102.2% and periphyton was 3.8 to 57.2%. Interestingly, there was a minimal change in adjusted mortality among these samples ranging from -1.7 to 1.2% in water and -4.5 to 1.4% in periphyton. There was no discernible trend in BLYR percentage and percent mortality or apoptotic-like cells in either water or periphyton samples.

Our final experiment was to determine the feasibility of diluting out interference without losing EEQ_{BLYES} sensitivity (Table 3). A decrease in interference was reported for each dilution in each sample (regardless of medium); however only three samples (all were filtered water) were diluted above our interference threshold. There was not a subsequent linear change in EEQ_{BLYES} as samples were diluted. In filtered water, 75% of the samples increased EEQ at 50% dilution while all were decreased at the 75% dilution. One periphyton sample increased from below detection to quantifiable only at the 75% dilution while all diluted 50% were never reported above detection.

3.2. Environmental Samples

The average BLYR output ratio grouped by site is presented in Figure 6. There was no significant difference among sites in either filtered water or periphyton during the reconnaissance

sampling period. None of the filtered water samples exhibited interference while all of the periphyton samples showed significant reduction in BLYR output (Figure 6A). During the biweekly sampling time period, both filtered and unfiltered water fractions at the North Branch Cumberland (MS4) were significantly lower than those from either of the South Branch Potomac sites (Figure 6B). There were no differences among sites in periphyton.

Minimal variability was noted among months in filtered water samples (Figure 7). There was a slight decrease in the average output ratio in the months of October and December as compared to some of the sites. In general, samples collected in the spring and summer had less interference than those collected in the fall and winter. There were no differences in unfiltered water or periphyton samples. There were no differences among filtered and unfiltered samples.

The individual sample temporal data among filtered water, unfiltered water and periphyton are presented at the three intensive sites in Figures 8-10. In general there was low sample variability in the two water fractions in the two South Branch sites and considerable variability in the North Branch site (numerous yet randomly distributed samples below the interference threshold). Considerable variability in periphyton samples was also found for all three sites. A similar overall trend among sample mediums were observed in the remaining reconnaissance sampling sites as well. The mean and range of standard deviations among all sites were 1.95% (0 – 9.19%), 1.56% (0 – 4.24%), and 2.50% (0 – 32.17%) for filtered water, unfiltered water and periphyton, respectively. Despite the individual sample variability, the average output ratio for all three mediums in subsequent results is reported below.

The final analysis was to compare the sample site variability between season/year. These data are presented in Figures 11-13. During the reconnaissance period, the Monocacy river near Frederick, Maryland was significantly higher than Shenandoah River at Millville, West Virginia,

the South Branch at Petersburg, West Virginia, the South Branch near Springfield, West Virginia, the North Fork of the South Branch near Seneca Rocks, West Virginia, the North Branch at Cumberland, Maryland, the mainstem of the Potomac river near Sharpsburg, Maryland, and mill creek near Romney, West Virginia. There were no differences in either filtered water (Figure 11A) or periphyton (Figure 13A) during the reconnaissance sampling period. During the biweekly sampling time period, the North Branch site at Cumberland was significantly lower than the two South Branch sites during the Fall 2014 and Winter 2015 (filtered water), and Winter 2015 and Fall 2015 (unfiltered water). An apparent site difference is noted in filtered and unfiltered water during the winter 2016; however this represents only one sampling event and statistical differences were not calculated. There were no differences in periphyton between sampling sites during the biweekly time period.

4. Discussion

The BLYES assay is used to measure relative estrogenicity as compared to an E2 standard curve. Utilizing the BYLR assay high concentrations of MeOH (>20%) caused interference above our threshold, however these are concentrations not likely to be utilized in the assays. The increased BLYR luminescence values did not correlate to cell density; thus the mechanism remains unclear. No reporter interference was reported in E2 standards < 0.25 ng/mL. This concentration corresponds to 9.2×10^{-9} M and is consistent with the upper bounds reported by Sanseverino et al. (2009). Based on their guidelines for data acceptance, it is recommended to use this value as the upper reporting limit with the BLYES assay.

The wide range of interference reported in environmental samples was not associated with mortality, apoptotic-like cells, or cell density. This interference may result in the underreporting of estrogenicity data (although the specific source remains unclear). Table 3

reports a decrease of the interference in environmental samples without a subsequent linear response in estrogenicity. In the absence of matrix effects, estrogenicity would be expected to be diluted out. However as interference decreased, estrogenicity increased in some samples which would suggest that at higher sample concentrations, the signal is being suppressed by the interference. Although the specific source of interference cannot be pinpointed, this may suggest a possible underreporting of estrogenicity due to reporter interference. This would need to be verified on a sample-by-sample basis.

A possible source for seasonal interference may be stream discharge. Figure 14 presents the mean daily discharge at the South Branch Potomac near Springfield, West Virginia (U.S. Geological Survey National Water Information System ID 01608070) and the North Branch Potomac River near Cumberland, Maryland (U.S. Geological Survey National Water Information System ID 01603000). Both sites experienced relatively higher discharge between the months of January and July (regardless of year) with episodic high flow events. Conversely, lower flows were observed in the late summer through early winter months. These lower discharges may result in a concentrating of EDCs or other compounds detrimental to strain *S. cerevisiae* BLYR. Kolpin et al. (2004) reported a decrease in organic wastewater contaminants in higher flow events which they believe resulted from the dilution of those compounds. Further research is required to quantify the temporal distribution of specific compounds at these sites.

An additional source of interference may be the compounds associated with land cover/land use (e.g. endocrine disrupting compounds (EDCs)). The land cover classified among each watershed was analyzed in chapter three and is represented here in Tables 4-6. In general, the North and South Branch sites are quite similar (heavily forested with localized agriculture). One noteworthy difference is the number of WWTPs in the North Branch Cumberland site;

however, Iwanowicz et al. (2009) documented that the negative reproductive effects in fish were not associated with WWTPs which would suggest additional influence from another source (such as agriculture). The Monocacy and three Shenandoah sites report a greater percentage of agricultural and developed land use. The agricultural contribution to in-stream EDCs are well documented (Alvarez et al. 2009; Ciparis et al. 2012; Fairbairn et al. 2016; Young et al. 2014). The estrogenic biological effects of pesticides are reviewed by Hayes et al. (2011). Disruption by atrazine extends to the invertebrate community as well (DeLorenzo et al. 2001). Sanseverino et al. (2009) reported toxic effects in select pesticides (such as atrazine) but not others (such as *o,p'*-DDT). They also report variable toxicity in other classes of compounds (pharmaceutical, hormone, etc.) Further research is necessary to quantify temporal distribution of these compounds and the possible association on BLYR reporter interference.

These environmental samples are the same which were assessed using EEQ_{BLYES} in chapter four. Based on a simple linear regression comparing the BLYR and EEQ_{BLYES} results across all sites and sample dates, there was a weakly significant correlation in these data ($p = 0.005$; $r^2 = 0.023$). Although this correlation is very weak, the interference may (in-part) influence the seasonal variability in EEQ_{BLYES} as reported in chapter four, Figure 2. Additionally, this interference may influence the site variance as reported in chapter four, Table 2. However, given the weak nature of the association, there are likely other factors resulting in the EEQ_{BLYES} trends.

The seasonal trend in interference is similar to the seasonal trend in total microcystins/nodularins as reported in chapter two. This association was weakly positive for all three sample mediums (as defined by Hinkle et al. (2003)) using Spearman's rank-order correlation. Both filtered water ($p = 0.012$; $\rho = 0.167$) and periphyton ($p < 0.001$; $\rho = 0.287$) were

significantly correlated with BLYR output; however there was not a significant association with unfiltered water ($p = 0.138$; $\rho = 0.173$). Although biomass was not quantified, the association may be evidence of reporter interference by cellular lysate. Molecular disruption has been documented in vertebrates by cyanobacterial lysate (Rogers et al. 2011; Rymuszka and Adaszek 2013; Štěpánková et al. 2011). Additionally, Štěpánková et al. (2011) showed select cyanobacterial species lysate to be cytotoxic. Although there was not an increase in dead or apoptotic cells among treatments, these lysates (particularly periphyton) may contain compounds which disrupt *S. cerevisiae* metabolism and thus, affect the output luminescence.

Overall, minimal interference was reported among water fractions. However, it is recommended to report both the EEQ_{BLYES} and associated BLYR output ratio as this would provide additional details which can aid in interpretation. For example it may provide insights into trend analysis; specifically in explaining deviations (outliers). Caution is recommended for reporting the EEQ_{BLYES} in periphyton samples. The interference in these periphyton samples was generally greater than those in either filtered or unfiltered water samples. In cases of extreme interference, it may be more appropriate to report qualitative (presence/absence) data as opposed to quantitative output; however further research is needed to determine what those threshold values should be. As suggested above, interference should be addressed on a sample-by-sample basis. As presented above, sample dilution resulted in a reduction of interference and a non-linear change in the corresponding EEQ_{BLYES} values. One possible solution would be to dilute these samples until the interference value is above the quantification threshold if the corresponding EEQ_{BLYES} value is not diluted out of the sample. The final EEQ calculation would simply need to be adjusted to account for the additional dilution. In situations when interference cannot be diluted out without also removing EEQ_{BLYES} , these samples should be reported with

the lowest possible interference value and flagged as they may be underreporting the total estrogenicity value.

This chapter has principally focused on interference below the critical output ratio threshold. However data are reported well over 100%. Among MeOH and DMSO, these values were not associated with increased cell density or apoptotic-like cells. This is confounded by the observation that these elevated percentages were reported at the lower concentrations of solvent. In practice, these values are not problematic as they should be consistent across all samples (e.g. environmental samples were all analyzed with 2.5% MeOH). However this does not address what may be driving these higher output ratios relative to media-only controls. Further research is needed focusing on what may be driving these relatively higher luminescence values in our reporter.

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Figures

Figure 1. The location of all 15 sites included in this study. All sites were located in the states of West Virginia, Maryland, and Virginia within the Potomac River watershed (inset map).

Figure 2. Gating strategy using flow cytometry to identify dead cells (here depicted by cells exposed to 50% MeOH and report 93.5% mortality). A-B. Primary gating of cells A) in focus and B) single cells. C-D. Secondary gating of cells C) dead cells based on intensity of propidium iodide and D) frequency. E-F. Example of cell types based on propidium iodide staining E) positive (dead) and F) negative (live).

Figure 3. Gating strategy using flow cytometry to identify apoptotic-like cells. A-C identifies different cell populations such as A) Apoptotic-like cell identified as small and compact as compared to B and C which are two distinct populations of larger cells with normal morphology (represented here as propidium iodide positive).

Figure 4. Temporal response of BLYES among varying concentrations of absolute methanol.

Figure 5. Temporal response of BLYES among varying concentrations of absolute dimethyl sulfoxide.

Figure 6. Site in BLYR output ratio values measured in filtered water, unfiltered water, and periphyton samples from the A) reconnaissance and B) biweekly sampling periods. Error bars represent one standard deviation about the mean. The red bar represents the interference threshold.

Figure 7. Monthly variability in BLYR output ratio values measured in filtered water, unfiltered water, and periphyton samples. Error bars represent one standard deviation about the mean. Statistical differences in filtered water samples are represented above each bar. There were no significant differences in unfiltered water or periphyton. The red bar represents the interference threshold.

Figure 8. Temporal variability in BLYR output ratio values measured in filtered water samples from biweekly sampling sites. Error bars represent one standard deviation about the mean. The y-axis scale begins at 60% to focus on the range of values. The x-axis represents specific sampling dates as opposed to a continuous range of dates. The red bar represents the interference threshold.

Figure 9. Temporal variability in BLYR output ratio values measured in unfiltered water samples from biweekly sampling sites. Error bars represent one standard deviation about the mean. The y-axis scale begins at 60% to focus on the range of values. The x-axis represents specific sampling dates as opposed to a continuous range of dates. The red bar represents the interference threshold.

Figure 10. Temporal variability in BLYR output ratio values measured in periphyton samples from biweekly sampling sites. Error bars represent one standard deviation about the mean. The x-axis represents specific sampling dates as opposed to a continuous range of dates. The red bar represents the interference threshold.

Figure 11. Season/year variability in BLYR output ratio values measured in filtered water samples from A) the reconnaissance and B) biweekly time period. Error bars represent one standard deviation about the mean. The y-axis scale begins at 75% to focus on the range of values. The red bar represents the interference threshold.

Figure 12. Season/year variability in BLYR output ratio values measured in unfiltered water samples from all sites. Error bars represent one standard deviation about the mean. The y-axis scale begins at 75% to focus on the range of values. The red bar represents the interference threshold.

Figure 13. Season/year variability in BLYR output ratio values measured in periphyton samples from A) the reconnaissance and B) biweekly time period. Error bars represent one standard deviation about the mean. The red bar represents the interference threshold.

Figure 14. Daily discharge at the South Branch Potomac River near Springfield, West Virginia (U.S. Geological Survey National Water Information System ID 01608500) and the North Branch Potomac River near Cumberland, Maryland (U.S. Geological Survey National Water Information System ID 01603000).

Tables

Table 1. Site description for environmental samples. Latitude and longitude are reported in decimal degrees.

Basin	Site Name	Site Abbreviation	Sample Site ID	Latitude	Longitude	Reconnaissance Site	Biweekly Site
Monocacy River	Monocacy River near Mouth near Frederick, MD	Monocacy	LP3	39.38778841	-77.37984832	X	
North Branch of the Potomac River	Mainstem of the North Branch at Cumberland, MD	North Branch Cumberland	MS4	39.58690227	-78.73752192	X	X
Potomac River	Mainstem of the Potomac River near Sharpsburg, MD	Potomac Sharpsburg	MS5	39.41430527	-77.74631932	X	
South Branch of the Potomac River	North Fork of the South Branch at Seneca Rocks, WV	North Fork South Branch	NF1	38.83389380	-79.37185151	X	
	South Fork of the South Branch near Moorefield, WV	South Fork South Branch	SF1	38.97944841	-78.97721032	X	
	Mainstem of the South Branch at Petersburg, WV	South Branch Petersburg	SB1	38.99917201	-79.08622612	X	
	Mainstem of the South Branch near Moorefield, WV	South Branch Moorefield	SB3	39.10406921	-78.95770572	X	X
	Upper Mainstem of the South Branch	Upper South Branch	SB4	38.85888361	-79.27907259	X	
	Mainstem of the South Branch near Mouth near Springfield, WV	South Branch Springfield	SB9	39.50934621	-78.60014072	X	X
	Mill Creek near Dorcas, WV	Mill Creek Dorcas	MC1	38.96181921	-79.11440952	X	
	Mill Creek near Romney, WV	Mill Creek Romney	MC2	39.32716127	-78.79627972	X	
	Seneca Creek near Seneca Rocks, WV	Seneca Creek	SC1	38.83423291	-79.37175894	X	
North Fork of the Shenandoah River	North Fork of the Shenandoah River at Strasburg, VA	North Fork Shenandoah	SR3	38.97431587	-78.35507539	X	
South Fork of the Shenandoah River	South Fork of the Shenandoah River at Front Royal, VA	South Fork Shenandoah	SR5	38.92916661	-78.21976819	X	
Shenandoah River	Shenandoah River at Millville, WV	Shenandoah Millville	SR6	39.27474127	-77.78561892	X	

Table 2. BLYR output ratio and flow cytometry summary. MeOH and DMSO percent's are compared to the Media only positive control. The E2 and environmental samples were compared to the 2.5% MeOH samples. Dead cells are defined as propidium iodide positive. Percent dead cells were adjusted to correct for background as reported in the positive control. Standard deviation is presented as SD.

Sample Description	BLYR Percentage	BLYR Percentage SD	Adjusted Percent Dead Cells	Cell Density (cells/mL) 1×10^6	Percent Apoptotic-like Cells
MeOH 50% (Dead Control)	0.12%	0.03%	82.83%	8.07	4.11%
MeOH 20%	47.88%	8.03%	0.64%	8.31	0.35%
MeOH 10%	95.55%	5.71%	-0.47%	5.67	0.03%
MeOH 05%	114.83%	0.68%	0.56%	5.65	0.16%
MeOH 2.5%	117.58%	0.29%	0.78%	6.77	0.07%
MeOH 1.25%	117.14%	2.22%	-1.15%	5.94	0.05%
MeOH 0.625%	115.61%	1.35%	-3.40%	5.65	0.00%
MeOH 0.3125%	109.12%	2.79%	0.06%	6.39	0.00%
Media Only (Positive Control)	100.00%	0.05%	0.00%	5.31	0.04%
DMSO_5%	69.18%	1.76%	12.69%	6.92	0.09%
DMSO_2.5%	91.29%	1.95%	3.26%	6.25	0.09%
DMSO_1.25%	103.75%	0.59%	-2.16%	5.79	0.05%
DMSO_0.625%	109.21%	1.98%	0.63%	4.46	0.05%
DMSO_0.3125%	112.40%	0.39%	-0.27%	6.92	0.05%
DMSO_0.15625%	111.19%	0.95%	-0.31%	5.19	0.00%
DMSO_0.078125%	109.14%	0.30%	0.70%	4.71	0.11%
E2 Std 1 (10 ng/mL)	64.94%	1.11%	-2.86%	4.02	0.00%
E2 Std 2 (5 ng/mL)	61.47%	2.28%	0.81%	2.54	0.02%
E2 Std 3 (1 ng/mL)	78.77%	1.07%	-2.27%	2.86	0.02%
E2 Std 4 (0.5 ng/mL)	85.17%	0.83%	0.08%	3.67	0.04%
E2 Std 5 (0.25 ng/mL)	91.97%	3.45%	-2.68%	5.08	0.00%
E2 Std 6 (0.125 ng/mL)	97.51%	1.96%	-2.51%	4.02	0.02%
E2 Std 7 (0.0625 ng/mL)	99.06%	0.87%	-2.75%	4.81	0.00%
E2 Std 8 (0.03125 ng/mL)	97.93%	0.35%	-1.75%	3.83	0.06%

Sample Description	BLYR Percentage	BLYR Percentage SD	Adjusted Percent Dead Cells	Cell Density (cells/mL) 1*10⁶	Percent Apoptotic- like Cells
E2 Std 9 (0.01563 ng/mL)	95.95%	2.46%	-2.52%	4.44	0.00%
E2 Std 10 (0.00781 ng/mL)	98.35%	3.05%	-3.14%	5.67	0.02%
E2 Std 11 (0.00391 ng/mL)	98.61%	1.24%	-4.45%	5.79	0.00%
Filtered Water Sample 1	25.41%	3.09%	1.24%	4.70	0.04%
Filtered Water Sample 2	48.54%	0.34%	0.23%	4.46	0.04%
Filtered Water Sample 3	44.97%	0.82%	0.71%	5.43	0.11%
Filtered Water Sample 4	48.36%	0.77%	1.44%	4.15	0.02%
Filtered Water Sample 5	66.41%	1.04%	-0.36%	3.96	0.06%
Filtered Water Sample 6	68.29%	0.39%	0.73%	5.08	0.04%
Filtered Water Sample 7	86.91%	0.17%	-1.67%	4.79	0.00%
Filtered Water Sample 8	81.15%	0.07%	0.85%	5.18	0.04%
Periphyton Sample 1	3.21%	0.32%	-4.46%	20.64	0.05%
Periphyton Sample 2	10.37%	0.25%	1.35%	7.32	0.02%
Periphyton Sample 3	5.98%	0.01%	-0.99%	6.39	0.05%
Periphyton Sample 4	24.17%	1.35%	-1.83%	7.77	0.02%
Periphyton Sample 5	9.93%	0.11%	-3.76%	12.47	0.10%
Periphyton Sample 6	38.39%	0.19%	-2.80%	4.71	0.00%
Periphyton Sample 7	48.65%	0.60%	-3.89%	5.67	0.02%
Periphyton Sample 8	48.32%	0.06%	-2.79%	7.09	0.02%

Table 3. BLYR/BLYES dilution comparison. Samples were diluted to the appropriate concentration and compared to a 2.5% MeOH control. Data below the detection limit of the assay are presented as BD.

Sample ID	Percent Sample	BLYR	BLYES
Filtered Water Sample 9	100%	99.64%	0.119
	50%	106.02%	0.131
	25%	109.85%	0.106
Filtered Water Sample 10	100%	70.76%	0.015
	50%	82.76%	0.016
	25%	97.30%	0.012
Filtered Water Sample 11	100%	62.61%	0.016
	50%	79.59%	0.012
	25%	85.65%	0.012
Filtered Water Sample 12	100%	67.17%	BD
	50%	86.76%	0.005
	25%	92.17%	BD
Periphyton Sample 9	100%	45.27%	0.038
	50%	63.85%	0.055
	25%	73.34%	0.073
Periphyton Sample 10	100%	26.23%	BD
	50%	44.31%	BD
	25%	53.60%	0.008
Periphyton Sample 11	100%	1.18%	BD
	50%	5.42%	BD
	25%	23.57%	BD
Periphyton Sample 12	100%	46.65%	BD
	50%	73.42%	BD
	25%	83.27%	BD

Table 4. Spatial summary for each sample site. Wastewater treatment facilities presented as WWTP.

Watershed	Stream Name	Basin Area (km²)	Total WWTP's
Monocacy	Monocacy River near Frederick, Maryland	2119.41	24
North Branch Potomac	North Branch Potomac at Cumberland, Maryland	2544.65	28
South Branch Potomac	North Fork South Branch Potomac near Petersburg, West Virginia	443.07	0
	South Fork South Branch Potomac near Moorefield, West Virginia	673.08	1
	South Branch Potomac below Petersburg, West Virginia	2207.83	4
	South Branch Potomac below Moorefield, West Virginia	3115.99	5
	South Branch Potomac below Franklin, West Virginia	778.01	2
	South Branch Potomac near Springfield, West Virginia	3822.54	7
	Seneca Creek at Seneca Rocks, West Virginia	176.77	0
	Mill Creek (Grant County, West Virginia)	122.57	0
	Mill Creek (Hampshire County, West Virginia)	120.80	0
Mainstem Potomac	Potomac Mainstem Below Antietam Creek near Sharpsburg, Maryland	16229.73	87
Shenandoah River	North Fork Shenandoah River at Strasburg, Virginia	1981.05	7
	South Fork Shenandoah River at Front Royal, Virginia	4230.90	23
	Shenandoah River Mainstem near Millville, West Virginia	7852.31	37

Table 5. Land cover summary for each sample site. These percentages represent the full watershed upstream from each sampling site. The percentage of wetland/open water are excluded.

Stream Name	Agricultural			Developed			Forested		
	2013	2014	2015	2013	2014	2015	2013	2014	2015
Monocacy River near Frederick, Maryland	53.05%	52.87%	49.68%	12.26%	13.00%	12.93%	34.09%	33.25%	36.43%
North Branch Potomac at Cumberland, Maryland	10.64%	10.50%	8.18%	7.83%	7.96%	8.45%	80.76%	80.52%	82.41%
North Fork South Branch Potomac near Petersburg, West Virginia	10.19%	11.63%	8.62%	3.88%	3.85%	3.79%	85.85%	84.47%	87.54%
South Fork South Branch Potomac near Moorefield, West Virginia	8.27%	8.77%	7.14%	3.43%	3.42%	3.46%	88.12%	87.59%	89.15%
South Branch Potomac below Petersburg, West Virginia	16.29%	17.54%	14.24%	3.68%	3.68%	3.68%	79.89%	78.61%	81.92%
South Branch Potomac below Moorefield, West Virginia	15.30%	16.42%	13.43%	3.82%	3.82%	3.83%	80.70%	79.54%	82.50%
South Branch Potomac below Franklin, West Virginia	20.89%	21.98%	18.76%	4.23%	4.13%	4.15%	74.81%	73.82%	77.01%
South Branch Potomac near Springfield, West Virginia	15.28%	16.32%	13.39%	3.77%	3.79%	3.82%	80.66%	79.54%	82.42%
Seneca Creek at Seneca Rocks, West Virginia	5.69%	6.59%	4.31%	2.46%	2.55%	2.46%	91.83%	90.82%	93.21%
Mill Creek (Grant County, West Virginia)	16.96%	18.14%	14.09%	3.77%	3.87%	3.81%	79.24%	77.92%	82.07%
Mill Creek (Hampshire County, West Virginia)	12.00%	12.68%	9.44%	4.33%	4.27%	4.33%	83.66%	83.00%	86.16%
Potomac Mainstem Below Antietam Creek near Sharpsburg, Maryland	22.62%	22.68%	20.58%	7.36%	7.67%	7.73%	69.54%	68.99%	71.04%
North Fork Shenandoah River at Strasburg, Virginia	34.90%	35.38%	33.78%	6.29%	6.29%	6.30%	58.60%	57.96%	59.51%
South Fork Shenandoah River at Front Royal, Virginia	33.99%	34.60%	33.13%	10.26%	10.30%	10.32%	55.39%	54.57%	55.99%
Shenandoah River Mainstem near Millville, West Virginia	34.28%	34.71%	33.11%	8.95%	9.00%	9.02%	56.37%	55.71%	57.27%

Table 6. Land cover summary for each sample site. These percentages represent the land cover/land use within a 30 meter buffer on either side of streams within each watershed upstream from each sampling site. The percentage of wetland/open water are excluded.

Stream Name	Agricultural			Developed			Forested		
	2013	2014	2015	2013	2014	2015	2013	2014	2015
Monocacy River near Frederick, Maryland	39.23%	38.90%	33.79%	11.06%	11.49%	11.50%	47.17%	45.68%	50.71%
North Branch Potomac at Cumberland, Maryland	7.91%	7.32%	5.54%	10.90%	11.01%	11.02%	77.96%	76.93%	78.57%
North Fork South Branch Potomac near Petersburg, West Virginia	15.42%	17.42%	12.15%	8.02%	8.02%	7.94%	76.19%	74.38%	79.70%
South Fork South Branch Potomac near Moorefield, West Virginia	10.18%	10.46%	7.71%	6.58%	6.52%	6.60%	82.56%	82.23%	84.83%
South Branch Potomac below Petersburg, West Virginia	24.44%	25.50%	20.07%	8.04%	8.01%	8.02%	66.89%	65.88%	71.19%
South Branch Potomac below Moorefield, West Virginia	21.57%	22.49%	17.60%	7.64%	7.59%	7.63%	69.96%	69.04%	73.72%
South Branch Potomac below Franklin, West Virginia	33.46%	33.40%	28.48%	9.65%	9.49%	9.50%	56.64%	56.82%	61.74%
South Branch Potomac near Springfield, West Virginia	21.09%	21.87%	17.08%	7.38%	7.35%	7.38%	69.93%	69.06%	73.62%
Seneca Creek at Seneca Rocks, West Virginia	3.36%	3.95%	2.51%	8.02%	8.05%	8.08%	88.53%	87.94%	89.38%
Mill Creek (Grant County, West Virginia)	18.95%	19.61%	13.76%	7.30%	7.10%	7.24%	73.73%	73.22%	78.96%
Mill Creek (Hampshire County, West Virginia)	16.66%	15.85%	10.87%	10.41%	10.43%	10.40%	72.91%	73.69%	78.60%
Potomac Mainstem Below Antietam Creek near Sharpsburg, Maryland	19.65%	19.66%	16.69%	8.71%	8.84%	8.86%	69.36%	68.56%	71.42%
North Fork Shenandoah River at Strasburg, Virginia	32.06%	32.56%	30.04%	7.15%	7.12%	7.11%	59.31%	58.05%	60.44%
South Fork Shenandoah River at Front Royal, Virginia	31.58%	32.37%	30.15%	10.70%	10.62%	10.69%	55.27%	53.99%	56.01%
Shenandoah River Mainstem near Millville, West Virginia	30.02%	30.57%	28.24%	9.25%	9.22%	9.25%	58.10%	56.98%	59.16%