

Cyanobacterial Toxins and Liver Cancer

BY

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world today. Over the past decade, the incidence of HCC in Canada has been increasing despite efforts to mitigate disease causing factors such as viral hepatitis B and C infection. Fresh water cyanobacteria are known to release potent hepatotoxins, such as microcystin-LR (MCLR), into drinking and recreational water sources, which have been associated with liver toxicity and HCC. Despite government efforts to limit MCLR contamination, chronic low-dose exposure continues to occur.

The purpose of this thesis was to gain an understanding of the potential relationship between chronic low-dose MCLR exposure and HCC. The research contains five areas of investigation; 1) *In vitro* effects of low-dose MCLR exposure on various hepatocellular cell types, 2) *in vitro* effects of low-dose MCLR exposure on hepatic stellate cells and fibrosis, 3) *in vivo* effects of chronic low-dose MCLR exposure on HCC initiation and promotion, 4) *in vivo* effects of chronic low-dose MCLR exposure on hepatic fibrosis and 5) national and provincial epidemiological analysis investigating the potential association between cyanobacterial hepatotoxins and liver cancer. Our methodology involved; 1) exposing WB-F344, PLC and CFSC-2G hepatic cell lines to varying concentrations of MCLR and observing changes in cell proliferation, protein expression, wound healing and malignant transformation, 2) continuously exposing adult male mice to 1 µg/L of MCLR in their drinking water for seven months, and observing changes in body weight, liver histology, liver enzymes, hepatic fibrosis and HCC development, 3) conducting a national HCC epidemiological study, and determining the relative risk of developing HCC from up-stream

factors contributing to cyanobacterial contamination of drinking water sources and 4) conducting a provincial epidemiological study that involved collecting liver cancer incidence data from the Manitoba cancer registry from 1985 to 2007, and temporally and spatially analyzing the association between cancer incidence and MCLR measured in Manitoba lakes.

The *in vitro* results indicate that chronic low-dose exposure to MCLR does not significantly induce cellular characteristics associated with a malignant and/or pro-fibrotic phenotype. *In vivo*, there were no significant changes in liver pathology, plasma chemistry, histology or tumor development compared to negative controls. Epidemiological analyses conducted at the national and provincial levels revealed similar trends. HCC incidence in Canada was associated with urban residence, immigration and hepatitis B. However, HCC incidence was not found to be influenced by the up-stream cyanobacterial predictive factors; agriculture, cattle and swine density. In Manitoba, the increasing incidence of liver cancer did not temporally or spatially associate with MCLR contamination sites within the province.

In conclusion, these results are reassuring in that they suggest that chronic low-dose exposure to MCLR does not initiate or promote the development of HCC at the cellular, systemic or epidemiological levels.

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Abbreviations

3MC: 3-methyl cholanthrene
Adam17 (Cd156b): a disintegrin and metallopeptidase domain 17
Akt1: thymoma viral proto-oncogene 1
Angpt2: angiopoietin 2
AR: Androgen receptor
Bax: BCL2-associated X protein
Bcl2: B cell leukemia/lymphoma 2
Bcl2l1 (Bcl-x): BCL2-like 1
Bid: BH3 Interacting Domain Death Agonist
Birc2 (c-IAP2): baculoviral IAP repeat-containing 2
Birc5: baculoviral IAP repeat-containing 5
BW: Body weight
Casp8 (Flice): caspase 8
Ccl5 (Rantes): chemokine (C-C motif) ligand 5
Ccnd1: cyclin D1
Ccnd2: cyclin D2
CD: Census division
Cdh1 (E-Cadherin): cadherin 1
Cdh13: cadherin 13
Cdkn1a (p21Cip1/Waf1): cyclin-dependent kinase inhibitor 1A (P21)
Cdkn2a (p16Ink4): cyclin-dependent kinase inhibitor 2A
Cflar (Casper): CASP8 and FADD-like apoptosis regulator
CFSC-2G: Human hepatic stellate cell line
Ctnnb1: catenin (cadherin associated protein), beta 1
CSC: Cancer stem cell
Cxcr4: chemokine (C-X-C motif) receptor 4
Dab2ip: disabled 2 interacting protein
DEN: Diethylnitrosamine
Dlc1: deleted in liver cancer 1
DMEM: Dubelco's modified eagles medium
DNA: Deoxyribonucleic acid
ECM: Extracellular matrix
EGF: Epidermal growth factor
Egfr: epithelial growth factor receptor
ELISA: Enzyme linked immune-assay
EpCAM: Epithelial cell adherence molecule
Ep300: E1A binding protein p300
ERK: Extracellular signal-Regulated Kinase
Fadd: Fas (TNFRSF6)-associated via death domain
Fas (Tnfrsf6): Fas (TNFRSF6) binding factor 1
Fhit: fragile histidine triad gene
Flt1: FMS-like tyrosine kinase 1
Fzd7: frizzled homolog 7

Gadd45b: growth arrest and DNA-damage-inducible 45 beta
GIS: Geographical information system
GSH: Glutathione
GST: Glutathione-s-transferase
Gstp1: glutathione S-transferase, pi 1
H2DCF-DA: Dichlorofluorecein diacetate
HBsAg: Hepatitis B virus surface antigen
HBV: Hepatitis B virus
HBVx: Hepatitis B virus X gene
HCC: Hepatocellular carcinoma
HCV: Hepatitis C virus
Hepes buffer: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer
Hgf: hepatocyte growth factor
Hhip: Hedgehog-interacting protein
Hras1: Harvey rat sarcoma virus oncogene 1
Igf2: insulin-like growth factor 2
Igfbp1: insulin-like growth factor binding protein 1
Igfbp3: insulin-like growth factor binding protein 3
IL-1: Interleukin-1
Itgb1: integrin beta 1 (fibronectin receptor beta)
Irs1: insulin receptor substrate 1
i.p: intraperitoneal injection
JNK: c-Jun NH2-terminal Kinases
Kdr: kinase insert domain protein receptor
L: liter
Lef1: lymphoid enhancer binding factor 1
LW: Liver weight
M: Molar
MAPK: mitogen activated protein kinase pathway
MC: Microcystin
MCLR: Microcystin-LR
Mcl1: macrophage stimulating 1 receptor
MEK: MAP/ERK Kinase
Met: met proto-oncogene
mg: milligram
ml: milliliter
mM: millimolar
Msh2: mutS homolog 2 (E. coli)
Msh3: mutS homolog 3 (E. coli)
Mtdh: metadherin
Myc: V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
N: Nitrogen
NAC: n-acetyl-L-cystein
Nfkb1: nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105
ng: nanogram

Nras: neuroblastoma ras oncogene
OA: Okadaic acid
OATP: Organic anion transporter protein
Opcml: opioid binding protein/cell adhesion molecule-like
P: Phosphorous
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PDGF: platelet derived growth factor
Pdgfra: platelet derived growth factor receptor, alpha polypeptide
Pin1: protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1
PLC/PRF/5: Human hepatoma cell line
PP1: Serine/threonine phosphatase inhibitor 1
PP2A: Serine/threonine phosphatase inhibitor 2A
Pten: phosphatase and tensin homolog
Ptk2: PTK2 protein tyrosine kinase 2,
Ptgs2 (Cox2): prostaglandin-endoperoxide synthase 2
Pycard (Asc): PYD and CARD domain containing
Rac1: RAS-related C3 botulinum substrate 1
Rassf1: Ras association (RalGDS/AF-6) domain family member 1
Rb1: retinoblastoma 1
Reln: reln gene
RHA: Regional Health Authority
Rhoa: ras homolog gene family, member A
RIPA: Radioimmunoprecipitation assay
RNA: Ribonucleic acid
Rpm: revolutions per minute
ROS: Reactive oxygen species
RR: Relative risk
Runx3: runt related transcription factor 3
SDS: Sodium dodecyl sulfate
Sfrp2: secreted frizzled-related protein 2
SMAA: Smooth muscle alpha actin
Smad7: SMAD homolog 7
Smad4: SMAD family member 4
Socs1: suppressor of cytokine signaling 1
Socs3: suppressor of cytokine signaling 3
Stat3: signal transducer and activator of transcription 3
TASO: Thioacetamide-S-oxide
Tcf4: transcription factor 4
Tert: telomerase reverse transcriptase
Tgfa: transforming growth factor alpha
TGF-β: Transforming growth factor beta
Tgfb1: transforming growth factor, beta 1
Tgfb2: transforming growth factor, beta receptor II
Tlr4: toll-like receptor 4

TNF- α : Tumor necrosis factor alpha
TNF- β : Tumor necrosis factor beta
Tnfrsf10b (Dr5): tumor necrosis factor receptor superfamily, member 10b
Tnfsf10: tumor necrosis factor (ligand) superfamily, member 10
TPA: 12-O-Tetradecanoylphorbol-13-acetate
TAA: Thioacetamide
Tris: (hydroxymethyl) aminomethane
Trp53: transformation related protein 53
Vegfa: vascular endothelial growth factor A
WB-F344: Rat hepatic stem cell line
WST-1: tetrazolium salt WST-1[2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium]
Wt1: Wilms tumor 1 homolog
Xiap: X-linked inhibitor of apoptosis
Yap1: yes-associated protein 1
 μ g: microgram
 μ g/L: microgram per litre
 μ l: microliter

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer related death in the world today. HCC tends to occur in chronically inflamed and fibrotic livers resulting from chronic hepatitis B (HBV) or hepatitis C (HCV) infections, alcoholism, fatty liver or exposure to environmental toxins such as aflatoxin. Within Canada, despite new and effective treatments for HBV and HCV, the incidence of HCC has been progressively increasing over the last two decades (Dyer, Peltekian et al. 2005; Canadian Cancer Society's Steering Committee on Cancer Statistics 2012): While the epidemic of obesity and fatty liver can account for some of the increase, whether environmental contaminants also contribute to increasing disease prevalence remains to be determined.

Cyanobacteria are ubiquitous to all aquatic environments, contributing to a balanced ecosystem. Over the past two decades growth of cyanobacteria within fresh water sources has been increasing in severity within Canada and globally (Carmichael 2008). Waterbody eutrophication, driven primarily by agriculture activities, urbanization and industrial contaminants, promotes cyanobacterial bloom (cyanobloom) formation (Piehler 2008). Cyanobacteria can release toxic secondary metabolites known as cyanotoxins into the environment, posing a serious threat to drinking water quality and human health (Carmichael 1992). Cyanotoxins, particularly hepatotoxins, are commonly found within the environment, toxic and can accumulate up the food chain (Christoffersen 1996; Harada and Tsuji 1998). To limit oral exposure, the World Health Organization (WHO) issued a safe allowable limit of 1 µg/L applicable for all cyanobacteria hepatotoxins (WHO 1998; WHO 2003).

Microcystins (MCs) are a class of toxic secondary metabolites produced predominantly by fresh water cyanobacteria. MCs are potent hepatotoxins, able to cause liver damage and promote tumor development through oxidative stress, serine/threonine phosphatase inhibition and genotoxicity (Guzman, Solter et al. 2003; Botha, Gehringer et al. 2004; Bouaicha and Maatouk 2004; Gaudin, Le Hegarat et al. 2009). There are over 80 known variants of MCs, microcystin-LR (MCLR) being one of the most potent variants identified (Campos and Vasconcelos 2010).

Acute or chronic exposure to high concentrations of MCLR is associated with a variety of adverse health effects including; nausea, vomiting, gastroenteritis and severe hemorrhagic hepatitis (Duy, Lam et al. 2000; Carmichael 2001). However the role chronic low-dose exposure to MCs plays in the course of chronic liver disease is unclear.

Epidemiological Studies in China, Serbia and United States reported increased incidence of HCC in regions chronically exposed to cyanobacterial contaminated drinking water (Yu, Zhao et al. 2001; Fleming, Rivero et al. 2002; Burns 2008; Svircev, Krstic et al. 2009). In China, populations endemic with hepatitis B virus and exposed to aflatoxin in food, had an increased incidence of primary liver cancer (PLC) in regions deriving their drinking water from cyanobacterial contaminated sources; pond and ditch water, compared to similar populations deriving their drinking water from cyanobacterial-free sources; deep wells (Harada, Oshikata et al. 1996; Ueno, Nagata et al. 1996; Yu, Zhao et al. 2001). Similar findings were observed in Central Serbia and United States (Florida), whereby PLC incidence was significantly increased in regions testing positive for recurrent

cyanobacterial blooms in drinking water reservoirs (Fleming, Rivero et al. 2002; Svircev, Krstic et al. 2009).

Within Canada, a national cyanotoxin monitoring program does not currently exist. In provinces where cyanobloom formation is recurrent, cyanobacterial monitoring and water treatment programs are in effect for rural and urban centers, to minimize high-dose exposure to cyanotoxins for urban centers. However, it is unknown to what extent rural and remote locations monitor for cyanobacteria.

Despite treatment of drinking water, low concentrations of MCs may be present. Therefore investigation into the health effects associated with chronic low-dose exposure on the liver is required at the cellular, animal and epidemiological levels within Canada, investigating the long-term effects on chronic liver disease development.

2. Rationale and Hypothesis

Within Canada the incidence rates of hepatocellular carcinoma (HCC) have been significantly increasing over the past two decades. The underlying factors promoting increased HCC incidence are unknown, however environmental contaminants such as MCLR may be contributing. At high concentrations, MCLR is characterized as a potent hepatotoxin and tumor promoter (Duy, Lam et al. 2000). However it remains to be determined to what extent, low concentrations of MCLR impact on HCC development.

In vitro studies: At the cellular level, MCLR has been observed to mediate the malignant transformation of immortalized human colorectal crypt cells and WRL-68 cells following chronic low-dose exposure (Xu, Qin et al. 2012). However, there is limited evidence on the impact of MCLR on hepatic stem cells, well differentiated hepatic tumor cells and hepatic stellate cells. **We hypothesize that a low concentration ($> 1 \mu\text{L}$) of MCLR will induce malignant transformation of immortalized hepatic stem cells (WB-F344 hepatic stem cells), enhance the growth of well differentiated hepatic tumor cells (PLC/PRC/5 hepatoma cells) and/or contribute to a tumor promoting environment, by activating hepatic stellate cells (CFSC-2G hepatic stellate cells) to acquire a pro-fibrogenic phenotype.**

In vivo studies: At the systemic level, the tumor promoting ability of MCLR has been observed under a variety of conditions. Tumor formation was observed following intraperitoneal (i.p) injections of MCLR at concentrations of 10 and 25 $\mu\text{g/kg}$ body weight in rats over an 8-10 week period, following chemical initiation with diethylnitrosamine (DEN) or in conjunction with partial hepatectomy (Nishiwaki-Matsushima, Ohta et al. 1992;

Ohta, Sueoka et al. 1994). It has yet to be determined whether chronic exposure to low-dose MCLR alone in drinking water initiates or promotes HCC development. **Therefore, we hypothesize that chronic low-dose (1 µg/L) exposure to MCLR in drinking water will initiate or promote HCC in mice over a 7 month period. We further predict that mice exposed to both MCLR and co-carcinogen, thioacetamide, will result in a greater number and size of tumors in treated animals.**

Fibrogenesis is the process of wound healing and repair in the liver, initiated in response to hepatic injury and inflammation. Prolonged hepatic injury deregulates this process, resulting in deposition of extracellular matrix proteins and fibrosis. Prolonged fibrosis cultivates a loss of liver function to establish cirrhosis. One of the primary risk factors for HCC development is cirrhosis (Guo and Friedman 2007; Okuda 2007). Therefore, understanding the role MCLR plays in the initiation and promotion of fibrogenesis is critical, when investigating possible mechanisms involved in HCC pathogenesis. To date few studies have investigated the impact of chronic low-dose MCLR exposure on initiation and progression of hepatic fibrosis. **We hypothesize that mice chronically exposed to MCLR alone and in conjunction with thioacetamide, will present with a more advanced stage of fibrosis/cirrhosis, compared to water alone and thioacetamide alone controls.**

Epidemiological studies: Studies from China, Serbia and United States observed higher HCC incidence in regions exposed to cyanobacterial contaminated drinking water (Yu, Chen et al. 1995; Ueno, Nagata et al. 1996; Yu, Chen et al. 1998; Fleming, Rivero et al. 2002; Svircev, Krstic et al. 2009). In Canada, exposure to high concentrations (> 1 µg/L) of

MCLR in drinking water is rare, particularly in urban areas. However, the potential exists for the population to be exposed to low concentrations ($\leq 1 \mu\text{g/L}$) of MCLR over time, particularly in rural or remote communities lacking consistent cyanobacterial monitoring programs. **We hypothesize that nationally, areas with suspected cyanobacterial contamination (measured through up-stream predictive markers), will have a higher incidence of HCC, particularly in rural areas. We further predict that this trend will be observed at the provincial level in Manitoba where data on MCLR concentrations exist. Here, areas in close proximity to MCLR contamination sites, will have a higher incidence of HCC.**

3. Objectives

3.1. *In Vitro* Studies

The *in vitro* study involved the use of three distinct cell lines; WB-F344, PLC/PRF/5 and CFSC-2G cells, each utilized to investigate the effect of MCLR on different aspects of HCC development. WB-F344 cells are non-malignant immortalized hepatic stem cells that were used to investigate the ability of low-doses of MCLR to induce malignant transformation of hepatic stem cells. PLC/PRF/5 cells are human hepatoma cells, which were used to investigate the ability of low-dose MCLR to promote cancer cell growth characteristics. CFSC-2G cells are inactive hepatic stellate cells, which were used to investigate the ability of low-dose MCLR to activate hepatic stellate cells and promote fibrogenesis. The specific objectives for studies involving each cell line are listed below.

3.1.1. *Malignant Transformation of Non-Malignant Hepatic Stem Cells*

- 1) Determine whether low-concentrations of MCLR increase the level of oxidative stress and serine/threonine phosphatase 2A inhibition in WB-F344 hepatic stem cells.
- 2) Determine whether low-concentrations of MCLR exposure alters the proliferative activity of WB-F344 hepatic stem cells.
- 3) Determine whether low-concentrations of MCLR exposure induces malignant transformation of WB-F344 hepatic stem cells.
- 4) Measure protein expression of HCC biomarkers in WB-F344 hepatic stem cells chronically exposed to low-concentrations of MCLR.

3.1.2. *Enhancement of Malignant Hepatocyte Growth*

- 1) Determine whether low-concentrations of MCLR increases the level of oxidative stress and serine/threonine phosphatase 2A inhibition in PLC hepatoma cells.
- 2) Determine whether low-concentrations of MCLR alters the proliferative activity of PLC hepatoma cells.
- 3) Determine whether low-concentrations of MCLR alters the invasiveness of PLC hepatoma cells.

3.1.3. *Fibrogenesis*

- 1) Determine whether low-concentrations of MCLR increases the level of oxidative stress and serine/threonine phosphatase 2A inhibition in CFSC-2G hepatic stellate cells.
- 2) Determine whether low-concentrations of MCLR exposure alters the proliferative activity of CFSC-2G hepatic stellate cells.
- 3) Determine whether low-concentrations of MCLR exposure alters the expression of the hepatic stellate cell activation marker, smooth muscle alpha actin.

3.2. *In Vivo Studies*

The *in vivo* study investigated whether chronic low-dose exposure to the allowable limit of MCLR (1 µg/L) in drinking water could 1) initiate the development of HCC, 2) promote the growth of existing HCC development and 3) initiate fibrogenesis in CD1 male adult mice following 7 months exposure. Thioacetamide (TAA) (carcinogen) was administered within drinking water alone and in conjunction with MCLR as the positive control.

3.2.1. Tumor Initiation

- 1) Quantify the number of liver tumors in mice chronically exposed to low-dose MCLR alone in drinking water for 7 months versus water alone.
- 2) Measure changes in animal weight, liver weight and percent liver weight to body weight in mice chronically exposed to low-dose MCLR alone in drinking water for 7 months.
- 3) Measure liver biochemistry; alanine transaminase (ALT) and total bilirubin, in plasma isolated from mice chronically exposed to low-dose MCLR alone in drinking water for 7 months versus water alone.
- 4) Histologically assess for changes in liver architecture, inflammation and tumor development in mice chronically exposed to low-dose MCLR alone in drinking water for 7 months compared to water alone.

3.2.2. Tumor Promotion

- 1) Quantify the size of liver tumors in TAA & MCLR treated mice compared to controls (water alone, MCLR alone and TAA alone).
- 2) Measure changes in animal weight, liver weight and percent liver weight to body weight in TAA & MCLR treated mice compared to TAA alone controls.
- 5) Measure liver biochemistry; alanine transaminase (ALT) and total bilirubin, in plasma isolated from TAA & MCLR treated mice compared to controls.
- 6) Histologically assess for changes in liver architecture and inflammation in TAA & MCLR treated mice compared to controls.

3.2.3. Fibrogenesis

- 1) Measure liver biochemistry; alanine transaminase (ALT) and total bilirubin, in plasma isolated from MCLR alone and TAA & MCLR treated mice compared to water and TAA alone controls.
- 2) Histologically assess for changes in liver architecture, inflammation and fibrosis in MCLR alone and TAA & MCLR treated mice compared to water and TAA alone controls.

3.3. Epidemiological Studies

Two epidemiological studies were conducted to further evaluate the association between low-dose exposure to MCLR and HCC development. The Canadian epidemiological study was conducted first in order to gain a national perspective on HCC incidence, risk factors and surrogate markers for cyanobacterial contamination between 1996 to 2004. That study was followed up by a provincial epidemiological study that investigated the association between HCC incidence and MCLR contaminated fresh water sources within Manitoba between 1985 to 2007.

3.3.1. National Epidemiological Study

- 1) Determine whether a geographical association exists between HCC incidence, socio-demographic HCC predictors and surrogate markers of cyanobacterial contamination in Canada between 1996 and 2004.
- 2) Determine the relative risk of developing HCC based on proximity to surrogate markers for cyanobacterial contamination.
- 3) Determine the relative risk of developing HCC for rural and urban locations in Canada between 1996 and 2004.

- 4) Determine the geographic distribution of HCC incidence across Canada between 1996 and 2004.

3.3.2. Provincial Epidemiological Study

- 1) Characterize the longitudinal and spatial distribution of liver cancer incidence in Manitoba between 1985 to 2007.
- 2) Characterize the longitudinal and spatial distribution of cyanobacterial toxins in fresh water sources in Manitoba between 1985 to 2007.
- 3) Determine whether an association exists between liver cancer incidence and cyanobacterial contaminated fresh water sources in Manitoba between 1985 to 2007.
- 4) Determine the relative risk of developing HCC based on proximity to MCLR contaminated fresh water sources.

4. Literature Review

4.1. *Chronic Liver Disease*

Chronic liver disease (CLD) (a common precursor to HCC) is characterized by chronic inflammation, regeneration and fibrosis, ultimately leading to liver failure and often hepatocellular carcinoma (HCC) (McCaughan, Gorrell et al. 2000). A key step in the progression of HCC is the development of extensive fibrosis or cirrhosis. Fibrogenesis is mediated by hepatic stellate cells (HSCs), a population of fibroblast cells located within the perisinusoidal space of the liver. In healthy liver tissue, HSCs typically remain quiescent, until activated by chronic liver injury and inflammation, causing them to differentiate into proliferative and secretory myo-fibroblasts (express smooth muscle alpha actin protein (SMAA)) (Sato, Suzuki et al. 2003; Parsons, Takashima et al. 2007).

Endogenous mediators of HSC activation include a variety of factors such as; alterations to extracellular matrix (ECM) composition (Type IV collagen, fibrinogen, and urokinase type-1 plasminogen activator), hepatocyte apoptosis, recruitment of inflammatory cells (lymphocytes or polymorphonuclear cells), release of inflammatory cytokines (tumor growth factor-beta1: TGF- β 1 and tissue inhibitor of metalloproteinase – 1: TIMP 1 and 2) and reactive oxygen species (ROS) by Kupffer cells and activated HSCs. TGF- β 1 in particular, is a key mediator of fibrogenesis, functioning to promote HSC proliferation and transdifferentiation into ECM synthesizing myofibroblasts (Li and Friedman 1999; Li, Liao et al. 2008).

Activated HSCs accumulate at sites of tissue damage and mediate the dynamic process of ECM remodeling. The hallmark of fibrogenesis is the deposition of ECM protein, type-1 collagen, which replaces hepatocytes to form fibrous scar tissue. Progression of fibrosis and deposition of scar tissue eventually distorts hepatic architecture, leading to the formation of regenerative nodules, which defines hepatic cirrhosis (Moreira 2007; Parsons, Takashima et al. 2007). Histologically, cirrhosis is characterized by the conversion of normal liver architecture into regenerative nodules encompassed by fibrous tissue, leading to portal hypertension and often HCC. The risk factors for cirrhosis have a consistent theme; they all induce a state of chronic inflammation and tissue damage of the liver and the etiologies include; chronic HBV and HCV infections, alcohol abuse, obesity, heavy metals (iron and copper), immunologic, genetic factors and toxins (Bruix, Boix et al. 2004; Schuppan and Afdhal 2008).

The pathological features of cirrhosis involve the replacement of normal parenchyma with fibrous scar tissue, portal hypertension and hepatic dysfunction. Once cirrhosis has been established, the liver progresses through preneoplastic (5-10 years) and dysplastic stages (3-5 years) prior to developing HCC. Hepatic preneoplasia is a stage of cirrhosis, whereby hepatic stem cells and hepatocytes display an altered phenotype and gene expression profile (Llovet, Burroughs et al. 2003; Okuda 2007). During this stage, transforming growth factor alpha (TGF- α) and insulin-like growth factor-2 (IGF-2) are up-regulated, stimulating stem cell and hepatocyte proliferation which leads to hepatic dysplasia (Tsai, Yauk et al. 1988; Scharf, Schmidt-Sandte et al. 1998; Hufnagl, Parzefall et al. 2001). Hepatic dysplasia marks the proliferative phase of cirrhosis. Structural gene changes (amplifications, mutations, deletions) that developed slowly during the preneoplastic stage,

now accumulate more quickly. The formation of dysplastic nodules provides the foundation for altered stem cells and hepatocytes to evolve into a malignant phenotype. HCC develops in only a fraction of dysplastic nodules, requiring multiple mutations/alterations of genes and chromosomes (Farazi and DePinho 2006; Schuppan and Afdhal 2008).

4.1.1. Cancer Stem Cells

Stem cells (SCs) are defined as progenitor cells, capable of self-renewal and differentiation into specialized tissue types. There are two general types of stem cells; embryonic and adult. Embryonic stem cells are pluripotent cells, derived from an early-stage embryo (blastocyst). Adult stem cells, otherwise known as somatic stem cells, are undifferentiated cells found in small amounts within all developed tissue throughout the body. In mature tissue, adult stem cells undergo self-renewal to regenerate damaged tissue, producing new cells from the organ of origin (Reya, Morrison et al. 2001). In some instances, the organ can undergo almost complete regeneration. For example, the liver is a unique organ that possesses the ability to regenerate following injury. In rodents who have undergone partial hepatectomy to remove two-thirds liver mass, the liver regenerates to its original mass in approximately 10 days (Michalopoulos and DeFrances 1997; Fausto 2004).

The cancer stem cell (CSC) model arose over 40 years ago, due to the presence of CSCs in various solid tumor types such as liver, colon, breast, prostate, melanoma, brain, pancreatic, head and neck cancer (Yao and Mishra 2009). It was proposed that cancer arose from tissue specific stem cells that had undergone deregulation of self-renewal pathways (Till and Mc 1961; Wicha, Liu et al. 2006). According to the CSC model, a tumor

is comprised of a heterogeneous cell population, consisting of a small group of self-renewing CSCs which give rise to a second larger group of non-tumorigenic and partially differentiated cancer cells, forming the bulk of the tumor. Under the CSC model, it is thought that only the CSC population possesses the characteristics required for initiation and maintenance of the tumor (Klonisch, Wiechec et al. 2008). It is further postulated that because stem cells are the only long-lived cell type within any tissue type and they undergo self renewal, they are the best candidate for accumulation of carcinogenic mutations (Dalerba, Cho et al. 2007).

There are three potential sources of stem like cells that the liver utilizes for regenerative purposes; de-differentiated hepatocytes, progenitor cells and bone marrow cells (BMCs). In response to CLD and fibrosis, intrahepatic (de-differentiated hepatocytes or progenitor cells) or extrahepatic (BMCs) hepatocytes are susceptible to genomic mutations. Mutated stem cells give rise to a pool of self-renewing cells, which do not enter the post-mitotic state, and are able to accumulate further mutations. Tumor formation has been observed in these highly purified liver stem cells following activation of Wnt/ β -catenin and B lymphoma Mo-MLV insertion region 1 homolog (BMI-1) signaling pathways; two major self-renewal regulators of stem cells (Wei, Fabre et al. 2000; Wong, Fan et al. 2001; Cui, Zhou et al. 2003; Park, Qian et al. 2003).

As HCC progresses, cancer cells are able to invade tissue and metastasize to other areas of the body; lungs, adrenal glands, lymph nodes, bones and to a lesser extent the central nervous system. Metastatic HCC has a poor prognosis (Kummar and Shafi 2003; Bruix, Boix et al. 2004). The five year survival rate of HCC is approximately 10% due in part to the

delays in diagnosis, the extent of the underlying liver disease which limits the use of chemotherapeutic agents and resistance to anti-cancer therapies (Hertl and Cosimi 2005).

4.2. *Hepatocellular Carcinoma*

There are several subtypes of primary liver cancers (PLCs) including HCC, hepatoblastoma, intrahepatic cholangiocarcinoma (bile duct carcinoma), hemangioepithelioma, angiosarcoma, and others. HCC accounts for approximately 85-90% of all PLCs (El-Serag and Rudolph 2007). The worldwide incidence of HCC is roughly 500 000 to 1 million new cases per year, with the highest incidence being in China, Africa and Japan (Venook, Papandreou et al. 2010).

HCC related mortality accounts for approximately 600,000 deaths annually, making HCC the third leading cause of cancer related death worldwide, and the fifth most common cancer (Venook, Papandreou et al. 2010). Eastern and southeastern Asia and central and eastern Africa have the highest HCC age-standardized incidence rates. According to the GLOBOCAN database, 2002 statistics indicate that China alone accounts for 55%, Europe ~9% and North America 5% of HCC within the world (Parkin, Bray et al. 2005).

In Canada, an evaluation of the long-term trends in mortality rates for liver cancer ranging from 1970 to 2007, revealed that age-standardized mortality rates/100,000 have increased from 1.8 to 3.1 cases/100,000 for males (fluctuating by 1.6%/year) and 0.7 to 0.85 cases/100,000 for females (fluctuating by 0.4%/year) (Kachuri, Ellison et al. 2013). Across Canada HCC incidence varies from coast to coast with the highest rates being observed in Southern Quebec, British Columbia, Alberta and Ontario. The sex-difference

and unique distribution of the disease may be explained in part by geographic exposure to risk factors, primarily HBV or HCV chronic infection (elSaadany, Tepper et al. 2002; El-Serag and Rudolph 2007).

4.3. *Cyanobacteria*

Blue-green algae otherwise known as cyanobacteria are a group of photoautotrophic bacteria that are a natural component of most illuminated environments; aquatic and terrestrial. Cyanobacteria are an essential component of many ecosystems. Due to their ability to fix nitrogen, produce carbon and generate oxygen, cyanobacteria play a vital role in the earth's nitrogen, carbon and oxygen cycles (Whitton. B.A. and M. 2012).

The existence of cyanobacteria dates back to over 3.5 billion years, having the distinction of being the most wide spread, genetically diverse and oldest photosynthetic organisms on earth. Cyanobacteria are thought to have changed the course of life on this planet, by converting the early reducing atmosphere into an oxygen rich environment, thereby stimulating biodiversity and selecting for aerobic metabolism and eukaryotic photosynthesis (Dutkiewicz, Volk et al. 2006; Sessions, Doughty et al. 2009).

Certain species of cyanobacteria are toxigenic, capable of synthesizing and releasing toxic secondary metabolites (cyanotoxins) into the environment. Toxin production varies between cyanobacterial strains of the same species, and can undergo spatial and temporal variance within the same bloom. Toxins are stored within the cell until released into the environment due to cell lysis and death or passively in response to stimuli such as; predation, nutrient levels, cell to cell communication, (Oh, Lee et al. 2000; Vézic, Rapala et

al. 2002; Ross, Santiago-Vázquez et al. 2006; Wang, Qin et al. 2010). Passive release of MCs into the environment is facilitated by the MycH exporter, which is a member of the ATP binding cassette (ABC) transporter superfamily (Tillett, Dittmann et al. 2000; Pearson, Hisbergues et al. 2004). It is proposed that the MycH exporter theoretically binds to the ADDA region of MCs to facilitate active transport of the compound to the extracellular environment (Saurin, Hofnung et al. 1999; Pearson, Hisbergues et al. 2004).

The production and passive release of toxins is also influenced by cellular circadian rhythms, regulated by a 24 hr light: dark cycle (Cordeiro-Araújo and Bittencourt-Oliveira 2013). Enhanced light intensity increases MC biosynthesis through transcription of mycB and mycD genes, within a large multifunctional enzyme complex (Tillett, Dittmann et al. 2000; Wiedner, Visser et al. 2003). Under low and medium light intensities, MC production is continuous, and is exported in response to threshold light intensities (Kaebernick, Neilan et al. 2000; Pearson, Hisbergues et al. 2004).

The presence of toxigenic cyanobacteria within freshwater sources negatively impacts water quality and biodiversity (WHO 1998; WHO 2003). Common toxigenic cyanobacteria species that dominate fresh water sources include but are not limited to; *Microcystis*, *Oscillatoria*, *Schizothrix*, *Nostoc*, *Aphanizomenon*, *Anabeana*, *Synechocystis*, *Cylindrospermopsis* and *Planktothrix* (WHO 2003).

4.3.1. Cyanobacterial Blooms

Excessive proliferation of cyanobacteria leads to the formation of cyanobacterial blooms (cyanoblooms), which is a high biomass aggregate of cyanobacteria. Blooms present on the water's surface are referred to as surface scum or surface blooms. Toxigenic

cyanobacterial species typically form blooms: *Microcystis* is commonly associated with bloom formation (Havens 2008). Toxin concentrations can be highest during bloom formation due to the high number of toxin producing cyanobacterial cells present, however, cyanobacterial cell density is not always an accurate indicator of toxin load (Sinclair and Hall 2008). Bloom formation can have far-reaching impacts on ecosystem health causing a variety of problems including; sunlight restriction to deeper layers of water column, depletion of bioavailable oxygen (hypoxia/anoxia), toxin release, decreased recreational value of water body and contamination of drinking water sources. Hypoxic/Anoxic conditions can further alter the benthic and bacterial community, changing the overall landscape of aquatic habitats (Havens 2008; Sinclair and Hall 2008).

Conditions that select for cyanobacterial growth include; eutrophication (elevated nitrogen and phosphorous), sunlight, elevated pH, low turbulence and increasing temperatures (Sinclair and Hall 2008). Bloom formation can be sporadic, however in many instances blooms develop during the warmest summer months (Havens 2008).

4.3.2. Cyanobacterial Blooms in North America

Over the past decade, the presence of toxigenic blooms in fresh water sources has increased across North America (Stone and Bress 2007; Carmichael 2008). Water basins with recorded bloom formation generally remain problematic for many years until the underlying cause of bloom formation resolves. Within the United States, persistent toxigenic blooms have been occurring in many states, most noticeably Florida, Nebraska and New York (Boyer 2008; Burns 2008; Carmichael 2008; Walker, Lund et al. 2008).

Provinces reporting persistent blooms include; Manitoba, Alberta, Ontario and Quebec (Orihel, Bird et al. 2012). Lake Winnipeg in Manitoba is the worlds' 10th largest fresh water lake. The lake has an abundant array of aquatic life and plays a critical role in Manitoba's tourism, commercial fishing, and hydroelectric generation industries. Due to eutrophication from agricultural, industrial and residential activity, the lake has become contaminated with nitrogen fixing cyanobacteria since the 1990's: limiting its use for commercial and recreational activities. In 2011, new standards were announced to reduce nutrient loading in municipal and industrial waste water in Manitoba, with a focus to restore the health of Lake Winnipeg (Bunting 2011; Manitoba 2011).

The appearance of toxigenic *Microcystis* and *Planktothrix* blooms has been reported since 1995 in spatially distinct regions around Lake Erie in Ontario. It was recognized in the early 1960's that eutrophication from human activity was changing the lakes' ecological profile. As a result, The Great Lakes Water Quality Agreement was signed in 1972 with the intention of reducing nutrient loading in the Laurentian Great Lakes. Although eutrophication was reduced by 60% and bloom formation decreased, recurrence of toxigenic blooms was observed the 1990s and continues today (Rinta-Kanto and Wilhelm 2006; Boyer 2008; US Environmental Protection Agency 2009).

Lake Champlain is a large fresh water lake that extends between the province of Quebec and the states of Vermont and New York. Since 2000, reports of toxigenic cyanoblooms have been observed in the shallow bays of the lake. Weekly monitoring programs such as the Lake Champlain Committee and The Lake Champlain Basin Program have been put into

effect, to routinely monitor for changes in cyanobacterial activity and cyanotoxin deposition (Ballinger 2011; Lake Champlain Committee 2011).

A recent study conducted by Orihel et al 2012, documented the presence of MCLR within 246 Canadian lakes from 2001 to 2011. MCLR concentrations were reported high, above the potable water and recreational guideline values for MCLR, in numerous eutrophic lakes with low nitrogen to phosphorous ratios (N:P). The lowest concentrations of MCLR were observed under conditions of high N:P, whereas maximum MCLR concentrations were prevalent in hypereutrophic lakes (Orihel, Bird et al. 2012).

In Canada, cyanobacteria and MCs are inconsistently monitored across the country, particularly in rural and remote communities. Water monitoring programs are carried out at the federal, provincial and municipal levels. Since 2010, 328 water monitoring stations have been set up across Canada. Utilizing these stations, a long-term water quality monitoring program has been established, which measures physiochemical water parameters such as; pH, alkalinity, temperature, major ions, nutrients and metals. The program is administered through federal-provincial-territorial, interprovincial and international agreements, and monitors for parameters set out within the Canadian Water Act (which does not make reference to blue-green algae or cyanobacteria) (Environment Canada 2013).

4.4. Cyanobacterial Hepatotoxins

The three main classes of toxins produced by cyanobacteria are; hepatotoxins, dermatotoxins and neurotoxins (Carmichael 1992; Codd, Morrison et al. 2005). Hepatotoxins

are of primary interest, as they pose the greatest risk to human and animal health. Hepatotoxins are highly stable within the environment as they are water soluble and resistant to heat and chemical breakdown. Due to their toxicity, prevalence in the environment and ability to accumulate up the food chain, hepatotoxins are the main focus for water quality testing and human exposure research (Christoffersen 1996; Smith and Haney 2006).

There are 3 types of hepatotoxins produced by cyanobacteria; microcystins (MCs), nodularins (NODs) and cylindrospermopsin (CYN) (Carmichael, Beasley et al. 1988 (Carmichael, Beasley et al. 1988; Harada 2004). MCs are found largely in fresh water sources, and are the only cyanotoxin that is routinely monitored for in residential drinking water. Nodularins are found in brackish water, and therefore do not pose as great a risk to human health. Cylindrospermopsin originally was classified as more of a tropical toxin, however within recent years the organism producing the compound has migrated into more temperate climates (Sinclair and Hall 2008).

4.4.1. Food web accumulation and Impact

Within the environment, wildlife (aquatic and terrestrial animals) can be exposed to cyanotoxins through ingestion of cyanobacteria, contaminated food and water. MCs have been found to accumulate in zooplankton, muscles, crustaceans, marine biota, fish and vertebrates (Smith and Haney 2006). Invertebrates such as zooplankton and crab larvae which feed directly on cyanobacterial colonies act as vectors involved in transferring MC through the aquatic food web (Williams, Craig et al. 1997). In the laboratory setting, pumpkinseed sunfish exposed to zooplankton rich with MC (50 ng/kg/day) for a period of

9 days accumulated toxin within their liver and muscle tissues. Despite a two week depuration period, high MC levels were still present in these tissues (Smith and Haney 2006). In a similar study, Nile tilapias were fed MC for a period of 12 weeks to observe the effects of chronic dietary exposure on growth, feed utilization and toxin accumulation. Increasing concentrations of MC directly correlated with increased concentrations of toxin within muscle tissue: concentrations in excess of the WHO's recommended tolerable daily intake of 40 µg/kg/ body weight were recorded (Zhao 2006).

4.4.2. Drinking Water Guidelines

In response to the global cyanobacterial epidemic, the World Health Organization established a provisional safe allowable limit of 1 µg/L for hepatotoxins in drinking water, taking into account both free and bound toxin. The derived value was based on acute oral toxicity studies conducted in pigs to predict toxicity and tumorigenicity of MCs in humans. The study involved exposing growing pigs to extract containing MCs from *Microcystis aeruginosa* in drinking water for 44 days. Microcystin-YR (MCYR) was identified as the dominant compound within the extract, which contained a minimum of 7 MC variants. The daily oral exposure doses were calculated to be 280, 800 or 1310 µg/kg of body weight per day (bw/day). No liver injury was identified through histopathology and serum enzyme levels in the 280 µg/kg/bw/day group, however liver injury was apparent in the 800 and 1310 µg/kg/bw/day groups. To determine the guideline value, risk assessment calculations were performed using study data, incorporating additional safety factors and accounting for potential tumor promotion. The safe guideline value of 1 µg/L of MC for oral consumption, corresponds to 5000 cyanobacteria cells/mL (Falconer, Burch et al. 1994; WHO 1998).

Due to the lack of oral toxicity data relating to nodularin and cylindrospermopsin, the guideline value of 1 µg/L is applied uniformly to all cyanobacterial hepatotoxins (Falconer, Burch et al. 1994; WHO 1998; WHO 2003). Numerous countries have also developed their own supplementary policies for drinking, recreational and agricultural water to limit cyanotoxin and cyanobacterial exposure (Burch 2008).

4.5. *Microcystins*

MCs are cyclic heptapeptides possessing two variable L-amino acids, three D-amino acids (alanine, methylaspartic acid and glutamic acid) and two uncommon amino acids (3-amino-9-methoxy-2,6,8-trimethyl-10phenyldeca-4,6-dienoic acid (ADDA) and N-methyldehydroalanine (Mdha) (Harada, Oshikata et al. 1996). Variation of MCs can occur in all seven amino acids, however, most substitutions take place at the two variable L-amino acid regions. The amino acid identity of the two variable amino acid regions, determines the microcystin congener (Botes, Kruger et al. 1982; Botes, Tuinman et al. 1984; Botes, Wessels et al. 1985; Harada, Fujii et al. 1996). Despite the variation between MC congeners, all possess the hydrophobic amino acid ADDA (Namikoshi, Sivonen et al. 1992; Namikoshi, Sivonen et al. 1992; Namikoshi, Sivonen et al. 1992; Trogen, Annala et al. 1996; Sivonen and Jones 1999). To date, over 80 different known congeners of MCs have been identified, differing by their variable amino acid identity, degree of methylation, peptide sequence, epimerization and hydroxylation. Toxigenic cyanobacterial species can synthesize a variety of MC congeners simultaneously to produce a mixture of MCs, at varying concentrations, depending on environmental stimuli; temperature, pH, light

intensity, oxygen saturation, nutrient levels, etc (Wicks and Thiel 1990; Chorus and Bartram 1999; Sivonen and Jones 1999; Falconer and Humpage 2005).

All MCs share the same general mechanism of toxicity, but differ in their potency and organ specificity. Microcystin-LR (MCLR) is one of the most toxic and commonly observed MC variants found in the environment. The majority of scientific studies investigating the cellular accumulation and hepatotoxicity of MCs have been conducted using MCLR. Acute toxicity resulting from MCLR exposure can cause hepatic necrosis, hemorrhaging, fibrosis, liver failure, renal failure and gastroenteritis (Ashworth and Mason 1946; Falconer, Beresford et al. 1983; Falconer, Smith et al. 1988; Carmichael 2001; Milutinovic, Zorc-Pleskovic et al. 2006).

4.5.1. Cellular Bioaccumulation and Metabolism of Microcystins

Throughout the body there are a variety of transporter proteins that facilitate active transport of molecules across the cell membrane. Organic anion transporter polypeptides (OATP: human, oatp: animal) which belong to the solute carrier family, are a group of membrane proteins that which facilitate the transport of hydrophilic organic anions across the cell membrane. These transporters are expressed on the apical and basolateral sides of a variety of cell types including; liver, kidney, intestine and blood brain barrier (Kullak-Ublick, Hagenbuch et al. 1995; Glaeser, Bailey et al. 2007; Kalliokoski and Niemi 2009).

MCs lack the ability to permeate plasma membranes due to their hydrophilic properties and high molecular weight (between 990 and 1100 Da). The membrane penetrating capacity (surface activity) of radio-labeled microcystin was investigated in rat hepatocytes, human hepatoma cell line HepG2 and mouse fibroblast cell line NIH-3T3, and in all cell

types active transport was required (Eriksson, Toivola et al. 1990; Chong, Gu et al. 2000). Fischer et al (2005) first identified membrane transporters involved in microcystin transport using a *Xenopus laevis* oocyte expression system. It was observed that rat organic anion transporter protein (oatp) 1b2, human OATP1B1, 1B3 and 1A2 mediate cellular uptake of MC, whereas oatp1a1, 1a4 and OATP2B1 failed to mediate transport (Fischer, Altheimer et al. 2005). MC toxicity was attenuated in Oatp1b2 null mice (homologue of human OATP1B1 and 1B3) compared to wild type controls, further verifying the involvement of specific transporters in microcystin cellular uptake (Lu, Choudhuri et al. 2008).

The liver is the primary target for microcystins due to an abundance of microcystin transporting OATP1B1 and 1B3 (Nishiwaki, Ohta et al. 1994; Monks, Liu et al. 2007). OATP1B1 and 1B3 share 80% homology and are expressed in a variety of cell types including; liver, intestine, kidney and to a lesser extent in the blood brain barrier. OATP1A2 is expressed primarily within the blood brain barrier endothelium, blood-cerebrospinal fluid barrier epithelium and neurons, and to a lesser extent in the liver, kidneys and intestine (Kullak-Ublick, Hagenbuch et al. 1995; Chong, Gu et al. 2000; Glaeser, Bailey et al. 2007; Kalliokoski and Niemi 2009). The identity of the two variable amino acids of MC alter the affinity of the compound for OATPs; the higher the affinity the greater the toxicity (Feurstein, Kleinteich et al. 2010; Fischer, Hoeger et al. 2010).

Although the liver is the primary site of accumulation for MCLR, bioaccumulation of the toxin occurs throughout the body and is highly dependent on blood perfusion and OATP expression levels. Within aquatic organisms, MCs are known to accumulate up the food

chain, with high concentrations of toxin residing in the liver, intestines, brain, kidney, blood, gills and muscle of larger invertebrates (Robinson, Pace et al. 1991; Chen, Xie et al. 2007). Consumption of fish and other aquatic organisms exposed to MCs, should be done with caution, as there is the potential to exceed the safe allowable limit of MCs recommended by the WHO (WHO 1998; WHO 2003; Cazenave, Wunderlin et al. 2005).

Metabolic transformation of MCs for detoxification in the liver occurs by phase II metabolism (Beattie, Ressler et al. 2003). Phase II metabolism involves conjugating partially detoxified compounds with glutathione or cysteine, making them water soluble for biliary excretion (Ishikawa 1992). Soluble glutathione-S-transferases (GST) catalyze the conjugation of glutathione (GSH) compounds during phase II metabolism (Wang, Liang et al. 2006). GSTs form a large multigene enzyme superfamily, responsible for detoxification of xenobiotics, tissue damage debris and toxins. Three major families of GSTs have been identified in mammals; cytosolic GSTs, mitochondrial GSTs and microsomal GSTs (Mannervik, Helena Danielson et al. 1988; Hayes, Flanagan et al. 2005).

Glutathione (GSH) is one of the primary non-enzymatic antioxidants in the body, acting as the main defense against ROS (Sies 1999). Kondo et al (1992) first observed the synthetic conversion of MCs to glutathione and cysteine conjugates in shrimp, postulating that conjugation with glutathione plays a key role in metabolic detoxification of MCs (Kondo, Ikai et al. 1992; Kondo, Matsumoto et al. 1996). Conjugation of MCs with cysteine results from either direct conjugation or indirectly from GSH-MC degradation (Kondo, Ikai et al. 1992). In bighead carp, MC-cysteine conjugates were observed within the kidneys, indicating that the kidneys may play a role in detoxification as well as excretion of MCs

(Chen, Xie et al. 2007). The GSH-MCLR conjugate has been observed in a variety of aquatic organisms such as zebra fish eggs and adults, invertebrates and a macrophyte (Pflugmacher, Wiegand et al. 1998). Zegura et al (2006) observed an increase in reduced GSH levels in HepG2 cells following MCLR exposure, and increased expression of glutamate cysteine-ligase (rate limiting enzyme required for GSH synthesis). Additional DNA damage was not reported under normal or elevated GSH levels, indicating the importance of GSH in MCLR detoxification (Zegura, Lah et al. 2006). In humans, Buratti et al (2011) observed that specific GST polymorphisms catalyze GSH-MC conjugation and include; GSTA1-1, M1-1 and T1-1 (Buratti, Scardala et al. 2011).

4.6. *Hepatotoxicity of Microcystins*

MCs are classified as serine/threonine phosphatase inhibitors, oxidative stressors, genotoxic and tumor promoters (Nishiwaki-Matsushima, Nishiwaki et al. 1991; Nishiwaki-Matsushima, Ohta et al. 1992; Fujiki and Suganuma 2011). The underlying mechanism of MC hepatotoxicity include but are not limited to; protein hyperphosphorylation, loss of cellular membrane integrity, oxidative stress, DNA damage, alteration of cell survival and death pathways, lipid peroxidation, loss of mitochondrial membrane potential, apoptosis and stimulation of inflammatory pathways (Nishiwaki-Matsushima, Ohta et al. 1992; Matsushima-Nishiwaki, Shidoji et al. 1995; Dawson 1998; Bouaicha and Maatouk 2004; Chen, Cui et al. 2006; Campos and Vasconcelos 2010). MC hepatotoxicity is dose, route and time dependent, eliciting a differential effect within the liver based on variations of the aforementioned parameters (Guzman and Solter 1999; Runnegar, Seward et al. 1999; Chen, Shen et al. 2005; Jiang, Gu et al. 2011).

4.6.1. Serine/Threonine Phosphatase Inhibition

Protein phosphatases and kinases are enzymes that maintain cellular homeostasis by catalyzing the phosphorylation or dephosphorylation of cellular proteins to regulate their activity. Inhibition of these regulatory enzymes alters cellular protein activity, disrupting homeostasis (Shi 2009). The serine/threonine phosphatase 1 and 2A (PP1 and PP2A) inhibitory action of MCs, is a key process through which toxin related cellular toxicity has been observed (Runnegar, Berndt et al. 1995; Monks, Liu et al. 2007).

Phosphatase inhibition by MC is a two step process; initial inactivation followed by formation of irreversible conjugation adducts. Interaction of MCLR with PP1 and PP2A occurs independently, and by unique mechanisms. Binding of MCLR to PP1 occurs at three regions; the hydrophobic groove, C-terminal groove and catalytic site. Initially the ADDA region reversibly interacts with the phosphatase hydrophobic groove to inactivate the enzyme. The MeAsp carboxyl residue then blocks the PP1 catalytic centre by interacting with Arg96 and Tyr134. The Mdha region forms irreversible covalent bonds with the nucleophilic cysteine residue Cys-273 to fully inhibit enzyme activity (Goldberg, Huang et al. 1995; MacKintosh, Dalby et al. 1995).

Binding of MCLR to PP2A occurs at the catalytic site, the binding pocket and C-terminus. Binding at the catalytic site occurs above the two manganese atoms of the phosphatase enzyme. The ADDA region of MC strengthens this interaction, through hydrophobic binding at the binding pocket with Gln122, Ile123, His191 and Trp200 residues. The MCLR Mdha side chain forms a covalent linkage between its' terminal carbon and Cys269 of the enzyme. Additional binding between MCLR and PP2A occurs between the hydrophobic

region of MCLR and Leu243, Tyr265, Cys266, Arg268 and Cys269 of the PP2A binding pocket using van der Waals forces (Dawson and Holmes 1999; Xing, Xu et al. 2006).

Inhibition of PP1 and PP2A by MCLR has been observed using *in vitro* and *in vivo* systems. Hepatocytes derived from fed and fasted juvenile goldfish *Carassius auratus*, were treated with 10 mg/L of MCLR over 4 hrs. MCLR accumulated within the fasted hepatocytes quickest, however hepatic protein phosphatase activity was almost completely inhibited within both groups within the first hour of treatment (Malbrouck, Trausch et al. 2004). Cultured little skate (*Raja erinacea*) hepatocytes, exhibited a dose-dependent decrease in phosphatase activity following exposure to 0.1, 0.5, 1, 2 and 5 μ M of MCLR for 30 min. Significant inhibition of phosphatase activity was only observed in those groups treated with 2 and 5 μ M of MCLR (Runnegar, Seward et al. 1999).

In vivo, balb/c mice were exposed to 12, 23, 45 or 100 mg/kg bw through i.p injection, and hepatic nuclear extracts examined for PP1 and PP2A activity. Within 50 minutes of initial dosing, inhibition of nuclear protein phosphatases (PP1 and PP2A) occurred within the 100 mg/kg dose treatment group, quantified by measuring p53 phosphorylation (substrate for PP1 and PP2A) (Guzman, Solter et al. 2003). Male Sprague-Dawley rats were continuously exposed to 0, 3, 6 or 9 mg/day of MCLR over 28 days through i.p mini-osmotic pumps. Hepatic PP1 and PP2A activity decreased in a dose-dependent manner, with inhibition of PP2A being most pronounced (Solter, Wollenberg et al. 1998).

4.6.2. Oxidative Stress

Oxidative stress occurs when the pro-oxidative forces (ROS) overwhelm cellular anti-oxidant defenses resulting in DNA damage (strand breaks, purine oxidation or

condensation), protein oxidation and lipid peroxidation. Anti-oxidant defenses function by preventing free radical formation, donating electrons to stabilize free radicals or neutralizing oxidative reactions initiated by free radicals. Cellular anti-oxidant defenses are comprised of enzymatic and non-enzymatic mechanisms. Anti-oxidant enzymes include; superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione reductase (GR). Non-enzymatic mechanisms include; Vitamins E, C and A, glutathione, carotenes and ubiquinol 10 (Sies 1997). The physiological conditions that regulate oxidative stress are complex and tightly regulated. Tipping the balance to favor oxidative stress conditions, promotes pathogenic mechanisms of hepatocarcinogenesis.

MCLR mediated oxidative damage has been shown to occur through a variety of mechanisms including but not limited to; 1) generation of reactive oxygen species (ROS), 2) inhibition/depletion of cellular anti-oxidants and 3) lipid peroxidation, which indirectly generates ROS.

In vitro systems used to investigate this process include human hepatoma cell line (HepG2), human erythrocytes, fish cell lines RTG-2 and PLHC-1, lymphocytes and primary rat and mouse hepatocytes. *In vivo* studies investigating the same phenomena have been performed in rats, mice, mammals, fish and crustaceans (Bainy 1996; Ding, Shen et al. 1998; Ding, Shen et al. 2001; Gehringer, Govender et al. 2003; Zegura, Sedmak et al. 2003; Botha, Gehringer et al. 2004; Bouaicha and Maatouk 2004; Al-Jassabi and Khalil 2006; Gaudin, Huet et al. 2008).

The production of ROS in the presence of MCLR has been verified in rat primary hepatocytes using a dichlorofluorescein diacetate (H2DCF-DA) fluorescent probe, and carp

liver (*Cyprinus carpio*) by measuring hydroxyl radical generation (OH) (Bouaicha and Maatouk 2004; Jiang, Gu et al. 2011). Zegura et al (2004) reported a similar finding in HepG2 cells, indicating that increased ROS may be indirectly due in part to a depletion of cellular ROS scavengers (superoxide dismutase, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL)(Zegura, Lah et al. 2004). It was further observed that pre-treatment of HepG2 cells with ROS scavengers prevents DNA damage and purine oxidation following MCLR treatment (Zegura, Sedmak et al. 2003; Zegura, Lah et al. 2004; Zegura, Lah et al. 2006).

In vivo, freshwater clams (*Diplodon chilensis*) exposed to microcystin for 6 weeks experienced an increase in anti-oxidant enzyme activity (SOD, CAT, GST), increased lipid peroxidation and decrease in GSH (Sabatini, Brena et al. 2011). Primary rat hepatocytes exposed to 1.25 mg, 12.5 mg and 125 mg of lyophilized *Microcystis aeruginosa* (cells/mL) extract for 24 hrs, experienced a time and dose-dependent increase in lipid peroxidation, ROS production and leakage of lactate dehydrogenase (Ding, Shen et al. 1998). It is suspected that binding of MCLR to aldehyde dehydrogenase is one proposed mechanism by which MCLR induces lipid peroxidation and ROS generation (Chen, Cui et al. 2006).

4.6.3. Genotoxicity

MC genotoxicity stems from its ability to induce DNA damage and inhibit DNA repair mechanisms (Bainy 1996; Botha, Gehringer et al. 2004; Bouaicha and Maatouk 2004; Al-Jassabi and Khalil 2006; Lankoff, Bialczyk et al. 2006). MCs are able to directly interact with DNA and inhibit key DNA repair mechanisms; nucleotide excision repair (NER) and non-homologous end joining (NEJ) of double stranded breaks (DSB) (Lankoff, Bialczyk et al.

2006; Shi, Guo et al. 2011). Using analysis and atomic force microscopy, Shi et al (2011) revealed that MCLR interacts with DNA directly through a non-intercalating minor groove binding mode, causing changes in DNA morphology (Shi, Guo et al. 2011).

One of the first studies investigating the genotoxic potential of MC involved the use of bacterial systems. A cyanobacterial extract containing MCs was collected from the Missouri reservoir in 1978 and caused a mutagenic response in *Salmonella typhimurium* TA1537 (Collins, Gowans et al. 1981). In a similar study, cyanobacterial extracts containing MC derived from the Sulejow water reservoir in Poland, induced a genotoxic response in *Escherichia coli* PQ37 as determined by the SOS chromotest system. Extracts containing higher MC content showed greater genotoxicity, however pure MCLR displayed decreased genotoxicity compared to the extracts (Mankiewicz, Walter et al. 2002) .

The ability of MCLR to directly induce DNA strand breaks, fragmentation and adduct formation was investigated both *in vitro* and *in vivo*, using a variety of cell and animal types. Lakshmana et al (1998) observed an increase in DNA damage and fragmentation in the baby hamster kidney (BHK-21) and mouse embryo fibroblast (MEF) cell lines following exposure to 100 and 1.0 ug/ml concentrations of MCLR for 3 hours (Lakshmana Rao, BhaTAAcharya et al. 1998). Single strand breaks within human hepatoma (HepG2) cells, human colon adenomacarcinoma (CaCo-2) cells, human lymphocytes and primary rat hepatocytes were observed using the comet assay in response to MCLR exposure at 1 ug/ml, 0.01 ug/ml, 1-4 ug/ml and 5 ug/ml respectively (Ding, Shen et al. 1999; Zegura, Sedmak et al. 2003; Palus, Dziubaltowska et al. 2007; Zegura, Volcic et al. 2008). Of note,

despite some of the cell lines not expressing the required oatp transporters, MCLR still has genotoxic potential within multiple cell types.

In vivo genotoxicity studies identified the same trend as observed *in vitro*. Hepatocytes derived from male and female Swiss albino rats showed increased DNA fragmentation following i.p injection of either purified *M. aeruginosa* extracts or pure MCLR at varying concentrations. In these studies it was unclear whether DNA fragmentation was the result of cytotoxicity or genotoxicity (Rao and BhaTAAcharya 1996; Lakshmana Rao, BhaTAAcharya et al. 1998; Gupta, Pant et al. 2003). In male Fisher 344 rats, unscheduled DNA synthesis (UDS) was measured as a means to quantify DNA synthesis resulting from excision repair of damaged DNA following MCLR injection. After 2-4 and 12-16 hours i.v exposure to 12.5 and 50 ug/kg body weight (bw) MCLR, no DNA damage was observed (Gaudin, Le Hegarat et al. 2009). The same authors investigated the difference between i.p and oral administration in mice, concluding that genotoxicity occurs independent of administration route but is a matter of dose; however animals were more sensitive to i.p injections due possibly to differences in bio-availability of MCLR (Gaudin, Huet et al. 2008). In a recent study using juvenile whitefish, MCLR (0.05 or 0.5 mg/ fish) was force-fed to fish over 96 hours. DNA strand breaks were identified by comet assay within red blood cells, and shown to have undergone partial repair (Sotton, Devaux et al. 2012).

Genotoxicity of MCLR can also be induced following chronic sub-lethal exposure with little cytotoxic side effects. Huang (2009) reported that human-hamster hybrid AL cells experienced altered cellular ATP concentrations and colony formation in soft agar

following chronic exposure to MCLR at 0.1 ug/ml, displaying minimal cytotoxic side effects (Huang and Xu 2009).

Clastogenic mutagenicity involves the direct interaction of a compound with chromosomes to elicit strand breaks, deletions or mutations. Micronuclei formation occurs when a small fragment of a chromosome is not incorporated into the daughter nuclei during cell division, indicating the presence of a genotoxic compound. Clastogenicity was observed in the human lymphoblastoid TK cell line TK6 following a prolonged 24 hour exposure to MCLR, when micronuclei formation and the mutation frequency at the heterozygous thymidine kinase locus was increased: no additional point mutations were observed (Zhan, Sakamoto et al. 2004). In a study conducted by Ding et al (1999), it was reported that MCLR enhanced the production of bone marrow micronucleated polychromatic erythrocytes in male Kunming mice (Ding, Shen et al. 1999). However, no micronuclei formation occurred in male CBA mice erythrocytes harvested 42 hours after i.p injection with varying doses of sublethal and lethal doses of MCLR, as measured by the micronucleus assay (Abramsson-Zetterberg, Sundh et al. 2010). The mutagenic potential of MCs has not been fully elucidated, whereby only one study has truly described MCLR induced mutagenesis in mammalian cells. Suzuki et al (1998), reported a dose dependent increase in the base substitution at the K-ras codon and ouabain resistance mutation frequency in transformed human embryo fibroblasts following 6 day of MCLR treatment (7.5 – 15 ug/ml) (Suzuki, Watanabe et al. 1998).

4.7. *Carcinogenicity of Microcystins*

MCs are regarded as potent tumor promoters: having the ability to support cell survival and clonal expansion (Klein and Klein 1985). An association between HCC incidence and chronic oral exposure to MCs through drinking water sources has been reported in China, Serbia and the United States (Ueno, Nagata et al. 1996; Yu, Zhao et al. 2001; Fleming, Rivero et al. 2002; Svircev 2009). In the first such study, Ueno et al (1996) sought to identify some of the underlying environmental factors contributing to the high incidence of primary liver cancer (PLC) in China. Water samples taken between 1993 and 1994 from rivers, shallow and deep wells, ponds and ditches in Haimen city and Fusui, Guangxi province, were sampled for MC contamination using an enzyme linked immuno-assay (ELISA). High MC contamination frequency was reported in rivers, ponds and ditches, particularly in the month of June. It was recommended by the authors that an advisory maximum limit of 0.01 µg/L of MC be allowed in drinking water, indicating that intermittent MC exposure in conjunction with aflatoxin ingestion could be contributing to the high PLC rates in China (Ueno, Nagata et al. 1996).

In a follow-up study, Yu et al (2001) utilized Meta-analysis, ecological and epidemiological methods to further investigate the relationship between MC contaminated drinking water and the high PLC incidence in China. It was observed that communities endemic with HBV and ingesting aflatoxin, a known carcinogen, had a higher incidence of PLC if their drinking water was derived from MC contaminated ponds or ditches. In neighboring communities with a similar HBV and aflatoxin profile, the PLC incidence rates were lower if their drinking water was derived from cyanobacteria-free deep wells. The findings were confirmed using HBVx transfected mice, wherein the PLC rates were elevated

in those animals exposed to aflatoxin and cyanotoxin contaminated pond/ditch water (Yu, Zhao et al. 2001).

In Central Serbia, 9 out of 20 drinking water reservoirs were observed to be contaminated with persistent cyanobacterial blooms. Tap water from cyanobacterial contaminated reservoirs contained on average 2.5 µg/L of MCLR. In regions where drinking water sources were uncontaminated, PLC rates from 1980 to 1990 and 2000 to 2002 were ranging from 4.1% to 13.6% respectively. In regions with MC contaminated drinking water, PLC rates from 1980 to 1990 and 2000 to 2002 were 11.6% and 34.7% respectively. The study concluded that high-risk areas for PLC occurrence corresponded with cyanobacterial contaminated water sources, whereas low-risk PLC occurrence corresponded with regions receiving drinking water from non-cyanobacterial contaminated reservoirs (Svircev, Krstic et al. 2009). A study conducted in Florida, revealed a similar trend in PLC occurrence, whereby increased cancer rates were associated with cyanotoxin contaminated drinking water sources (Fleming, Rivero et al. 2002).

Based on previous studies investigating the carcinogenic properties of MCs, in particular MCLR, the International Agency for Research on Cancer (IARC) has labeled MCLR as a possible human carcinogen (Group 2B) (Duy, Lam et al. 2000). MCs are classified as tumor promoters (Falconer and Humpage 1996; Fujiki and Suganuma 2011). The underlying mechanisms of MC tumorigenicity are thought to be due to the toxins' ability to inhibit serine/threonine phosphatases 1 and 2A (PP1 and PP2A), induce oxidative stress

and genotoxicity (Ohta, Nishiwaki et al. 1992; Guzman, Solter et al. 2003; Bouaicha and Maatouk 2004).

Studies investigating the direct ability of MCLR to stimulate malignant transformation, initiate or promote tumor development have had mixed results. In a study conducted by Zhu et al (2005), immortalized non-malignant normal human colorectal crypt (NCC) cells were malignantly transformed following chronic exposure to MCLR, displaying constitutive activation of the AKT, p38, JNK and MAPK pathways (Zhu, Zhong et al. 2005). Falconer et al (1988) reported the induction of bronchogenic and abdominal carcinoma in Swiss albino mice after one year of orally ingesting extracts of *M. aeruginosa* (Falconer, Smith et al. 1988). Similarly, neoplastic liver nodule formation was observed in mice who received 100 i.p injections of 20 ug/kg bw of MCLR over 28 wks (Ito, Kondo et al. 1997).

Conflicting evidence has been reported, whereby limited liver nodules or GST-P positive foci were observed in male Fisher F344 rats exposed to i.p injections of MCLR without an initiator (diethylnitrosamine) over a course of 8 (1 ug/kg bw or 10 ug/kg bw) and 9 (25 ug/kg bw) weeks exposure (Nishiwaki-Matsushima, Ohta et al. 1992; Ohta, Sueoka et al. 1994). Suggesting that when administered alone, MCLR may require chronic high dosing in order to elicit a carcinogenic effect or the presence of a strong tumor initiator.

Other *in vivo* studies strongly support the tumor promoting properties of MCLR, particularly in male Fischer 344 rats. In a study by Nishiwaki-Matsushima et al (1992), liver cancer was initiated in rats treated with 200 mg/kg DEN with and without partial hepatectomy, and exposure to 10 ug/kg MCLR pre hepatectomy and 10, 25, 50 ug/kg MCLR

post-hepatectomy for 8 weeks. The number and size of glutathione-S-transferase placental (GST-p) form positive foci (a marker of malignancy) increased with increasing MCLR dose. However in DEN-uninitiated controls no GST-p foci were observed (Nishiwaki-Matsushima, Ohta et al. 1992). In a similar six week study, Sekijima et al (1999) initiated liver cancer in rats using a single i.v dose of 200 mg/kg of diethylnitrosamine (DEN) and two thirds partial hepatectomy, and two weeks later gave i.p injections of either 1 or 10 ug/kg of MCLR twice a week for six weeks. In both MCLR treatment groups, an increase in the number of GST-p form positive foci and neoplastic nodules within the liver were observed compared to uninitiated controls (Sekijima, Tsutsumi et al. 1999).

In a long-term 22 week study, C57 black mice were used to investigate the tumor promoting activity of MCs on duodenal and adenocarcinomas. Mice exposed to two doses of 40 mg/kg p.o of *N*-methyl-*N*-nitrosurea (MNU), followed by 0, 1.2 and 4.2 mg/kg/bw/day of *Microcystis* extract supplemented in their drinking water. Examination of tissues revealed no evidence of primary liver tumor formation, nor the presence of duodenal or lymphoid tumors (Falconer and Humpage 1996).

Oncogenes are genes that have the potential to cause cancer by overriding programmed cell death and promoting cellular proliferation (Weinstein and Joe 2006). MCLR has been observed to alter proto-oncogene expression in primary rat hepatocytes treated with 1 uM of MCLR. It was observed that following 6 hours of exposure to MCLR, proto-oncogenes; jun-B, c-fos, c-jun, jun-D, TNF- α , fos-B and fra-1 showed increased expression (Sueoka, Sueoka et al. 1997). The same study reported similar findings in HepG2 cells, whereby p21,

gadd45 α and mdm2 proto-oncogenes were elevated in response to 0.01 ug/ml MCLR for 6-12h.

In another study, the livers, kidneys and testis of male Fischer 344 rats were harvested after i.v injection of MCLR (86.5 equivalents/kg bw) for up to 24h. Li et al (2009) observed that early response oncogene expression; c-myc, c-fos and c-jun and TNF- α , was enhanced at the transcriptional and protein level in all organs (particularly the liver), and remained elevated for up to 24 h post treatment (Li, Xie et al. 2009). It was further suggested by the authors that due to PP1 and 2A inhibition, oncogene expression was stabilized at the mRNA level. In particular, TNF- α which is an endogenous tumor promoter and pleiotropic inflammatory cytokine typically produced by activated macrophages, was also produced in hepatocytes following treatment (Li, Xie et al. 2009).

5. Materials and Methods

5.1. In Vitro Studies

5.1.1. Chemicals

3-methylcholanthrene (3MC), 12-O-tetradecanoylphorbol-13-acetate (TPA), okadaic acid (OA), epidermal growth factor (EGF), transforming growth factor beta (TGF- β) and thioacetamide (TAA) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Endoporter was purchased from Gene-Tools (Philomath, OR, USA). Antibodies for Western Blot (anti-Epcam, anti-Cyclin D1, anti-smooth muscle alpha actin and sheep anti-mouse secondary antibody) were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA).

5.1.2. Cell Culture

WB-F344 cells were obtained from the Health Science Research Resources Bank (HSRRB) (2-11 Rinku-minamihama, Sennan-city, Japan). Cell lines were cultured in Minimal essential media (MEM), 2mM L-glutamine (Gibco Laboratories, Grand Island, NY, USA) supplemented with 7% fetal bovine serum (FBS) and 100 units/mL of penicillin and 100 ug/mL streptomycin (P/S). PLC/PRF/5 (PLC) cells were obtained from the American Type Cultures Collection (ATCC, Manassas, VA, USA). CFSC-2G cells were a generous gift from Dr. Y. Gong (University of Manitoba, Winnipeg MB). Both PLC and CFSC-2G cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), low glucose (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FBS and 100 units/mL of penicillin and 100 ug/mL streptomycin. All cells were incubated at 37°C in a humidifier in a 5% CO₂ atmosphere. All culture supplements, including Dulbecco's phosphate buffered saline (PBS), FBS, P/S and TrypLE were purchased from Invitrogen (Burlington, ON,

Canada) or Gibco Laboratories (Grand Island, NY, USA). All *in vitro* experiments utilized Endo-porter according to manufacturers instructions.

5.1.3. Protein Quantification

Cellular protein levels were quantified using the BCA protein quantification assay kit protocol (Thermo Scientific, Waltham, MA, USA). First, WB-F344, PLC and CFSC-2G cells were lysed using radioimmunoprecipitation (RIPA) lysis buffer plus protease inhibitor. Protein quantification reagent was prepared by mixing 50:1 BCA reagent A (BCA and tartrate in an alkaline carbonate buffer) with BCA reagent B (4% CuSO₄•5H₂O). In a 96 well plate, 100ul of protein quantification reagent was added to each well, along with 25ul of cell lysate. Samples were shaken for 1min, and incubated at 37°C for 30min. Readings were taken at an absorbance of 560nm (reference wavelength optical density 630nm) using a BioTek plate reader (BioTek Instruments, Winooski, VT, USA) against assay controls.

5.1.4. Serine/Threonine Phosphatase Inhibition Assay

Serine/threonine phosphatase inhibition was measured using a 96 RediPlate™ EnzChek Serine Threonine Phosphate assay kit (Life Technologies, Grand Island, NY, USA), according to manufacturer's instructions. Briefly, WB-F344, PLC and CFSC-2G cells were separately seeded at 200, 000 cells/well in MEM or DMEM supplemented medium in 6 well plates and allowed to aTAAch overnight. Medium was then replaced with medium containing various concentrations of MC-LR (0.1- 10,000 µg/L), okadaic acid (Sigma Aldrich, Oakville, ON, Canada) (positive control) or medium alone and incubated for a further 24 hrs. Following PBS washes, cells were lysed with ripa lysis buffer containing protease inhibitor. 50 µL of reaction buffer optimized for PP-2A (12 mL of 100 mM Tris-

HCl at pH 7.0 containing 0.2 mM CaCl₂, 250 µg/mL BSA, 0.1% Tween® 20 and 1 mM NiCl₂) and equal protein concentrations (1 µg/µl) were added to each well of a 96 well Rediplate microplate, and allowed to incubate for 30 min in the dark at 37°C. Fluorescence readings were measured using a BioTek Synergy 4 microplate reader (BioTek Instruments, Winoosky, VT, USA) at an excitation/emission maxima of 358/452.

5.1.5. Oxidative stress Assay

To measure the level of oxidative stress, WB-F344, PLC and CFSC-2G cells were plated in 96 well plates at a density of 10,000 cells per well in 100 µL of MEM or DMEM media and allowed to adhere overnight. The medium was then replaced with media containing various concentrations of MCLR (0.1, 1, 10, 100, 1000, 5000, 10000 µg/L), 0.1 µg/L of OA or media alone. After 24hrs of incubation at 37°C, CellRox Deep Red Reagent oxidative dye (Life Technologies, Grand Island, NY, USA) was added to each well at a concentration of 5µM and incubated at 37°C for 30 min. Cells were then washed 3x using sterile PBS. Readings were taken using an Zeiss Axio observer Z1 fluorescent microscope at a 40x objective against untreated cells containing PBS and 5µM of CellRox dye and analyzed using Zeiss AxioVision 4 software.

5.1.6. Cellular Proliferation Assay

To measure cellular proliferative activity of cells, the cellular proliferation reagent tetrazolim salt WST-1[2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] (WST-1) (Roche Applied Science, Laval, QC, Canada) was used. In brief, 96 well plates were seeded with WB-F344, PLC and CFSC-2G cells at a density of 2000 cells/well, 2000 cells/well and 4000 cells/well respectively in MEM or DMEM supplemented media and allowed to aTAAch for 4hrs. The medium was then replaced with

media containing various concentrations of MCLR (0.1, 1, 10, 100, 1000) at $\mu\text{g/L}$ and 100 ng/L of Epithelial growth factor (EGF) or 100 ng/L Peripheral Derived Growth Factor (PDGF). Following 24, 48, 72 and 96hrs of incubation at 37°C, the cells were washed with PBS and fresh media containing 10 μL of WST-1 reagent was added to each well. Cells were incubated for 2hrs at 37°C, and absorbance was read at 450nm (reference wavelength 630nm) using a BioTek plate reader (BioTek Instruments, Winoosky, VT, USA). Readings were compared against cell free blanks containing media and WST-1 reagent.

5.1.7. In Vitro Two-Stage Malignant Transformation

Malignant transformation of WB-F344 cells was identified by monitoring soft agar gels for colony formation. Cells were seeded at density of 5000 cells/ 25cm² tissue culture flask in complete MEM and allowed to attach for 24hrs. The medium was replaced with media containing 2mg/L of tumor initiator 3MC, MCLR alone (0, 0.1, 1, 10, 100, 1000 $\mu\text{g/L}$) or in combination (3MC + MCLR), and incubated for 72hrs at 37°C. The media was removed and the cells were washed with sterile PBS 3x, and incubated in fresh media for 4 days. The cells were washed again with sterile PBS 3x and media was added that contained 100 $\mu\text{g/L}$ of TPA or MCLR alone 0, 0.1, 1, 10, 100, 1000 $\mu\text{g/L}$). Cells were incubated for 14 days in the tumor promoting TPA containing media and changed every 2-3 days. After 14 days, the cells were washed 3x with sterile PBS and cultured for an additional 9 days in fresh complete medium, changed every 2 days. Concentrations of MCLR ($\mu\text{g/L}$) were added to the cultures in the following combinations; 1) 3MC + 0, 0.1, 1, 10, 100, 1000 MCLR $\mu\text{g/L}$, and 2) 0, 0.1, 1, 10, 100, 1000 MCLR $\mu\text{g/L}$ alone, starting after the 24hr attachment period. Cells from these cultures were then seeded into soft agar plates to monitor for malignant transformation. Initially 0.5 mL of 0.5% base layer agar was layered on the

bottom of 24 well plates. After the base layer of agar had set, 1,250 cells per well in 0.25 mL of 0.3% agar was added separately to each well to form the top layer. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 30 days. Once per week, 0.25 mL of 0.3% agar was added to each well. After 30 days, colonies of greater than 20 cells were counted under contra-phase microscope.

5.1.8. Wound Healing Assay

To measure the invasiveness of malignant hepatocytes, PLC cells were subjected to the wound healing assay. PLC cells were cultured in 6 well plates to confluence using Connaugh Medical Research Laboratories 1066 medium (CMRL). At confluence, the media was removed and cells were washed with sterile PBS. Using a 200 µL pipette tip, a linear wound was scraped across each well. To remove debris, cells were gently washed 2x with sterile PBS. PBS was discarded and replaced with fresh DMEM supplemented with various concentrations of MCLR (0.1, 1, 10, 100, 1000 µg/L) and 100 ng/L of PDGF. Wound size was measured using phase microscopy at 0, 24, 48, 72 and 96hrs in a blinded fashion.

5.1.9. Western Blot

Western Blot was used to measure the level of certain proteins expressed in treated cell types (WB-F344, PLC and CFSC-2G cells). Treated cells were washed 3x with PBS and harvested using 1x TrypLE Express (Gibco – Life Technologies Inc, Burlington, ON, Canada), with the digest terminated by adding complete medium. Cells were centrifuged at 1000 rpm for 5min at room temperature in order to remove excess media. Cells were lysed using RIPA lysis buffer containing protease inhibitor cocktail for 30min on ice, followed by sonication for 30 sec on ice. Samples were then centrifuged at 14,000 rpm for 30 min to remove cellular debris at 4°C. Protein was quantified in each sample using the BCA

method described previously. 20ug of protein per sample were mixed with the appropriate volume of 4x laemmli buffer (0.5 M Tris (pH 6.8), 26% Glycerol, 20% SDS, 1% Bromophenol Blue, 0.05% beta- mercaptoethanol), and boiled for 5min at 100°C. Samples were separated in a 10% precast polyacrylamide gel membrane (Thermo Scientific, Rockford, IL, USA), and run at 80-100 mV for 1hr in SDS-page running buffer (0.1M Tris, 0.1M HEPES, 3mM SDS, pH 8). Proteins were transferred to a 0.45 um nitrocellulose membrane (Bio-Rad) using SDS-page transfer buffer (0.25M Tris, 1.92M Glycine, 1% SDS, 20% methanol, H₂O) overnight at 4°C. Membranes were blocked overnight in blocking buffer (PBS, 1% Tween-20, 5% powdered milk) at 4°C. In the case of WB-F344 cells, membranes were incubated with anti-Epcam (1:500 diluted in PBS + 1% Tween-20) or anti-cyclin D1 (1:500 diluted in PBS + 1% Tween-20) primary antibody, whereas CFSC-2G cells were incubated with anti-smooth muscle alpha actin (smaa) (1:1000 diluted in PBS + 1% Tween-20) primary antibody, for 2hrs at room temperate. Membranes were washed in PBS + 1% Tween-20 and exposed to peroxidase-conjugated sheep anti-mouse secondary antibody (1:1000 diluted in PBS + 1% Tween-20) (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) for 1hr at room temperate. Bands were detected using the enhanced chemiluminescence system (Amersham-Pharmacia Biotech, Piscataway, NJ, USA).

5.1.10. Statistics

All statistical analysis was performed with Sigma Plot software applying one-way ANOVA analysis. All tests were performed in triplicate, representing results as the mean $\pm$ standard deviation (SD). A two-sided $P < 0.05$ was considered significant where $p < 0.001 = ***$, $p < 0.01 = **$ and $p < 0.05 = *$.

5.2. In Vivo Studies

5.2.1. Animal Model

Eighty male CD1 adult mice weighing between 20-24 grams (g) were obtained from the University of Manitoba Central Animal Care Facility (University of Manitoba, Winnipeg, MB, Canada) and allowed to acclimate at the Health Sciences Centre Animal Care Facility (University of Manitoba, Winnipeg, MB, Canada) for two weeks prior to use. All experiments were approved by the University of Manitoba Animal Ethics Committee.

Animals had free access to commercial laboratory chow and water, and were maintained on a 12 hour light:dark cycle. MCLR (approximately 95% pure) was purchased from Alexis, dissolved in minute amounts of ethanol and diluted in water to $1\mu\text{g/L}$. Thioacetamide (TAA) was purchased from Sigma, and dissolved in water to 300 mg/L (0.03% w/v). MCLR and TAA were combined together in water at $1\mu\text{g/L}$ and 300 mg/L respectively. Twenty mice from each group were assigned to various treatment groups (24hr, 28 week control, 24hr, 28 week MCLR treatment, 24hr, 28 week TAA treatment and 24hr, 28 week TAA & MCLR treatment groups) immediately prior to dosing and given free access to food and water during the study. MCLR and TAA were added at the specified concentrations to the drinking water of the treatment groups for the duration of the study.

Animals were weighed prior to dosing, and then once a week until the end of the exposure period to monitor for weight changes. At 28 weeks, all mice from each group were anesthetized using 5% isoflourane, exanguinated through a coronary puncture and euthanized by cervical dislocation.

5.2.2. Plasma Chemistries

Whole blood was collected prior to cervical dislocation from the heart, and allowed to stand at room temperature for 30min to allow for clotting. Plasma was separated from samples following centrifugation at 4000 rpm for 20min at 4°C and stored at -80°C. Concentrations of total bilirubin and alanine aminotransferase (ALT) were measured using the Total Bilirubin DiscretPak (Product no. C314)) and DiscretPak ALT (SGPT) (Product no. C164) assay kits according to the supplied protocols (Catachem, Inc., Oxford, CT, USA). Samples were read using a Biotek Powerwave XS absorbance reader and analyzed using the Biotek Gen5 Software Pak (BioTek Instruments, Winoosky, VT, USA).

5.2.3. Gross Pathological Analysis and Histological Preparation

The liver was removed, patted dry, weighed, visually inspected and photographed. A portion of the liver was preserved in RNA-later (Ambion Inc., Austin, TX, USA) as per the manufacturer's instructions. An additional piece was preserved in 10% neutral buffered formalin for histological processing and analysis. The remainder of the liver was flash-frozen in liquid nitrogen, and stored at -80°C for future analysis. Livers preserved in buffered formalin were further processed; embedded in paraffin, sliced at 5 µm and stained with hemotoxylin and eosin (H&E) and Picric Acid Sirius Red, for light microscopic analysis.

5.2.4. Liver Section Selection and RNA Isolation

At the end of the *in vivo* study, portions of livers were removed and immediately placed in “RNA later” (Ambion, Austin, TX, USA), and stored at -80°C. Four livers from each group were randomly selected for RNA isolation. Total RNA was isolated from approximately 100 mg of mouse liver tissue by homogenizing the sample on ice in 2 ml of Trizol reagent (Life Technologies, Burlington, ON, Canada), followed by sonication for 30 seconds on ice. RNA was further purified using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, USA) according to manufacturer’s instructions. The quality and quantity of RNA was analyzed using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

5.2.5. cDNA Synthesis and PCR Arrays

First strand cDNA was generated from 1 µg of each RNA sample using the RT² First Strand kit (Qiagen, Valencia, CA, USA) according to manufacturer’s instructions. The liver cancer gene expression profile of each sample was analyzed using a commercially available Liver Cancer pcr array kit (SA Biosciences, Frederick, MD, USA) according to the manufacturer’s instructions, which included five housekeeping genes and seven RNA quality controls to account for variability. Relative changes in gene expression and statistical analyses were calculated using the web-based RT² Profiler PCR array data analysis software (SA Biosciences, Frederick, MD, USA) for custom pcr arrays. Gene tables, spreadsheets and graphs were also generated using the software template formulas. To ensure reproducibility, RNA from four liver samples per treatment group were analyzed

and those from the negative control group were treated as “normal”. A complete list of genes analyzed and their functions is presented in Table 1.

Table 1. Mouse liver cancer pcr array gene table

Regulation and Function	Gene
Genes Up-Regulated during hepatocellular carcinoma	Bcl2, Birc5, Ccnd1, Cflar (Casper), Fzd7, Igf2, Irs1, Lef1, Mcl1, Met, Nfkb1, Pin1, Ptk2, Rac1, Rhoa, Smad7, Stat3, Tcf4, Tgfa, Tgfb1, Tnfsf10 (Trail), Xiap
Genes Down-Regulated during hepatocellular carcinoma	Bax, Bid, Casp8 (Flice), Cdkn1a (p21Cip1/Waf1), Cdkn2a (p16Ink4), Dlc1, Fadd, Fas (Tnfrsf6), Hhip, Igfbp1, Igfbp3, Itgb1, Pten, Rb1, Smad4, Socs1, Socs3, Tgfbr2, Tnfrsf10b (Dr5)
Signal Transduction: <i>Classical WNT</i>	Ccnd1, Cdkn2a (p16Ink4), Ctnnb1, Fzd7, Lef1, Mtdh, Pin1, Rb1, Sfrp2, Tcf4.
<i>TGFβ</i>	Smad4, Smad7, Tgfb1, Tgfbr2
<i>PI3K/AKT</i>	Akt1, Irs1, Pten
<i>IGF/IGFR</i>	igf2, Igfbp1, Igfbp3, Irs1
<i>Hippo</i>	Yap1
<i>Ras/Raf/MEK/ERK</i>	Cdkn1a (p21Cip1/Waf1), Cdkn2a (p16Ink4), Dlc1, Hras1, Nras, Rac1, Rassf1, Rb1, Rhoa, Trp53
<i>EGFR</i> <i>MET/HGF</i>	Adam17 (Cd156b), Cdh13, Egf, Egfr, Tgfa, Tgfb1 Met, Hgf
<i>Small GTPase-Mediated</i>	Cdh13, Cdkn1a (p21Cip1/Waf1), Cdkn2a (p16Ink4), Dab2ip, Dlc1, Hras1, Nras, Rac1, Rassf1, Rb1, Reln, Rhoa, Trp5
Epithelial to Mesenchymal Transition	Ctnnb1, Hgf, Lef1, Smad4, Smad7, Tgfb1.
Cell Cycle	Birc5, Ccnd1, Ccnd2, Cdkn1a (p21Cip1/Waf1), Cdkn1b (p27Kip1), Cdkn2a (p16Ink4), E2f1, Ep300, Gadd45b, Igf2, Myc, Pten, Rassf1, Runx3
Apoptosis	Bax, Bcl2, Bcl2l1 (Bcl-x), Bid, Birc2 (c-IAP2), Birc5, Casp8 (Flice), Cflar (Casper), Dlc1, E2f1, Ep300, Fadd, Fas (Tnfrsf6), Fhit, Gadd45b, Gstp1, Mcl1, Msh2, Pycard (Asc), Runx3, Socs3, Tert, Tnfrsf10b (Dr5), Tnfsf10 (Trail), Wt1
Immune / Inflammatory Response	Ccl5 (Rantes), Cxcr4, Nfkb1, Ptgs2 (Cox2), Rac1, Tgfb1, Tlr4
Adhesion / Proteolysis	Adam17 (Cd156b), Akt1, Cdh1 (E-Cadherin), Cdh13, Cflar (Casper), Dlc1, Egfr, Ep300, Hgf, Itgb1, Lef1, Opcml, Pten, Pycard (Asc), Rac1, Reln, Rhoa, Smad7, Socs1, Socs3, Tgfb1
Angiogenesis	Angpt2, Casp8 (Flice), Cdh13, Ctnnb1, Egf, Flt1, Kdr, Pdgfra, Pten, Ptk2, Tgfbr2, Vegfa
DNA Damage	Ccnd1, Cdkn1a (p21Cip1/Waf1), Msh2, Msh3, Trp53, Xiap.

Source: (Qiagen 2013)

5.2.6. Statistics

For the immunohistochemical analysis of liver tissue, H&E slides were graded for inflammation according to metavir inflammation scoring scale using the following units; 0 = no inflammation, 1 = minimal inflammation/occasional spotty necrosis, 2 = mild inflammation/little hepatocellular damage, 3 = moderate inflammation with noticeable hepatocellular damage and 4 = severe inflammation with prominent diffuse hepatocellular damage. Picric Acid and Sirius Red stained slides were graded for fibrosis according to the metavir fibrosis grade scale using the following units; 0 = no scarring, 1 = minimal fibrosis confined to the portal tracts, 2 = fibrosis extending beyond the portal tracts, 3 = bridging fibrosis; fibrosis spreading and connecting to central veins or other portal tracts and 4 = cirrhosis or advanced scarring of the liver. Results were analyzed for significance using the Kruskal-Wallis one-way ANOVA on ranks method, comparing the treatment groups to the negative control.

Statistical analysis for the plasma chemistries was performed with Sigma Plot software applying one-way ANOVA analysis. All tests were performed in triplicate and results are presented as mean $\pm$ SD. A two-sided $P < 0.05$ was considered significant where $p < 0.001 = ***$, $p < 0.01 = **$ and $p < 0.05 = *$. Pcr array analysis was performed using the online Qiagen RT² Profiler PCR array data analysis software. Prior to analyzing gene expression, all arrays were normalized to the hrp gene internal control. Results with a value of $p < 0.05$ were considered significant.

5.3. National Epidemiological Study

5.3.1. Data Sources

Liver cancer incidence data were obtained from the National Cancer Registry for Canada for the years 1996 to 2004. These data were received in a highly aggregated form, with no age or gender breakdown, and case counts provided at the Census Division (CD) level. Population data were also supplied by the National Cancer Registry for the years 1996 to 2004 at the CD level. In Canada there are 284 CDs with a mean population of 108,304, a median population of 40,355, and a range of 1,397 to 2.5 million (1996 - 2004 average). For 92 CDs in which the liver cancer case count was less than 5, case counts were suppressed by the National Cancer Registry in order to protect confidentiality. Case counts for suppressed CDs were imputed in this study by calculating the expected number of cases. This was accomplished by applying national liver cancer rates for 1996 to 2004 to the age and gender specific population counts in each suppressed CD. Expected case counts were assumed to be a reasonable estimate if they were less than 5, since the actual case counts must be less than 5. Similarly, if the expected case count was greater than 5, knowing that actual case counts were less than 5 for suppressed CDs, a reasonable estimate was assumed to be 4. Since it was not possible to calculate directly age and gender standardized rates for this study (age and gender were not supplied with the case data), all analyses were restricted to using the 40+ population as a denominator in order to partially control for the effect of age on study results. Over 95% of liver cancer cases occur in the 40+ population (Nordenstedt, White et al. 2010).

Surrogate markers of potential cyanobacterial exposure included: agricultural activity (percent of land devoted to agriculture), cattle and swine densities. Established risk factors

for liver cancer included population based estimates of age, gender, incidence of hepatitis B and C, binge drinking, recent immigrant status and urban residence. The variables analyzed in the study, their sources, and methods of calculation are summarized in Table 2.

Table 2. Data sources of socio-demographic factors relating to liver cancer and cyanobacterial contamination in Canada.

Variable	Source	Method of Calculation
Land Area devoted to Agriculture (%)	2006 Canadian Census of Agriculture (accessed through the Data Liberation Initiative, University of Manitoba)	The reported area of land devoted to agriculture was divided by the total land area of each CD
Cattle Densities (no. of cattle/1000 sq. km)	2006 Canadian Census of Agriculture (accessed through the Data Liberation Initiative, University of Manitoba)	The reported number of cattle in each CD was divided by the land area of each CD.
Swine Densities (no. of swine/100 sq. km)	2006 Canadian Census of Agriculture (accessed through the Data Liberation Initiative, University of Manitoba)	The reported number of swine in each CD was divided by the land area of each CD
Population 40+ (%), 1996 - 2004	Population Data supplied by National Cancer Registry	No. of persons aged 40 plus divided by the total population, using population denominator data from 1996 - 2004 combined
Male Population (%), 2001	2001 Canadian Census (accessed through the Data Liberation Initiative, University of Manitoba) http://www12.statcan.ca/english/census01/home/index.cfm	Number of males divided by the total population
HepB Incidence, cases/100,000, 1989 - 2004	Notifiable Diseases On-Line http://dsolsmed.phac-sp.gc.ca/dsolsmed/ndis/index_e.html	Reported HepB incidence for the years 1989-2004 were averaged at the provincial level. The average provincial rate was then applied to CDs by province.
HepC Incidence, cases/100 000, 1989-2004	Notifiable Diseases On-Line http://dsolsmed.phac-spc.gc.ca/dsolsmed/ndis/index_e.html	Reported HepC incidence for the years 1989 - 2004 were averaged at the provincial level. The average provincial rate was then applied to CDs by province.
Binge Drinking (%), 40-64 yr olds, 2007	2007 Canadian Community Health Survey http://secure.cihi.ca/indicators/2007/en/datatables_maps07_e.html	Numerator (no. of binge drinkers 40-64 years of age) and denominator (no. of persons 40-64 years of age) by Regional Health Authority Area were re-scaled to Census Division using the areal interpolation extension in Arc-GIS
Recent Immigration (%), 2001	2001 Canadian Census (accessed through the Data Liberation Initiative, University of Manitoba) http://www12.statcan.ca/english/census01/home/index.cfm	Derived directly from reported census values
Urban/rural	2001 DMTI Population Counts by City (accessed through the Data Liberation Initiative, University of Manitoba) http://www.dmtispatial.com/	CD were classified as Urban if they contained at least one City with a population of 50,000 or more in 2001

5.3.2. Statistical Methods

To visualize spatial trends in liver cancer rates and its predictors, thematic maps were generated using ArcGIS 9.3 (ESRI 2009). Crude incidence rates for the 40+ population were calculated for 1996 to 2004 combined and used for mapping. Smoothed rate estimates were generated using the Bayesian smoothing function in the GeoDa software (GeoDa Center for Geospatial Analysis and Computation 2006). The GeoDa software was set to access data from the five nearest CDs for smoothing. The spatial scan statistic implemented using the Satcan 8 software (Kulldorff 2009), was used to confirm that the spatial patterns in liver cancer incidence identified in the visualization stage were real and not due to random spatial variation. The software was set to find high rate clusters containing a maximum of 50% of the study population and low rate clusters containing a maximum of 1% of the study population. Detected clusters were tested for significance using 999 Monte Carlo random simulations, with only clusters significant at the $p < 0.05$ level retained for mapping.

The Gini Coefficient was calculated using the EPIDAT 3.0 software package (Comesana 2005), to describe the degree to which liver cancer cases were distributed equally in relationship to the population at risk. The Gini coefficient was calculated from the ranked cumulative proportion of liver cancer cases and the population at risk across CDs. The ecological relationships between liver cancer and its predictors were assessed through the development of a partially adjusted (adjusting only for age and gender at the ecological level) negative binomial regression model using SAS 9.13 (SAS Institute Inc 2004). The negative binomial distribution was used to proactively deal with potential over-dispersion in the data. In these models, the count variable was incident liver cancer cases from 1996 to

2004 and the off-set variable was the population for the years 1996 to 2004 combined. Predictor variables were classified into tertiles across geographic areas using the Jenks natural breaks algorithm in ArcGis 9.3 and entered into the regression model as categorical predictors. Model outputs were expressed as relative risk, using the lowest rate category as the reference where possible.

5.4. Provincial Epidemiological Study

5.4.1. Data Sources

Liver cancer incidence data was obtained from the Manitoba Cancer Registry for the years 1985 to 2007. The total number of liver cancer cases recorded in Manitoba from 1985 to 2007 was 751.

The data were not aggregated, and had no age or gender breakdown. Case counts were made available at the area code level, and were grouped according Regional Health Authority (RHA). In Manitoba, there are 22 RHA's, inclusive of those within the City of Winnipeg; Winnipeg (Assiniboine South, Fort Gary, St. Vital, St. Boniface, Transcona, River East, Seven Oaks, Inkster, Point Douglas, Downtown and River Heights), Brandon, North Eastman, South Eastman, Interlake, Central, Assiniboine South, Parkland, Norman and Churchill/Burntwood .

To visualize temporal trends in liver cancer incidence over the 22 year study period, thematic maps were generated using ArcGIS 9.3 (ESRI 2009), dividing the liver cancer cases into 3-5 year blocks from 1985 to 2007; 1985-1989, 1990-1994, 1995-1999, 2000-2004 and 2005-2007. Maps expressed cancer incidence in the province within each

identified RHA as a rate per 100,000 people. The spatial scan statistic implemented using the Satcan 8 software (Kulldorff 2009), was used to confirm that the spatial patterns in cancer incidence identified in the visualization stage were real and not due to random spatial variation.

The Manitoba Water Stewardship provided the MCLR toxin exposure data. MCLR concentrations were measured in 72 different fresh water locations within the province between the periods of 2007 to 2011. MCLR concentrations were measured from fresh water samples that were taken from 30 cm to 1 meter below the surface of the water at various time points throughout the summer months; June to September. Fresh water samples were analyzed by ALS Laboratories (Winnipeg, MB, Canada), using Microcystin Quantiplate Elisa Plate kits (Envirologix, Portland, ME, USA), to determine the total MCLR concentration within the water. The samples were measured for MCLR toxin load, cyanobacterial cell number and cyanobacterial species present.

The average MCLR concentration from each sampling location was calculated, and assigned a low ($0.2 - < 1 \mu\text{g/L}$), medium ($1 - < 10 \mu\text{g/L}$) or high ($> 10 \mu\text{g/L}$) value. A buffer zone was assigned to each MCLR measured site, based on the values of MCLR measured; low ($0.2 - < 1 \mu\text{g/L}$) – buffer zone of 10 km, medium ($1 - < 10 \mu\text{g/L}$) – buffer zone of 20 km and high ($> 10 \mu\text{g/L}$) – buffer zone of 25 km. A buffer ring of was uniformly applied around all MCLR measured areas outside of the dedicated buffer zone, in order to pick up any subtle trends in cancer incidence related to MCLR exposure.

To geographically assess whether liver cancer incidence is associated with areas of MCLR contamination, multivariate maps were generated which overlapped MCLR buffer

zones and rings with cancer incidence data. For each 3-5 year period, the total number of cancer cases occurring within the MCLR buffer zone, ring and outside the buffer areas was calculated as a percent of total liver cancer cases. The liver cancer incidence rate within the MCLR buffer zone, ring and outside the buffer areas was calculated and compared for significance to the 1985 - 1989 reference period.

The association between cancer incidence and MCLR contamination was further assessed by calculating the mean distance of cancer cases in meters (m) to MCLR contamination sites. A straight-line (Euclidean Distance) distance dataset for each cancer point was generated, by calculating the distance to the nearest MCLR contamination site (only including $> 0.2 \mu\text{g/L}$) of each cancer case throughout each five year period. Uneven distribution of distance was accounted for by converting distance to a logarithmic transform. The values obtained were mapped and presented as the overall mean, median and minimum distance in m to MCLR. All values were calculated from the total number of liver cancer cases for the given period.

Finally, the relative risks of cancer incidence in relation to MC-LR exposure zones for each 3-5 year time period were also assessed and significance determined against the 1985 - 1989 reference period.

5.4.2. Statistical Analysis

Cancer incidence rates from 1985 to 2007 were generated by age-standardizing crude rates to the 2001 Canadian population statistics. Thematic maps were produced from the age-standardized incidence rates using ArcGIS 9.3 (ESRI 2009), grouping the rates into 3-5 year segments. Variation in age-standardized cancer incidence for each 3-5 year period was

compared to the 1985 – 1989 reference period, and the z-test was performed to determine significance.

A General Linear Model (GLM) was used to calculate whether variations existed in the logarithmic distance of cancer incidence to cyanobacterial exposure zones throughout each 3-5 year study period. To determine significance, t-tests with Bonferonni correction were performed using the 1985 - 1989 period as the reference group. The relative risk was calculated based on the number of cases occurring within the MCLR buffer zone, ring and outside the buffer areas, compared to the 1985-1989 reference period.

MCLR concentrations were measured over a four-year period (2007-2011) in a variety of locations in Manitoba lakes. Measurements were taken throughout the summer months when cyanobacterial growth occurs. To account for variability in MCLR measurements, toxin load and time, one MCLR average value and standard deviation were determined for each location sampled throughout the six year time period.

6. Results

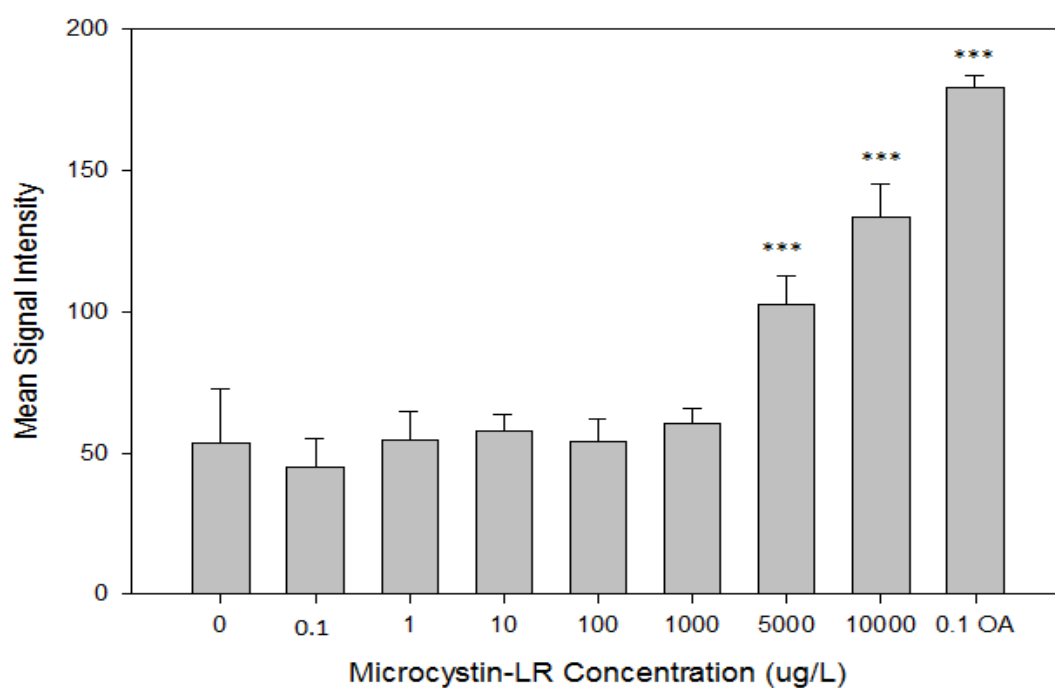
6.1. Malignant transformation of non-malignant hepatic stem cells

MCLR is a potent hepatotoxin, causing cellular damage, primarily through oxidative stress and serine/threonine PP1 and PP2A inhibition. The non-malignant hepatic stem cell line WB-F344 cells, were selected to investigate the impact of chronic exposure to low-concentrations of MCLR on oxidative stress, PP2A inhibition, proliferation and malignant transformation.

6.1.1. Oxidative Stress in WB-F344 Cells

One of the primary mechanisms of MCLR induced cellular cytotoxicity is through the induction of oxidative stress. Therefore we measured the level of oxidative stress to determine whether MCLR exerts these effects on WB-F344 cells. In response to a 24hr exposure to varying concentrations of MCLR (0.1 – 1000 µg/L), there was no increase in the oxidative stress response observed within WB-F344 cells compared to the negative control (buffer alone). However, at 5000 and 10000 µg/L concentrations of MCLR (well above the safe limit established by the WHO), significant increases were observed ($p < 0.001$). As predicted, the positive control okadaic acid (OA), caused a significant increase in oxidative stress ($p < 0.001$) (Figure 1).

Figure 1. Oxidative stress in WB-F344 cells following 24 hour exposure to varying concentrations of microcystin-LR.

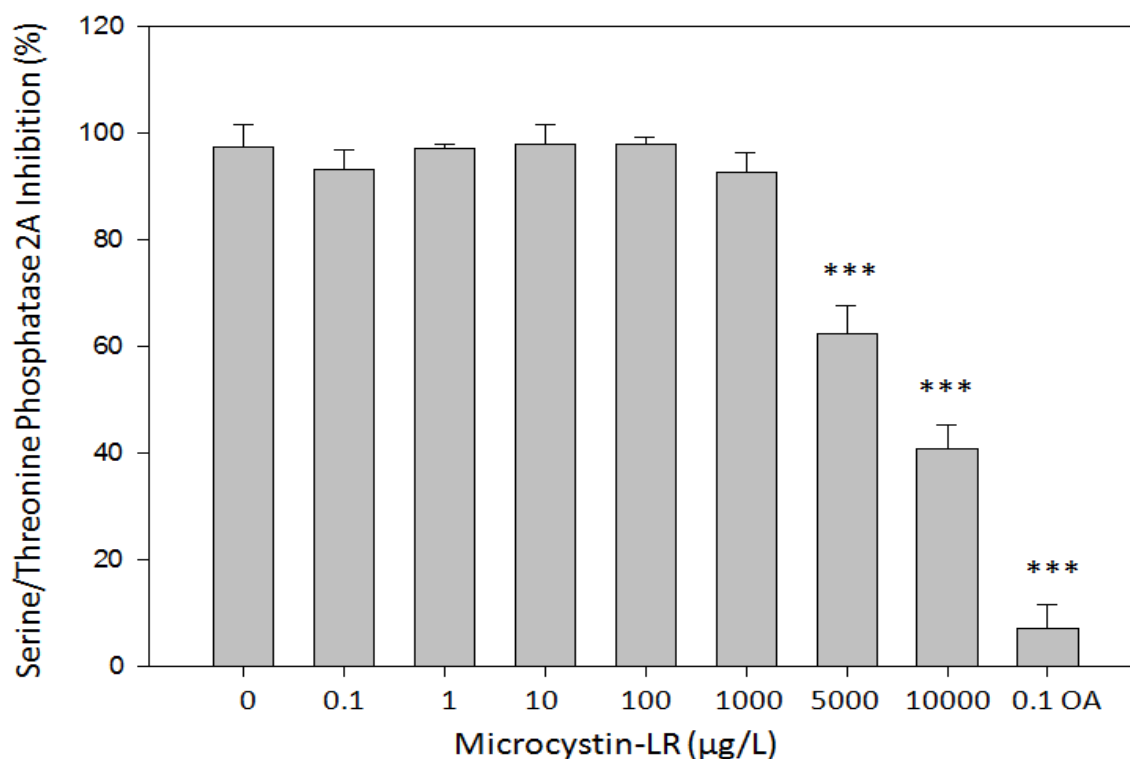


One-way ANOVA Compared to controls:
 $P < 0.001$ ***
 $P < 0.01$ **
 $P < 0.05$ *

6.1.2. Serine/Threonine Phosphatase Inhibition in WB-F344 Cells

It has been previously demonstrated that MCLR interferes with PP2A and PP1 within mammalian cells. However, following a 24 hr exposure to MCLR at varying concentrations (0.1 – 1000 µg/L), there was no significant change in serine/threonine phosphatase activity in WB-F344 cells. At the higher concentrations of 5000 and 10000 µg/L, PP2A activity was significantly inhibited (Figure 2). Okadaic acid (OA) was used as the positive control, and significantly ($p<0.001$) inhibited serine/threonine phosphatase 2A activity in the cell line following 24 hrs exposure.

Figure 2. Serine/threonine phosphatase 2A activity in WB-F344 cells following 24 hour exposure to varying concentrations of microcystin-LR.

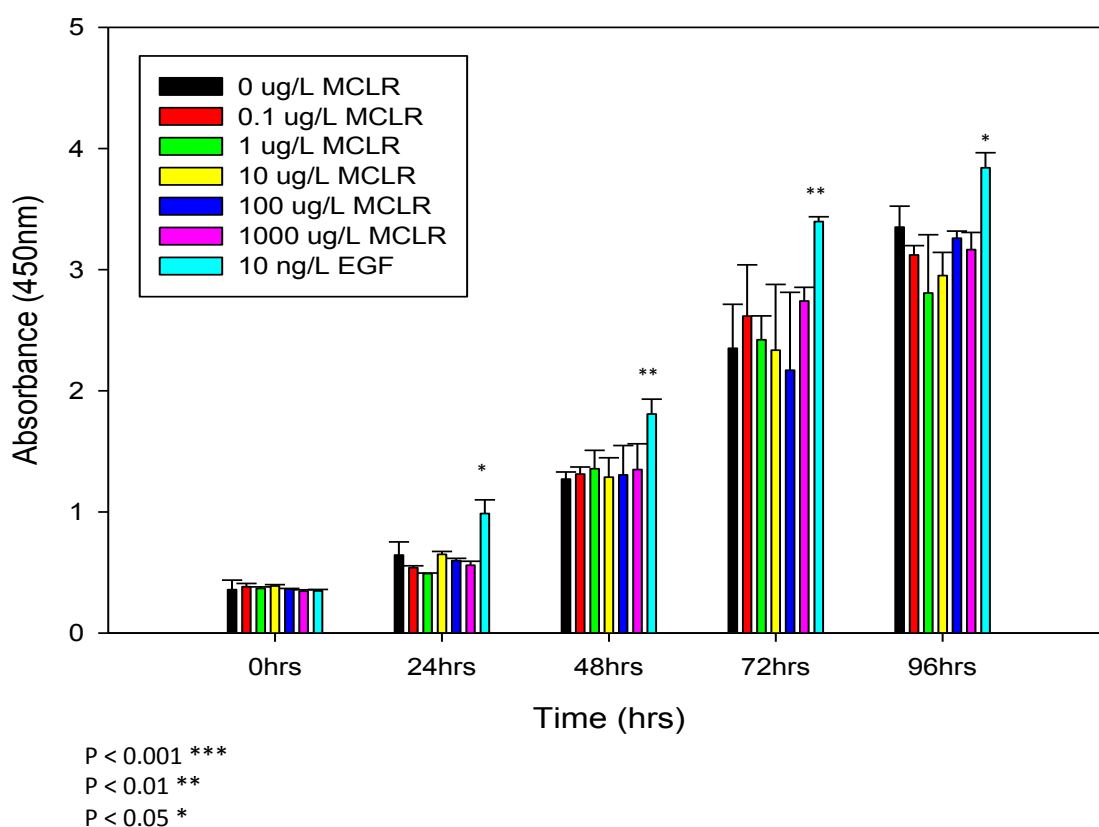


$p<0.001$ ***

6.1.3. Proliferative Activity of WB-F344 Cells

Increased proliferative activity increases the risk of mutagenesis. Thus, MCLR could contribute to malignant transformation by increasing stem cell proliferation. Our results revealed that low-concentrations of MCLR (0.1 – 1000 $\mu\text{g/L}$), when administered over a 96 hr time period, did not significantly alter the proliferative activity of WB-F344 cells (Figure 3). Epidermal growth factor (EGF), the positive control in these experiments, increased proliferative activity at all time points ($p < 0.05$).

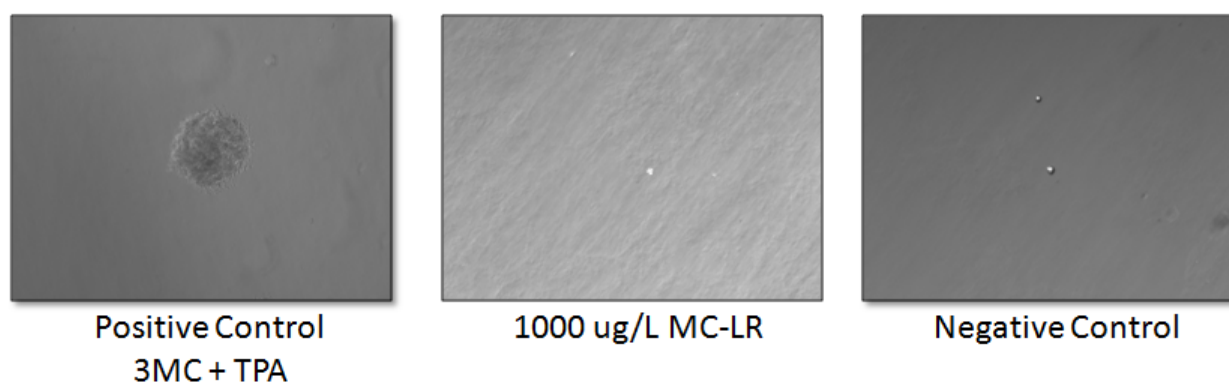
Figure 3. Proliferative activity of WB-F344 cells following 96 hours exposure to varying concentrations of microcystin-LR.



6.1.4. *Malignant transformation*

Because MCLR exposure could result in malignant transformation of hepatic stem cells, by activation of pathways independent of; oxidative stress, phosphatase inhibition or promotion of proliferative activity, WB-F344 cell colony formation in soft agar gel was determined in the presence and absence of MCLR. Here, a two-stage chemical carcinogenesis model was utilized to detect possible malignant transformative properties of MCLR at various concentrations (0.1-1000 µg/L) following 30 days of exposure to WB-F344 cells. Colony formation in soft agar was monitored over a 4 week period, revealing that MCLR (0.1-1000 µg/L) or in combination with 3MC alone, did not induce malignant transformation of non-malignant WB-F344 hepatocytes. The combination of 3MC and TPA (positive control) showed positive colony formation, measured by groups of cells of >20 cells per colony (Figure 4).

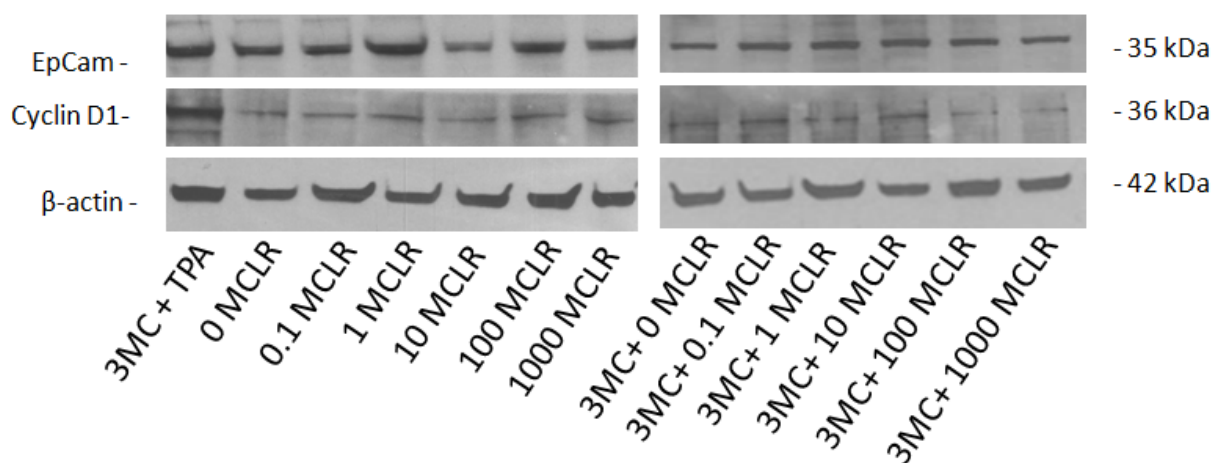
Figure 4. WB-F344 cell colony formation in soft agar gels following 30 days of exposure to varying concentrations of microcystin-LR.



6.1.5. Cyclin D1 and EpCAM Protein Expression

To further detect for signs of malignant transformation of WB-F344 cells exposed to varying concentrations of MCLR (0.1-1000 µg/L) for 30 days, we assessed the level of protein expression for two cell markers that are up-regulated in malignant cells: Cyclin D1 and EpCAM. WB-F344 cells were exposed to MCLR alone, and in combination with 3MC. Expression of Cyclin-D1 was unaltered in the presence of MCLR after 30 days exposure compared to negative controls. Cells co-treated with various concentrations of MCLR (0.1-1000 µg/L) and 3MC or TPA, also did not show any change in protein expression compared to negative controls. However, cells co-treated with 3MC and TPA (positive controls) expressed a significant increase in Cyclin-D1 and to a lesser extent EpCAM (Figure 5).

Figure 5. EpCAM and cyclin D1 protein expression in WB-F344 cells after 30 days exposure to varying concentrations of microcystin-LR.



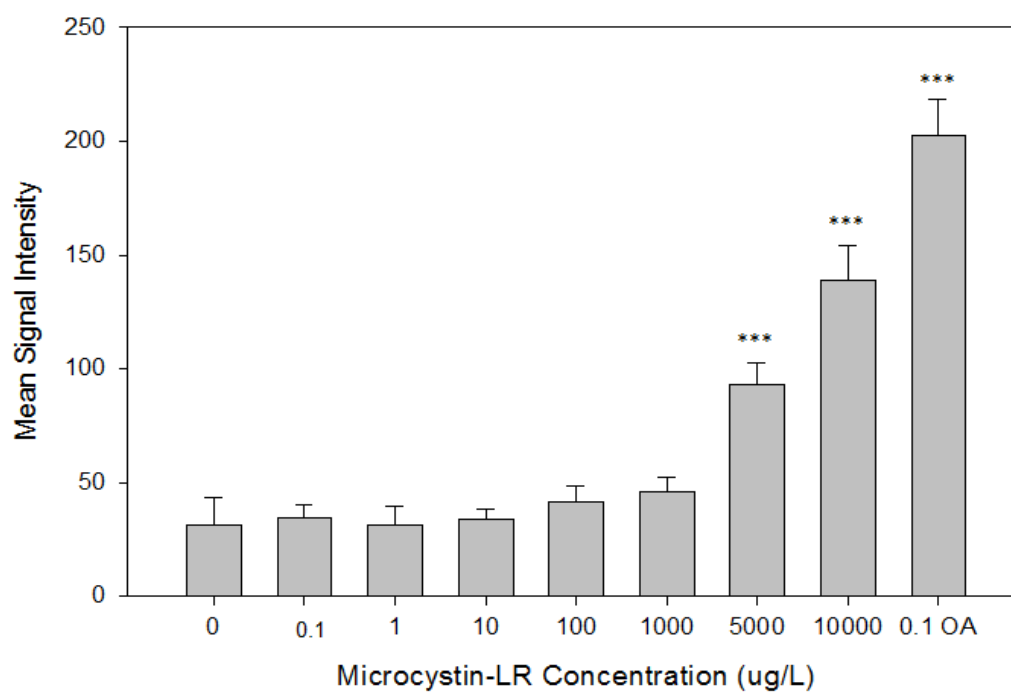
6.2. Enhancement of Malignant Hepatocyte Growth

MCLR is regarded as a potent tumor promoter. PLC cells were selected to determine whether exposure to low-concentrations of MCLR enhances the growth of malignant hepatocytes. To investigate potential enhancement of the malignant phenotype, PLC cells were exposed to varying concentrations of MCLR on oxidative stress, PP2A inhibition, proliferation and invasiveness.

6.2.1. Oxidative Stress in PLC Cells

To determine whether low-concentrations of MCLR are able to induce oxidative stress in malignant hepatocytes, PLC cells were exposed to varying concentrations of MCLR (0.1 – 1000 µg/L) for 24 hrs. We observed no increase in the oxidative stress after 24 hrs exposure for MCLR concentrations 0.1 – 1000 µg/L. However At the 5000 and 10000 µg/L of MCLR, significant increases ($p < 0.001$) in oxidative stress were documented. As predicted, okadaic acid (OA) (positive control), caused the greatest increase in oxidative stress ($p < 0.001$) (Figure 6).

Figure 6. Oxidative stress of PLC cells exposed for 24 hours to varying concentrations of microcystin-LR.

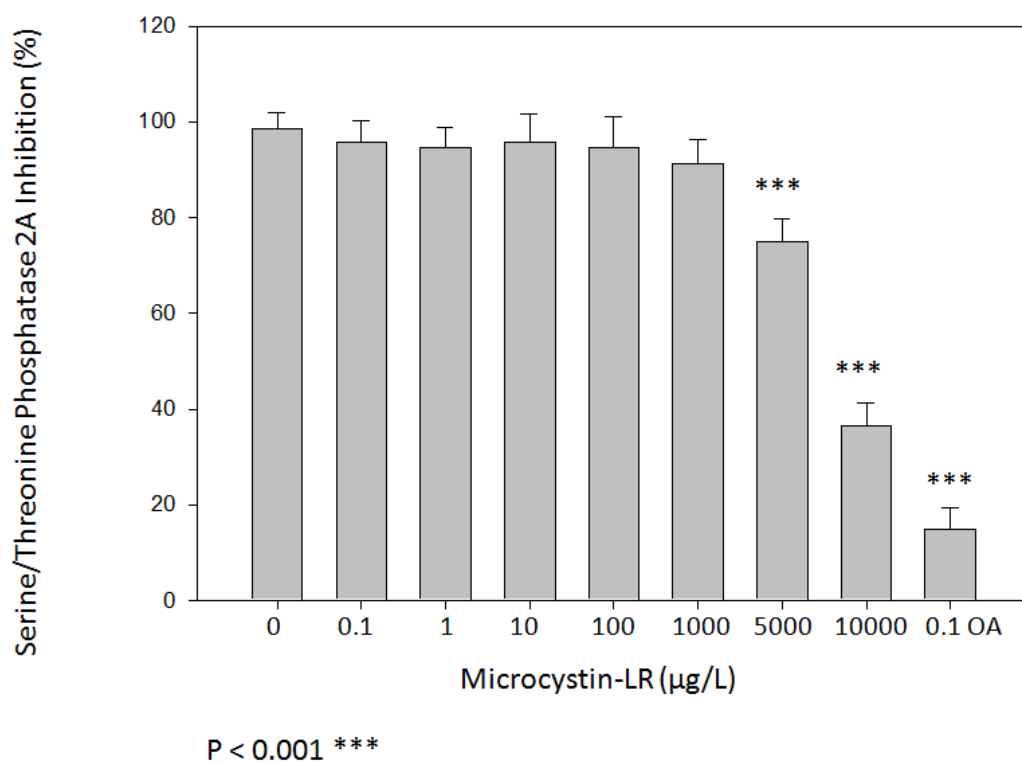


p < 0.001 ***

6.2.2. Serine/Threonine Phosphatase Inhibition in PLC Cells

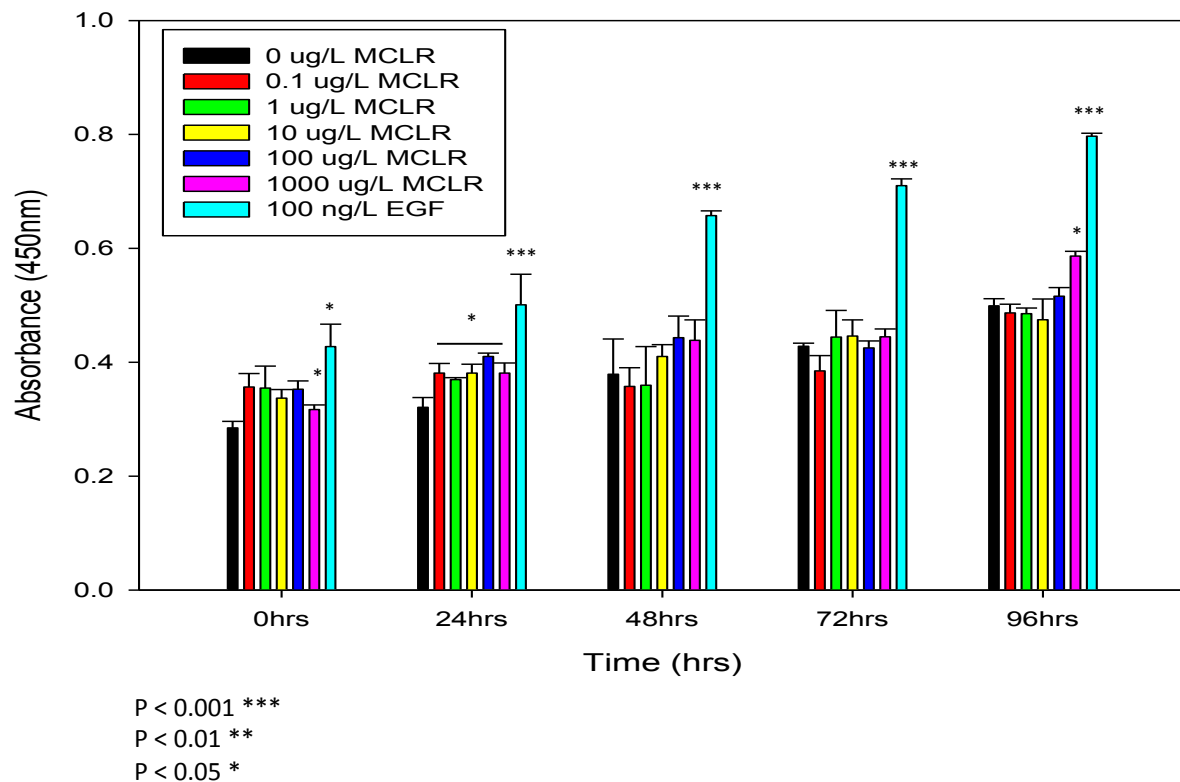
MCLR is a potent PP2A inhibitor, however it is unclear to what extent low-concentrations of MCLR are able to inhibit PP2A activity in malignant hepatocytes. Following a 24hr exposure to MCLR at varying concentrations (0.1 – 1000 µg/L), there was no significant change in PP2A activity in PLC cells observed. However, at MCLR concentrations of 5000 and 10000 µg/L, PP2A activity was inhibited. Similar to the WB-F344 experiments, OA was used as the positive control, and significantly decreased serine/threonine phosphatase 2A activity (Figure 7).

Figure 7. Serine/threonine phosphatase 2A activity in PLC cells following 24 hour exposure to varying concentrations of microcystin-LR



To determine whether low-concentrations of MCRL could alter the proliferative activity of PLC cells, and enhance the malignant phenotype, the proliferative activity of PLC cells was measured following 96 hrs exposure to MCLR. We observed that concentrations of MCLR (0.1 – 1000 $\mu\text{g/L}$), when administered over 96 hrs, did not consistently alter the proliferative activity of PLC cells. While proliferative activity was significantly increased at 24 hrs with all concentrations of MCLR (0.1-1000 $\mu\text{g/L}$), there was no dose-response effect and the increases were not sustained at 48, 72 or 96 hrs (Figure 8). As with WB-F344 cells, EGF served as the positive control, and increased proliferative activity of PLC cells at all time points.

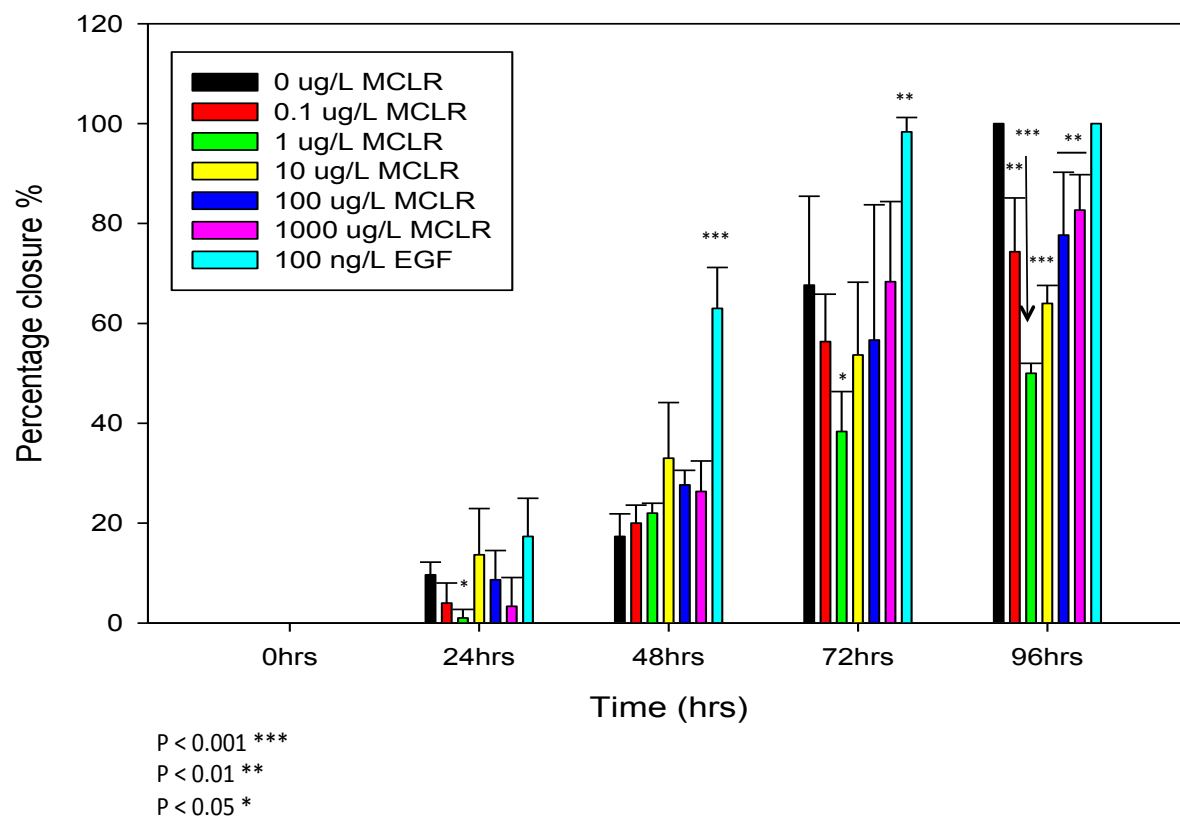
Figure 8. Proliferative activity of PLC cells exposed for 24 hours to varying concentrations of microcystin-LR.



6.2.4. *Invasiveness of PLC Cells*

The ability of MCLR to enhance the invasiveness of malignant hepatocytes, was measured by exposing PLC cells to various concentrations of MCLR over 96 hrs. The results of the wound healing assays revealed that low concentrations of MCLR (0.1 – 1000 µg/L), when administered over a 96hr period to PLC cells, had a varied effect on PLC cell invasiveness (Figure 9). At 24 hrs 0.1 and 1µg/L concentrations of MCLR inhibited wounded healing to a significant level compared to controls, whereas the remaining concentrations had no effect. At 48 hrs, all samples displayed similar wound healing compared to controls. However, at 72hrs wound healing was again inhibited for 0.1 and 1µg/L concentrations compared to controls. At 96 hrs, all MCLR concentrations (0.1 – 1000 µg/L) inhibited wound healing in PLC cells. Overall, these results can be interpreted as demonstrating that MCLR does not increase the invasiveness of PLC cells, as reflected by the wound healing assay.

Figure 9. Wound healing assay for PLC cells exposed for 96 hours to varying concentrations of microcystin-LR.



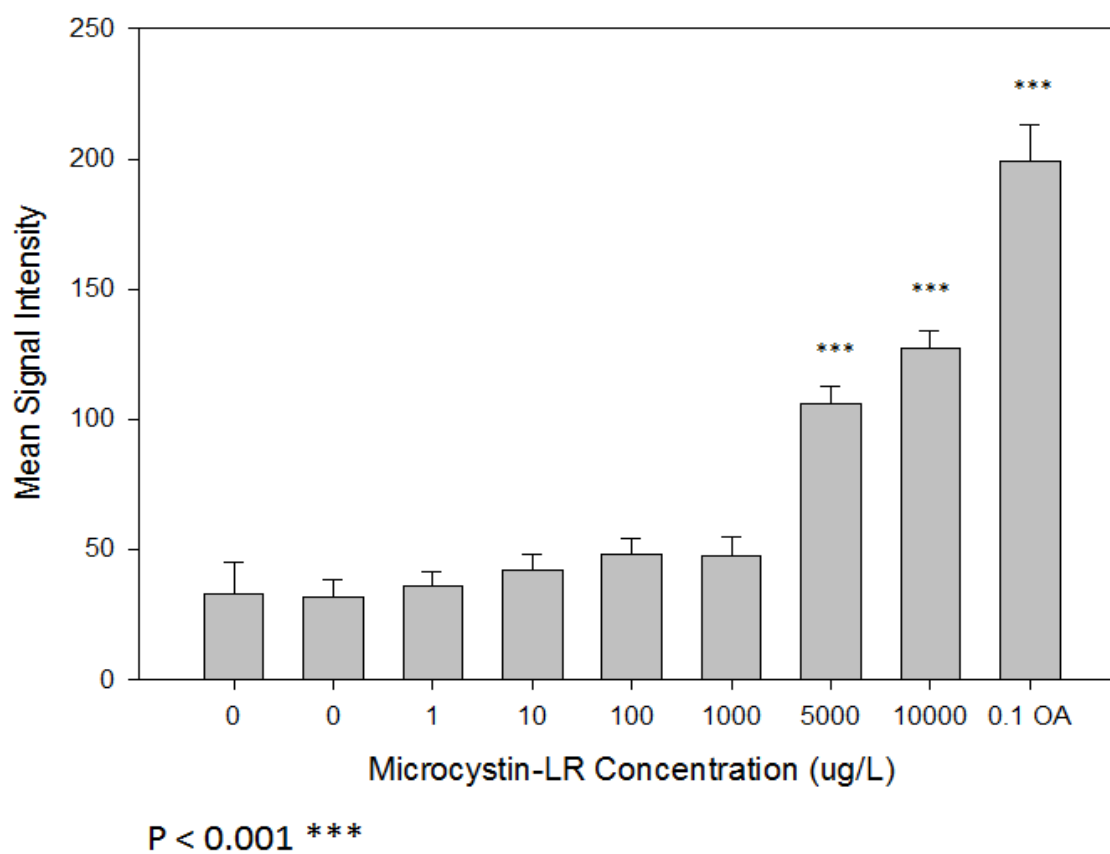
6.3. Fibrogenesis

Hepatic stellate cells are considered the principle mediators of hepatic fibrogenesis, becoming activated (perhaps via oxidative stress or by PP2A inhibition) in response to liver injury and inflammation. Thus, the purpose of these experiments was to determine whether MCLR promotes the development of HCC by activation of CFSC-2G cells and initiating fibrogenesis.

6.3.1. Oxidative Stress in CFSC-2G Cells

To determine whether exposure to low-concentrations of MCLR can induce oxidative stress in hepatic stellate cells, CFSC-2G cells were exposed to MCLR for 24hrs. Following exposure of CFSC-2G cells to varying concentrations of MCLR (0.1 – 1000 µg/L) for 24 hrs, there was no significant increase in the oxidative stress response observed. As with WB-F344 and PLC cells, at the 5000 and 10000 µg/L of MCLR, there was a significant increase in oxidative stress ($p < 0.001$), which increased with increasing MCLR concentration. OA (positive control), caused the most significant increase in oxidative stress ($p < 0.001$) compared to MCLR treated cells (Figure 10).

Figure 10. Oxidative stress of CFSC-2G stellate cells following 24 hour exposure to varying concentrations of microcystin-LR.



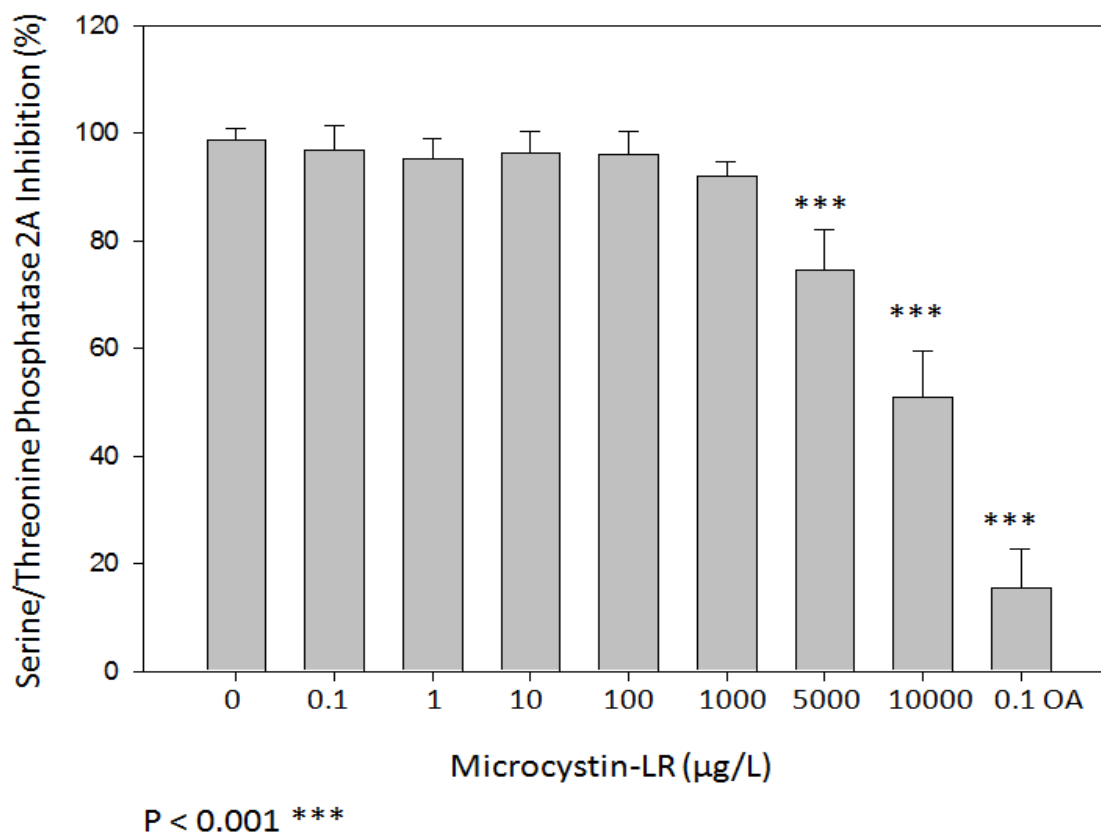
6.3.2. Serine/Threonine Phosphatase Inhibition in CFSC-2G Cells

To determine whether MCLR inhibits serine/threonine phosphatase 2A activity in hepatic stellate cells, CFSC-2G cells were exposed to varying concentrations of MCLR over 24 hrs.

Exposure of CFSC-2G cells to varying concentrations of MCLR (0.1 – 1000 µg/L), induced no significant changes in PP2A activity in CFSC-2G cells at 0.1-1000 µg/L MCLR exposure.

Inhibition of PP2A activity was observed at 5000 and 10000 µg/L of MCLR. As observed with WB-F344 and PLC cells, the positive control (OA), induced to greatest PP2A inhibition (Figure 11).

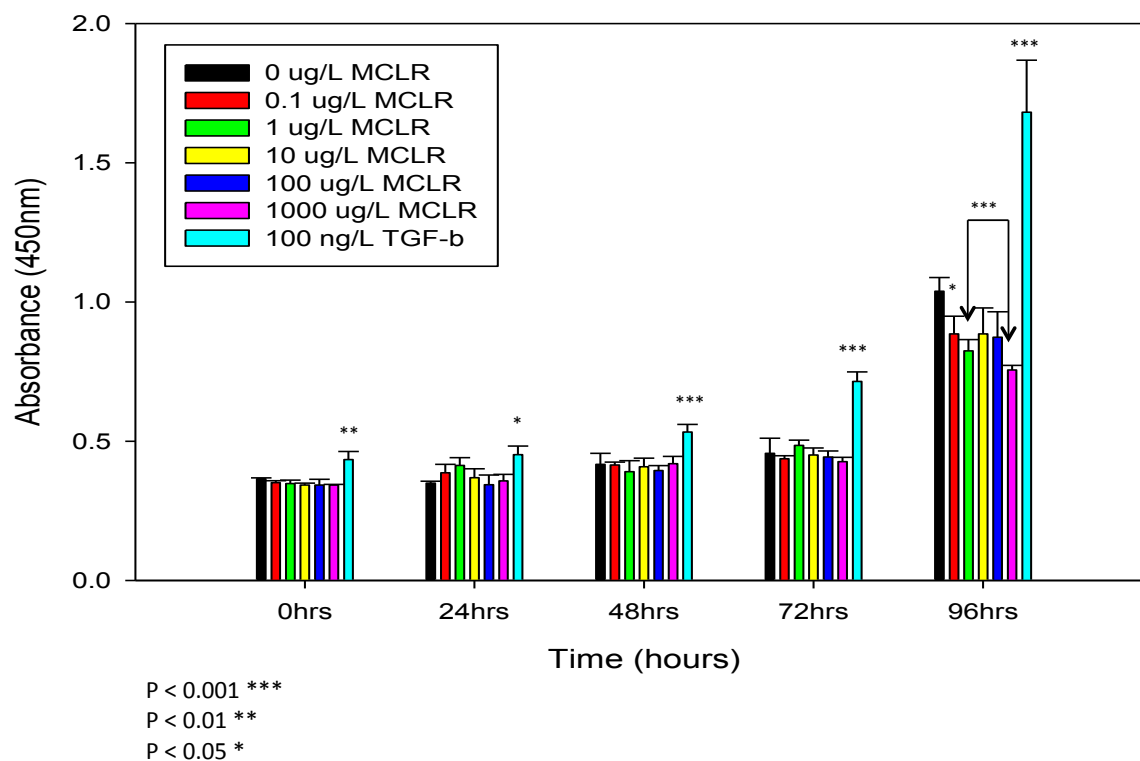
Figure 11. Serine/threonine phosphatase 2A activity in CFSC-2G cells following 24 hour exposure to varying concentrations of microcystin-LR.



6.3.3. Proliferative Activity of CFSC-2G Cells

A characteristic of activated hepatic stellate cells is they become proliferative. Therefore, CFSC-2G cells were exposed to low-concentrations of MCLR, to determine whether MCLR could activate them and enhance their proliferative activity. When administered over 72 hrs, MCLR (0.1 – 1000 µg/L) did not increase the proliferative activity of CFSC-2G cells. Indeed at 96 hrs, MCLR concentrations of 0.1, 1 and 1000 µg/L, significantly inhibited the proliferative activity of CFSC-2G cells. These findings suggest that MCLR has no consistent effect on the proliferative activity of CFSC-2G cells. Transforming growth factor - beta (TGF-β) (positive control) increased the proliferative activity of CFSC-2G cells at all time points (Figure 12).

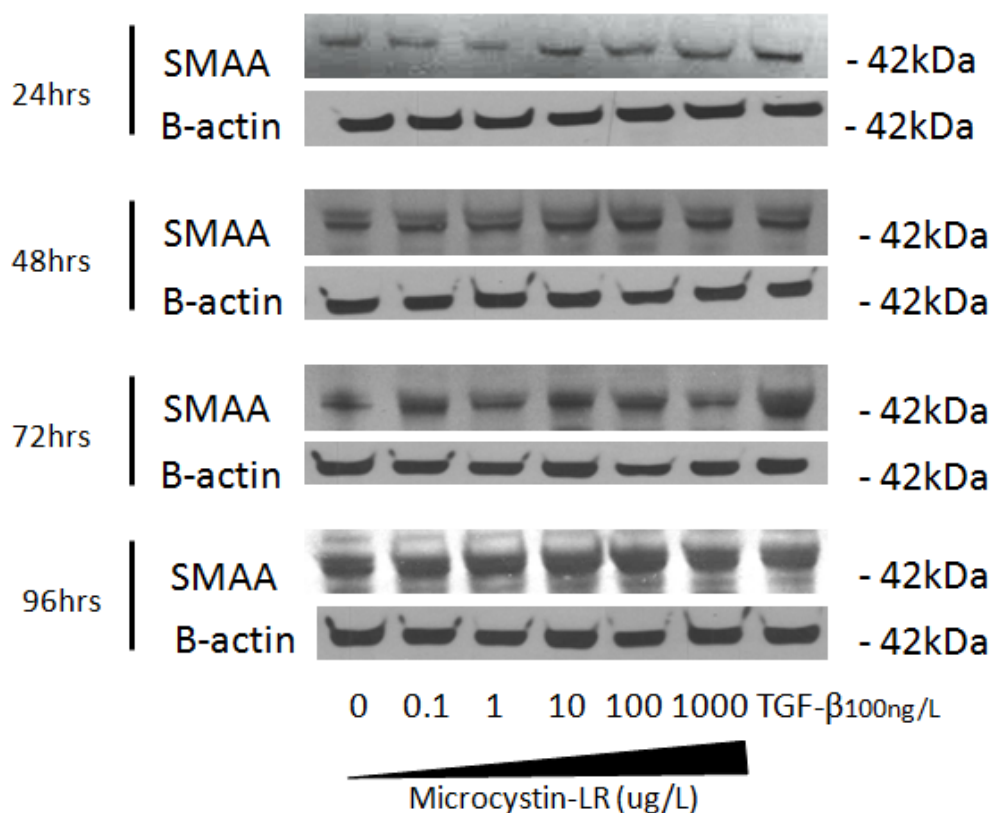
Figure 12. Proliferative activity of CFSC-2G stellate cells following 96 hours exposure to varying concentrations of microcystin-LR.



6.3.4. Smooth muscle alpha actin protein expression in CFSC-2G Cells

Smooth muscle alpha actin (SMAA) is a reliable indicator of HSC activation/transformation (Sato, Suzuki et al 2003). In response to MCLR exposure at various concentrations (0.1 – 1000 µg/L), CFSC-2G cells did not undergo activation compared to controls. Throughout the 96hr exposure period, cellular activation progressed at the same pace as negative controls (no MCLR) as measured by smooth muscle alpha actin (SMAA) protein expression visualized using Western blot analysis. TGF-β (positive control) showed an increase in SMAA expression within 24 hrs of exposure (Figure 13).

Figure 13. Smooth muscle alpha actin protein expression in CFSC-2G stellate cells exposed for 96 hours to varying concentrations of microcystin-LR.



6.4. *In Vivo Tumor Initiation, Promotion and Fibrogenesis*

In vivo studies have investigated the tumor initiating and promoting capabilities of MCLR using animal models of chronic liver disease; carbon tetrachloride (CCl₄) and diethylnitrosamine (DEN). These studies administered MCLR through i.p or intramuscular injection in order to by-pass digestive degradation of MCLR and increase circulating MCLR content. However, population exposure to MCLR occurs primarily through oral ingestion of low-dose MCLR in drinking water sources.

Thioacetamide (TAA) is a compound that requires oxidative bio-activation to become metabolically activated. TAA is first metabolized in the liver to form TAA-S-oxide (TASO) and then converted to TAA-S.S-dioxide (TASO₂) (Dashti, Jeppsson et al. 1989; Hajovsky, Hu et al. 2012). TASO₂ is the toxic metabolite, which binds to amine-lipids and proteins to induce oxidation and hepatic necrosis. TAA administered in drinking water at a concentration of 0.03% w/v, leads to the development of cirrhosis and HCC over a period of 5-7 months. TAA induced cirrhosis is achieved over a longer period of time compared to the CCl₄ model, and is associated with decreased animal mortality (Dashti, Jeppsson et al. 2008). TAA was selected as the model to induce cirrhosis and HCC, because; 1) it could be administered within drinking water along with MCLR, 2) the cirrhosis and liver tumors created more closely approximate to those seen in humans and 3) it is associated with decreased animal mortality compared to CCl₄ administration.

6.4.1. Body and Liver Weights

Over the course of the 7-month exposure period to MCLR, there was no significant change in body weight of the MCLR treated mice, (55.8 ± 5.29) compared to the water alone control group. There was a significant change in body weight observed in the two groups treated with TAA alone (47.4 ± 3.5) and TAA & MCLR treated (45.7 ± 4.1 g) groups. The liver weights of those animals from TAA groups were not significantly different from those of the MCLR or water alone groups, the average liver weight as a percent of body weight was significantly higher in the TAA alone (6.5 ± 0.2 %) and TAA & MCLR (6.0 ± 0.1 %) treated groups (Table 3).

Table 3. Gross pathological findings of male CD1 mice exposed for 7 months to microcystin-LR in drinking water.

Treatment Group	Body Weight (BW) (g)	Liver Weight (LW) (g)	LW as % of BW (%)	Tumor Number	Tumor Size (mm)
Control	53.8 ± 5.3	2.8 ± 0.3	5.0 ± 0.1	0	0
MCLR	55.8 ± 5.2	2.7 ± 0.6	4.8 ± 0.1	0	0
TAA	$47.4 \pm 3.5^*$	3.1 ± 0.6	$6.5 \pm 0.2^{**}$	4*** (2 regenerative nodules)	$6.3 \pm 5.9^{***}$
TAA & MCLR	$45.7 \pm 4.1^{**}$	2.9 ± 0.5	$6.0 \pm 0.1^{**}$	5***	$6.5 \pm 5.0^{***}$

P < 0.001***

P < 0.01**

P < 0.05*

6.4.2. Plasma Chemistry

There were no significant changes in serum alanine aminotransferase (ALT) or total bilirubin levels in the MCLR alone compared to water alone controls (Table 4). However, TAA alone (ALT = 68.71 ± 25.71 U/L and total bilirubin 0.21 ± 0.03 mg/dL) and TAA & MCLR (ALT = 69.12 ± 23.16 U/L and total bilirubin = 0.22 ± 0.04 mg/dL) treated animals had significantly higher ALT and total bilirubin levels than water alone controls. When the results of TAA alone group were compared to the TAA & MCLR treatment group, there were no significant differences in ALT or total bilirubin levels (Table 4 and Figure 14).

Table 4. Liver plasma chemistry (alanine aminotransferase and total bilirubin) and histology grade of CD1 male mice exposed for 7 months to microcystin-LR in drinking water

Treatment Group	ALT Levels (U/L)	Total Bilirubin Levels (mg/dL)	Inflammation Grade	Fibrosis Grade
Control	43.61 ± 17.48	0.11 ± 0.03	0.88 ± 0.89	0.06 ± 0.01
MCLR	48.35 ± 25.32	0.12 ± 0.05	1.16 ± 0.5	0.05 ± 0.015
TAA	$68.71 \pm 25.71^*$	$0.21 \pm 0.03^{**}$	$3.26 \pm 0.65^{**}$	$1.79 \pm 0.71^{**}$
TAA & MCLR	$69.12 \pm 23.16^*$	$0.22 \pm 0.04^{**}$	$2.57 \pm 0.99^{**}$	$2.0 \pm 1.03^{**}$

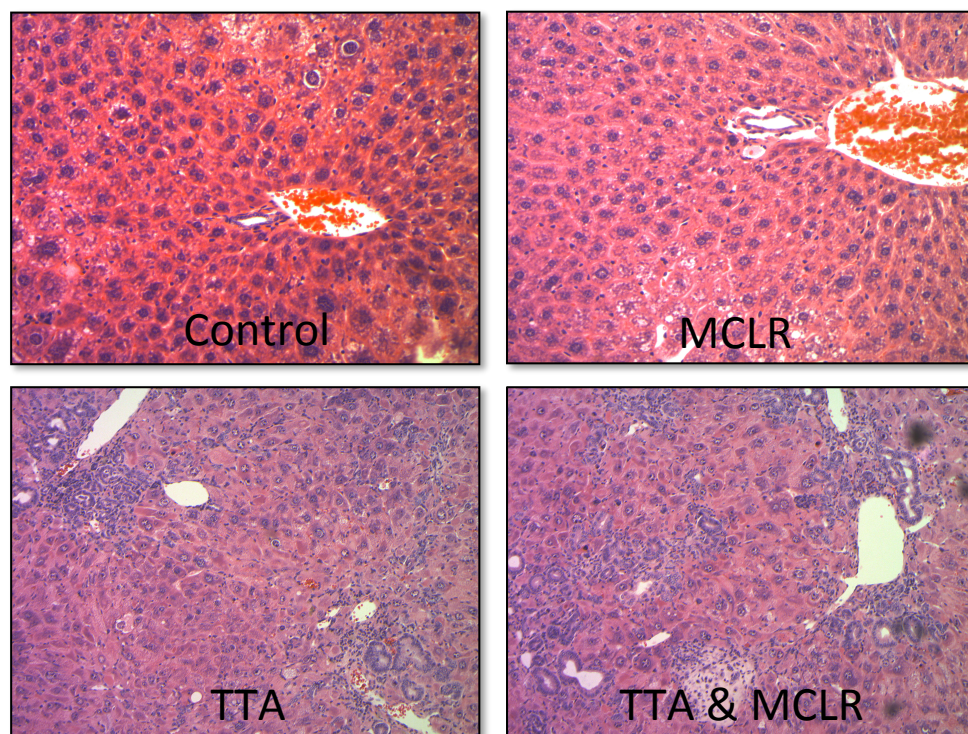
P < 0.001***

P < 0.01**

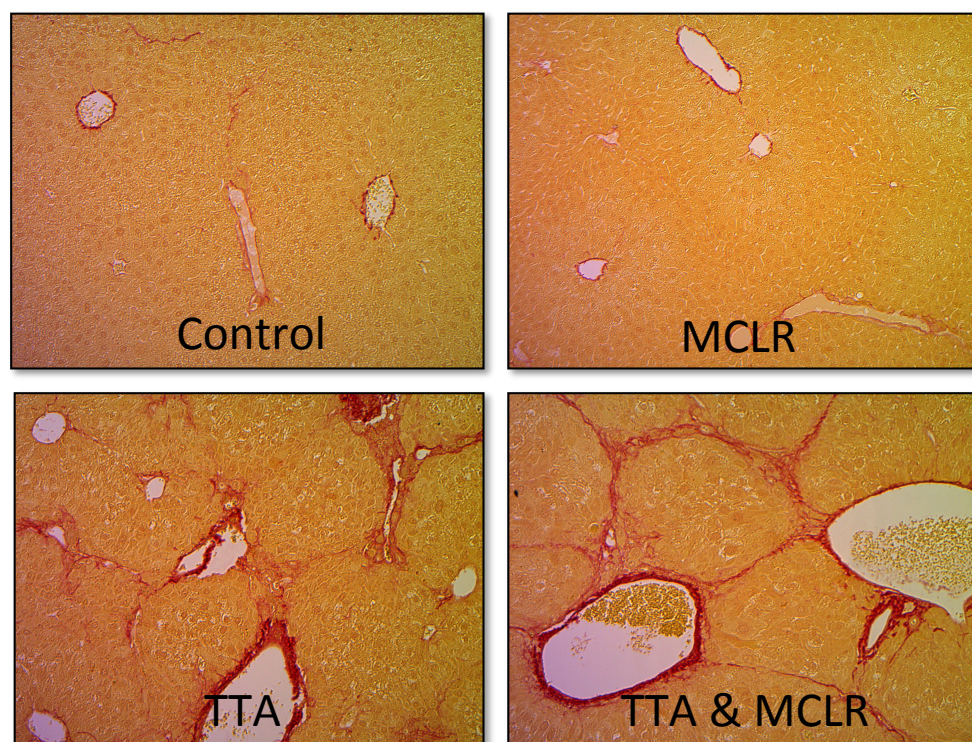
P < 0.05*

Figure 14. Histological stains of livers of male CD1 mice exposed to microcystin-LR for 7 months. a) Hemotoxylin and Eosin stain for inflammation and b) Picric Acid Sirius Red stain for fibrosis.

a)



b)



6.4.3. Liver Appearances and Histology

Upon inspection, the livers of mice in the water and MCLR alone groups appeared normal; red in color, supple and flexible consistency, while those livers from the TAA treatment groups appeared discolored, pale, hardened and cirrhotic.

Liver histology was examined for architecture, inflammation and fibrosis using H&E Picric Acid Sirius Red stained slides. As shown, the livers of mice from the water alone group had normal hepatic sinusoidal patterns and distribution of hepatocytes and lobules. Inflammation as measured using the metavir scoring system (0-4) of H&E stained slides, revealed only minor inflammation (<1) within this cohort. The degree of fibrosis was also minimal (<1 on a fibrosis scale of 0-4). For both inflammation and fibrosis, the MCLR alone exposed group was increased compared to water alone controls, but not to a significant extent ($p > 0.05$). In the TAA and TAA & MCLR treated groups, inflammation was significantly increased compared to water alone controls. The level of collagen deposition and fibrosis was also significantly increased in TAA treated groups compared to water alone controls. However, between TAA alone and TAA & MCLR treated groups there was no significant difference in collagen deposition. The average inflammation and fibrosis grade for the four treatment groups were as follows; water alone: 0.88 ± 0.89 and 0.06 ± 0.25 , MCLR alone: 1.16 ± 0.5 and 0.26 ± 0.45 , TAA alone: 3.26 ± 0.65 and 1.79 ± 0.71 and TAA & MCLR: 2.57 ± 0.99 and 2.00 ± 1.03 (Table 4 and Figure 14). These data indicate that long-term, low-dose MCLR exposure does not result in significant inflammation or fibrosis, nor does it increase the extent of existing inflammation or fibrosis in this model of chronic liver disease.

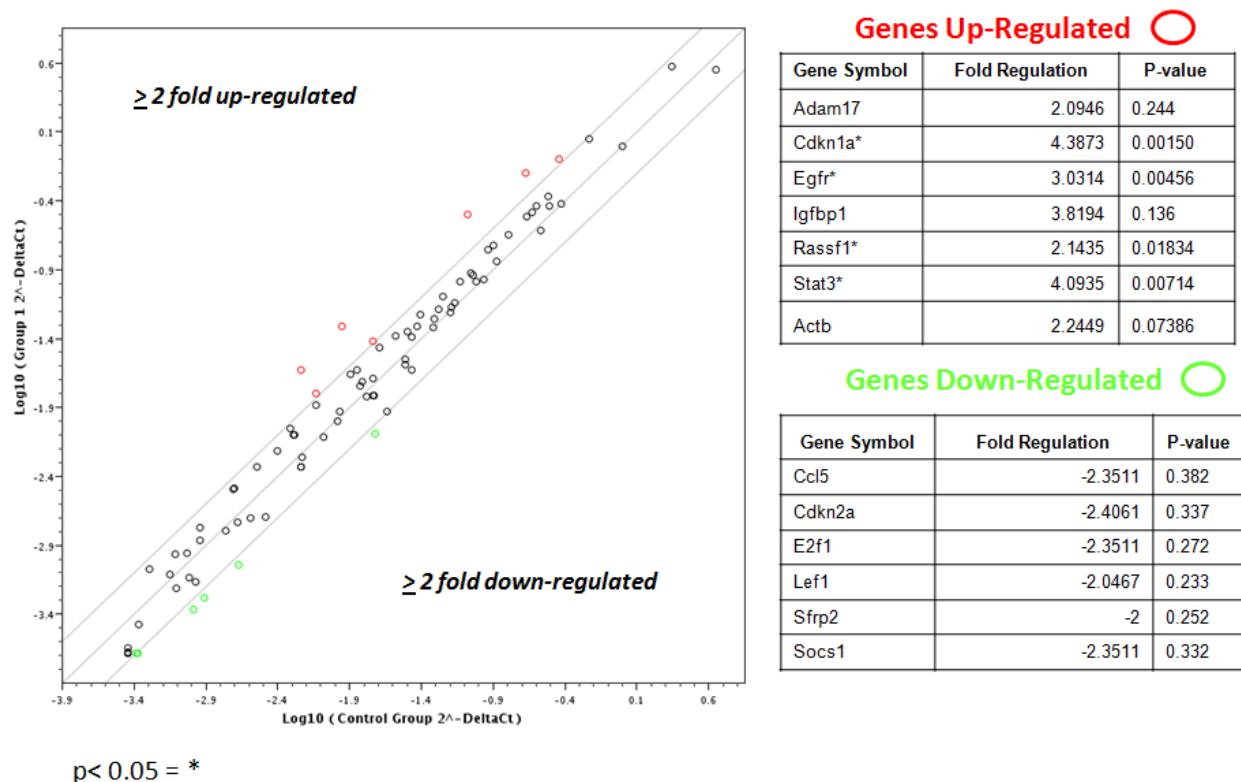
6.4.4. Tumor Formation

Resected livers were subjected to careful pathological examination for the presence of liver tumors immediately after removal. There were no tumors in the water alone or MCLR alone treated groups. Tumors were identified in 4 mice within the TAA alone group and 5 mice within the TAA & MCLR group. The tumors were poorly differentiated, but non-malignant (no signs of micro vascular or adjacent tissue invasion). The average tumor size did not significantly differ between the TAA alone (6.3 ± 5.9 mm) and TAA & MCLR (6.5 ± 5 mm) treatment groups (Table 3). Here again, the results suggest that long-term, low-concentration exposure to MCLR does not result in de novo tumor formation in healthy livers or increase the number or size of tumors developing in this animal model of hepatocellular carcinoma

6.4.5. Microarray Analysis

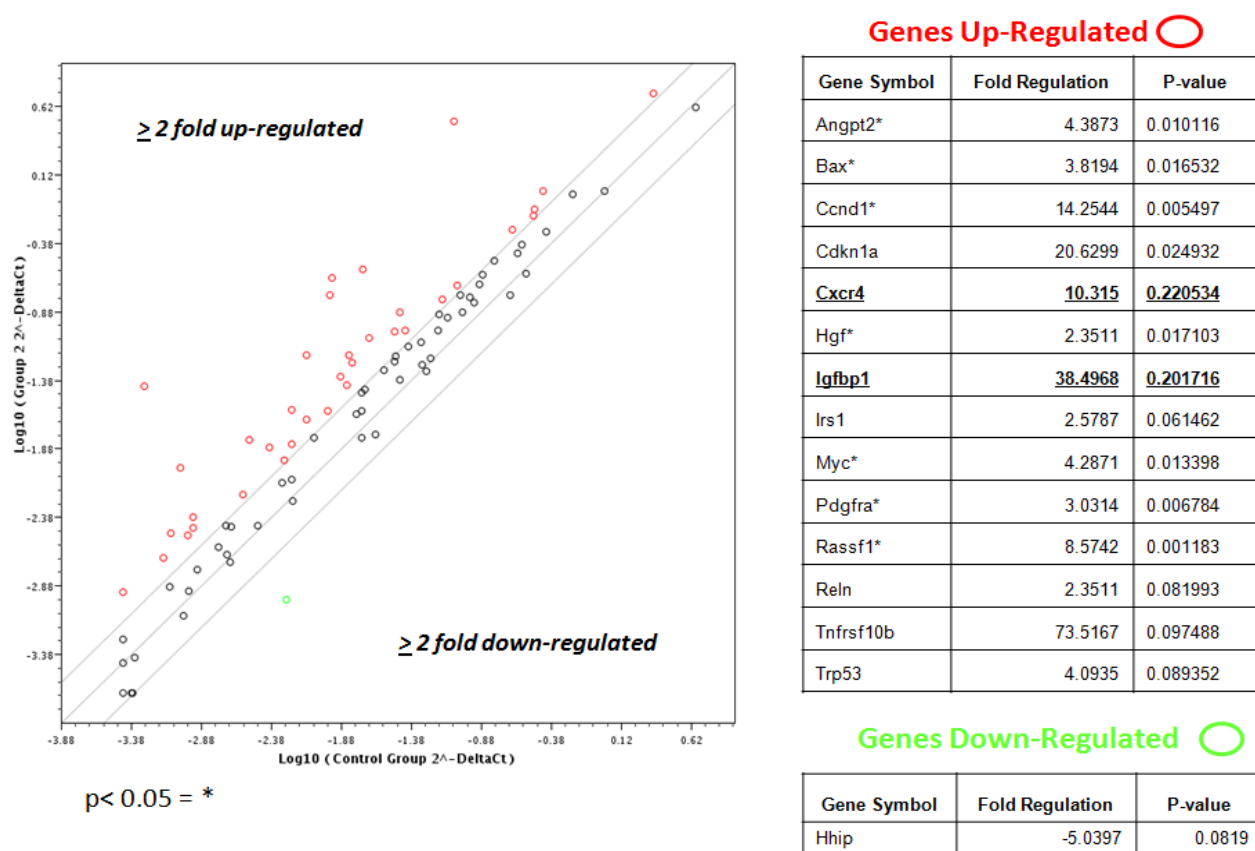
To further determine if chronic exposure to MCLR alters the expression profile of genes associated with HCC development, liver tissues were profiled using liver cancer pcr arrays. Analysis using the Qiagen pcr array software revealed statistically significant fluctuations in the expression of 4 genes in the MCLR alone treated group compared to water alone controls (Figure 15). Up-regulated genes were those associated with adhesion/proteolysis, transcription, insulin growth factor binding, and various signal transduction pathways: Cdkn1a, Stat3, Rassf1 and Edgfr. For the most part, gene de-regulation was relatively limited (less than 5 fold). Only Cdkn1a and Stat3 expression were > 4 fold higher in MCLR alone treated mice (Figure 15).

Figure 15. Hepatocellular carcinoma gene expression array for male CD1 mice exposed for 7 months to microcystin-LR in drinking water compared to negative control.



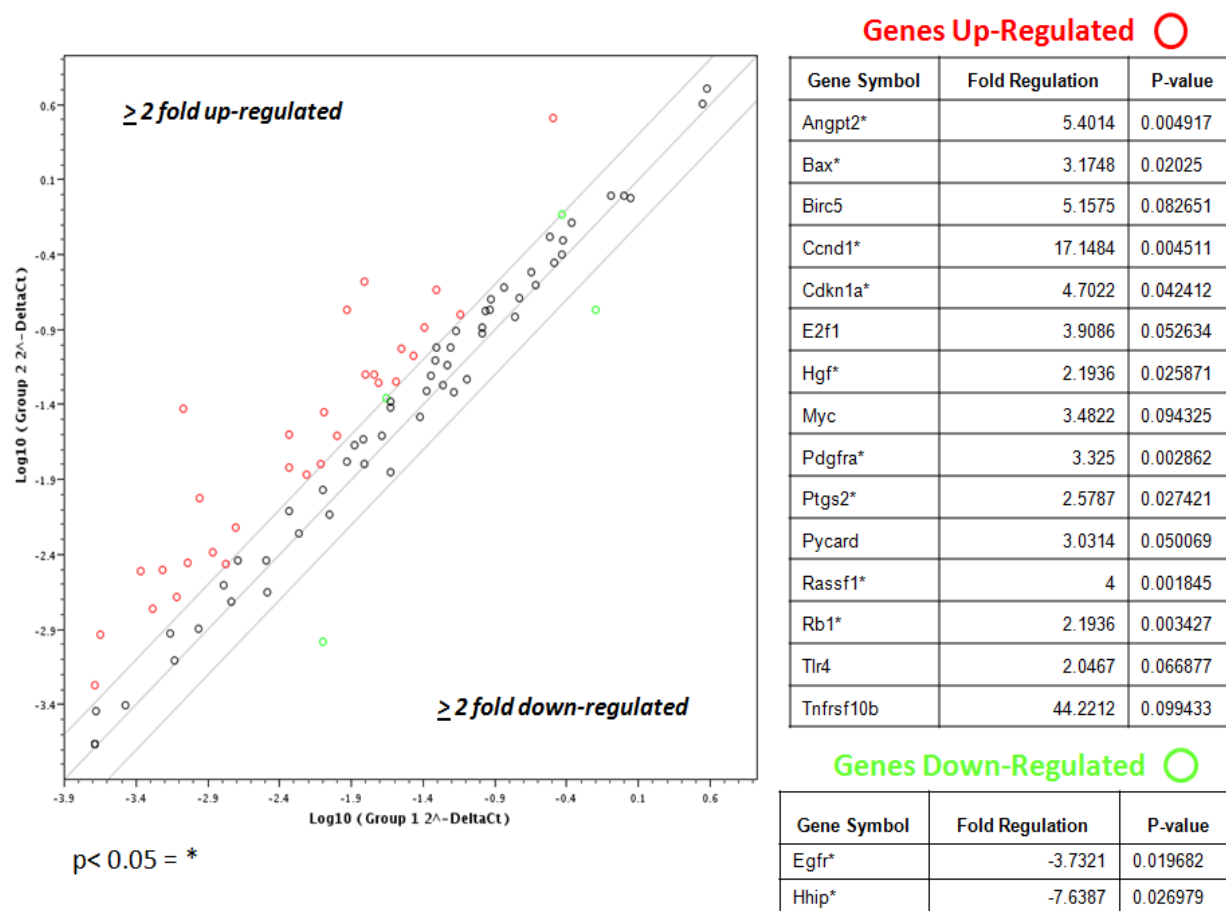
alone controls revealed significant alterations in the expression of 7 genes. Up-regulated genes were associated with; angiogenesis, apoptosis, cell cycle regulation, DNA damage and repair, adhesion and proteolysis, immune and inflammatory response, epithelial to mesenchymal transition and numerous signal transduction pathways including; classical WNT, P13/AKT, MET/HGF, RAS/RAF/ERK/MET and small GTPase mediated. The most marked changes in expression were observed in Ccnd1, Cxcr4, Cdkn1a, Angpt2, Tnfrsf10b, Igfbp1, Myc, Rassf1 and Hhip genes, having a > 4 fold fluctuation (Figure 16).

Figure 16. Hepatocellular carcinoma gene expression array for male CD1 mice exposed for 7 months to thioacetamide in drinking water compared to negative control.



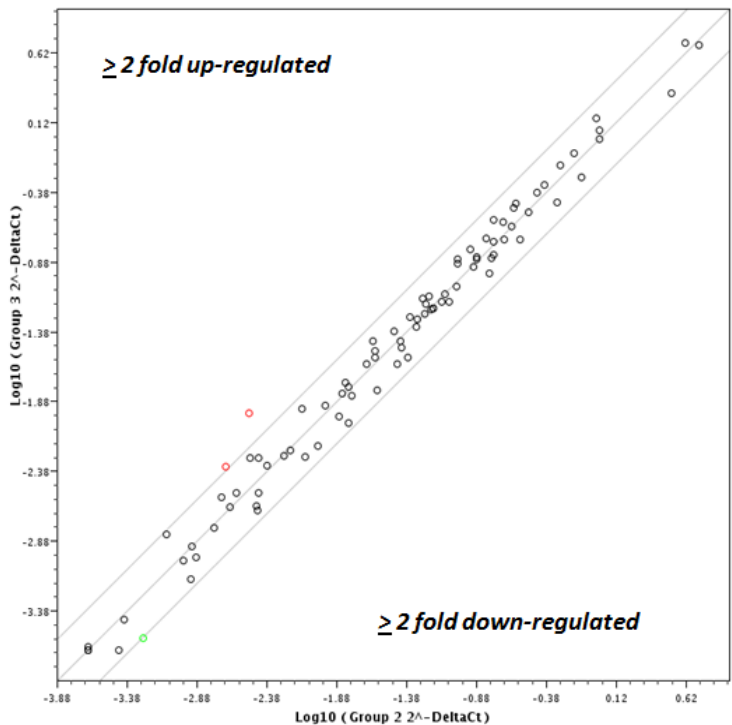
Using the same gene analysis parameters to compare the TAA alone to the MCLR alone treatment group revealed that there were 11 genes with significantly altered expression. Genes with > 2 fold altered expression were involved in; angiogenesis, apoptosis, DNA damage/repair, immune and inflammatory response, cell cycle regulation and various signal transduction pathways including; classical WNT, small GTPase mediated, RAS/RAF/ERK/MEK, P13K/AKT, insulin growth factor and MET/HGF. The most marked changes in expression observed in Ccnd1, Angpt2, Tnfrsf10b, Birc5 and Hhip genes, having a > 4 fold fluctuation (Figure 17).

Figure 17. Hepatocellular carcinoma gene expression array for male CD1 mice exposed for 7 months to thioacetamide versus microcystin-LR.



Profiling of genes comparing the TAA alone to the TAA & MCLR treated groups revealed similar changes in gene expression. Indeed, the only significant difference in expression was observed for the Ptgs2 gene, which was down-regulated 2 fold in the TAA alone group (Figure 18).

Figure 18. Hepatocellular carcinoma gene expression array for male CD1 mice exposed for 7 months to thioacetamide versus thioacetamide and microcystin-LR.



p< 0.05 = *

Genes Up-Regulated ○

Gene Symbol	Fold Regulation	P-value
Cdkn2a	3.4822	0.795109
Wt1	2.1435	0.214472

Genes Down-Regulated ○

Gene Symbol	Fold Regulation	P-value
Ptgs2*	-2	0.0239

6.5. Canadian Epidemiological Study

Across Canada the rates of liver cancer incidence has been steadily increasing for both males and females over the past two decades, despite efforts to limit risk factors (eg, neonatal HBV vaccinations, HCV anti-viral treatment, etc). Toxigenic cyanobacterial blooms continue to persist in fresh water sources across the country, releasing high concentrations of MCLR into the environment. Scientific evidence supports the role of MCLR as a tumor promoter at the cellular and systemic levels (Nishiwaki-Matsushima, Ohta et al. 1992; Ohta, Nishiwaki et al. 1992; Zhu, Zhong et al. 2005). Epidemiological studies from China, United States and Serbia have found an association between consumption of MCLR contaminated drinking water and increased HCC incidence in exposed populations (Ueno, Nagata et al. 1996; Fleming, Rivero et al. 2002; Svircev, Krstic et al. 2009; Li, Chen et al. 2011).

The Canadian epidemiological study was conducted in order to document the geographical relationship between liver cancer, cyanobacterial up-stream markers and other socio-demographic factors. The study collected cancer incidence, land utilization and socio-demographic data between 1996 and 2004, a period that coincides with increasing liver cancer incidence in Canada.

6.5.1. Distribution of Liver Cancer

Between the years of 1996 to 2004, 9,288 incident cases of liver cancer in the 40+ population were identified, having an annual crude rate of 7.55 cases/100,000 person (ranging from 2.8 to 12.6/100,000 person) years of exposure. In both the unsmoothed and smoothed thematic maps (Figure 19 and Figure 20), major urban centers posed the highest rates of liver cancer; Vancouver, Montreal, Calgary and Toronto, with Vancouver (1.7 times

greater), Montreal (1.74 times greater) and Toronto (1.6 times greater) having higher rate clusters than expected (Figure 21). The lowest liver cancer cluster rates appeared in Northern Saskatchewan, British Columbia, Ontario and the far North. The location of statistically significant ($p < 0.05$) high and low rate clusters of liver cancer were identified using a spatial scan statistic. Using this technique, Canadian liver cancer incidence was non-randomly distributed, with high rate clusters appearing in large urban centers and the lowest rates occurring primarily within the North, Maritimes and rural areas.

To further explore the distribution pattern of liver cancer in Canada, the Gini coefficient for liver cancer incidence from 1996-2004 was calculated, by plotting the ranked cumulative proportion of liver cancer incidence cases against the ranked cumulative proportion of the population at risk (Figure 22). The plotted data generates a Lorenz curve, and was used to calculate the Gini coefficient. The minimal deflection of the curve, generated a Gini coefficient of 0.196, indicating that liver cancer cases are fairly evenly distributed across Canada.

Figure 19. Canadian hepatocellular carcinoma Cases per 100,000 within the 40+ population measured between 1996 – 2004. The map depicts the un-smoothed geographic distribution of HCC cases.

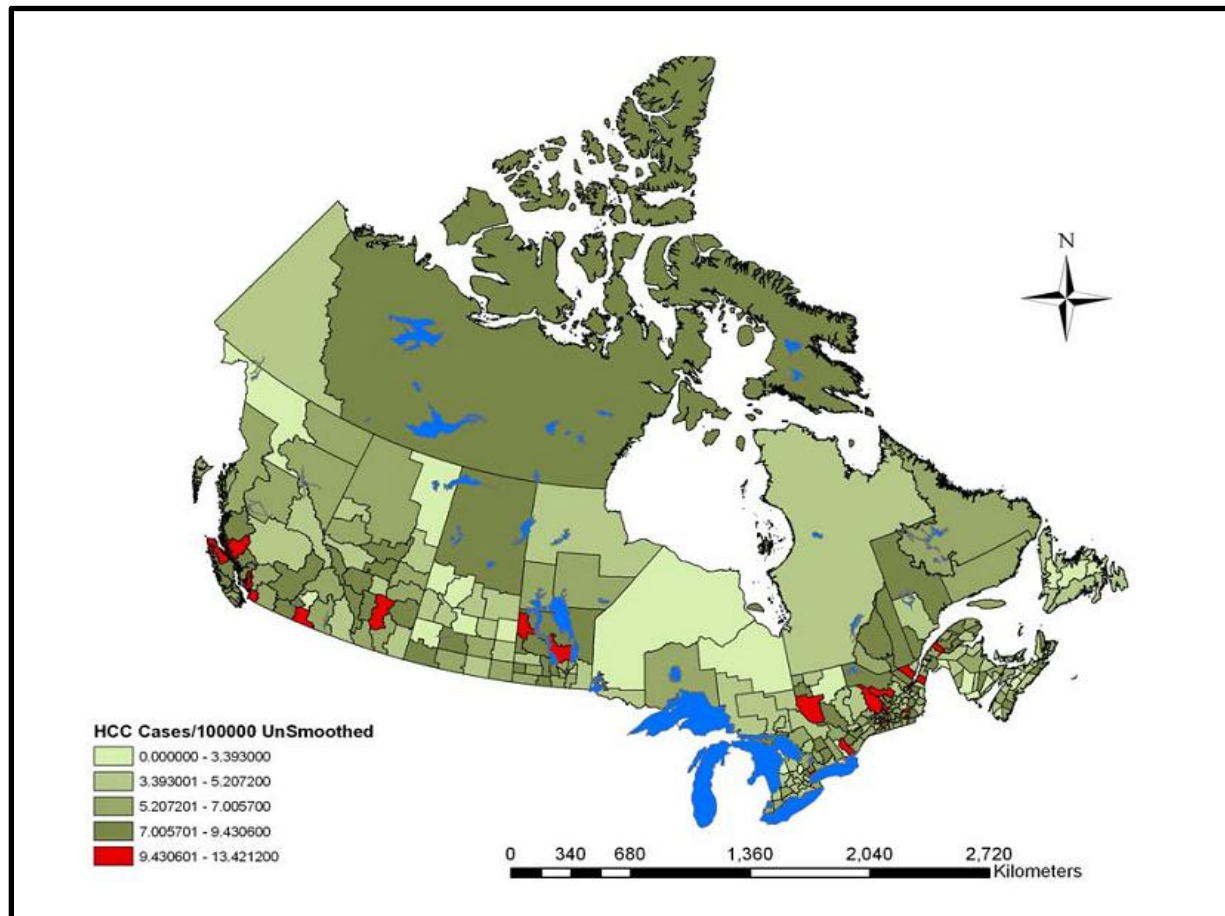


Figure 20. Canadian hepatocellular carcinoma Cases per 100,000 within the 40+ population measured between 1996 – 2004. The map depicts the smoothed geographic distribution of HCC cases, using the 5 nearest spatial empirical bayes (5 nearest neighbors).

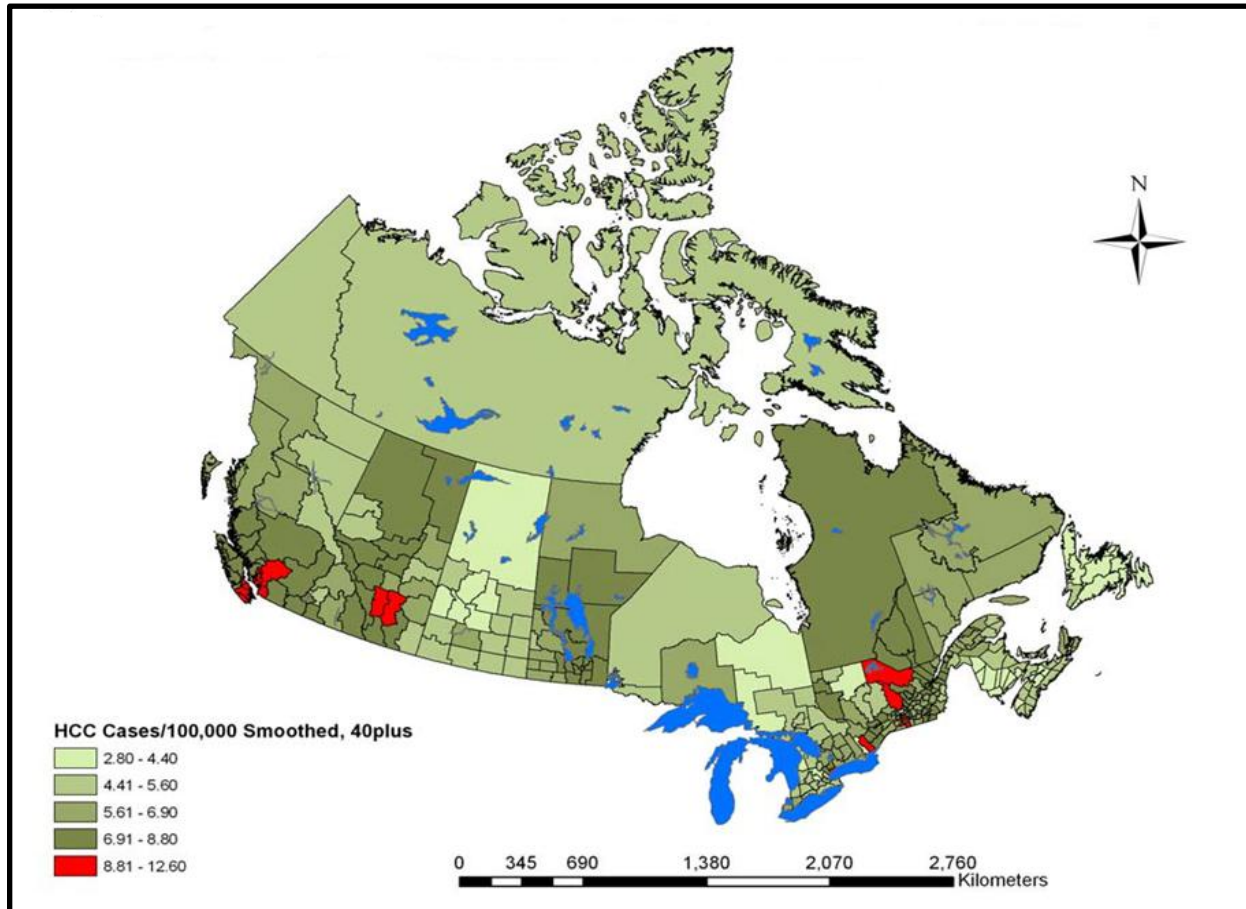


Figure 21. Cluster identification of hepatocellular carcinoma cases in Canada between 1996 and 2004, using spatial scan statistic (Satscan) software. Figure depicts regions of high, medium and low rate clusters.

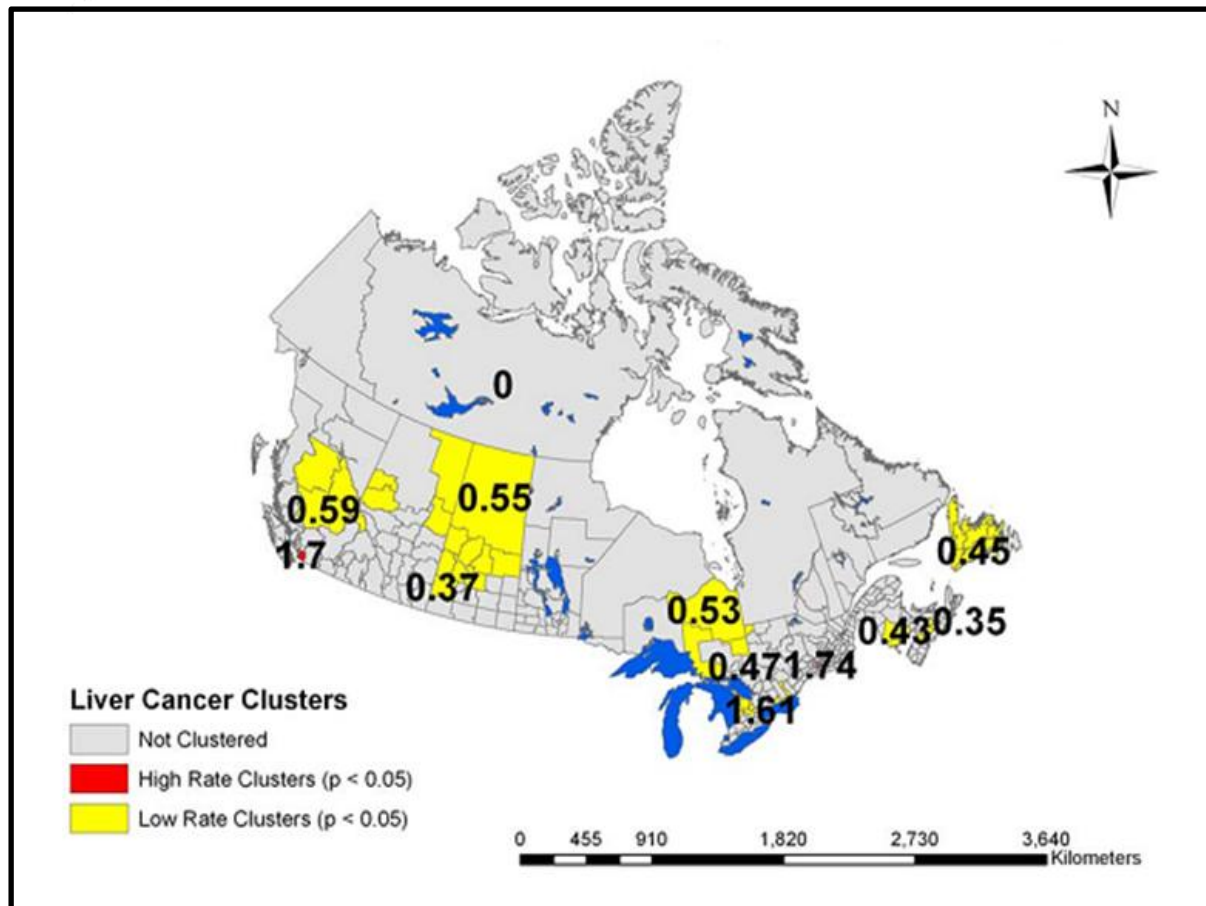
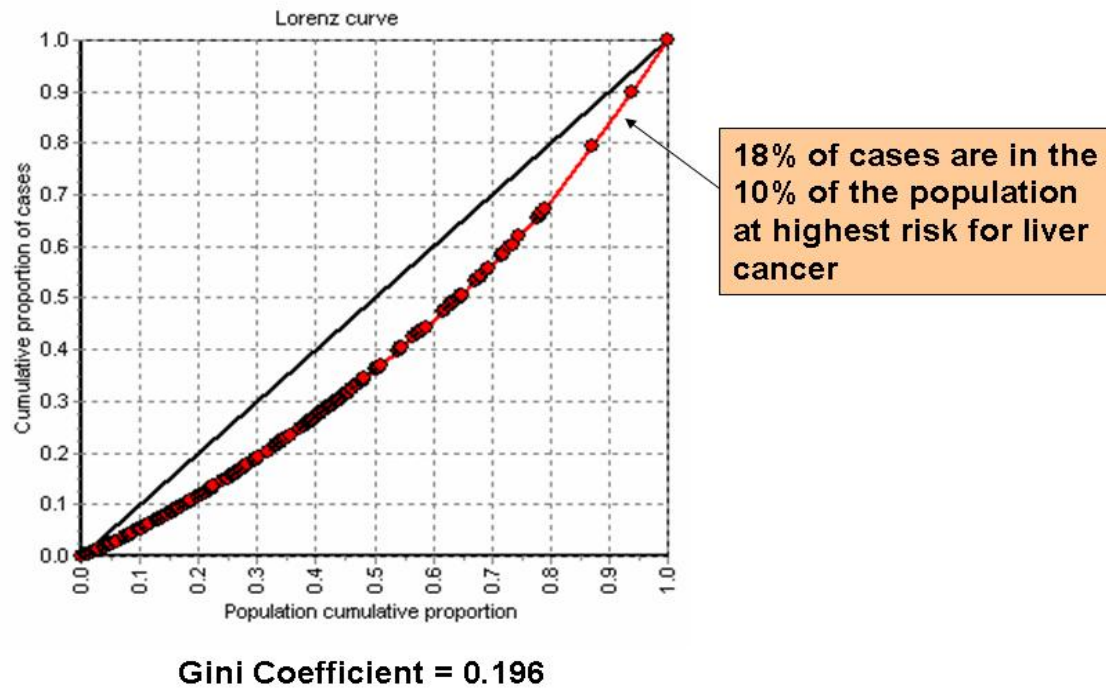


Figure 22. The Gini coefficient describes the spatial relationship between hepatocellular carcinoma incidence and geographic location.



6.5.2. *Socio-Demographic and Landscape Factors and Liver Cancer*

Socio-demographic and landscape factors were utilized to investigate further the relationship between liver cancer incidence, liver cancer risk factors and cyanobacterial predicting variables. Negative binomial regression analysis reveals that spatially, liver cancer incidence varies in accordance with a variety of socio-demographic factors in unpredictable directions (Table 5). To determine the risk of developing primary liver cancer (PLC), relative risk (RR) was calculated by dividing the incidence rate of the category of interest (middle and high range) by the rate of the reference category (lowest range, control), using a partially adjusted model (variables adjusted for age and gender). Overall (urban and rural combined), liver cancer incidence was higher in areas of urban residence (RR = 1.25, $p < 0.0001$) (Figure 23), recent immigration (RR = 1.89, $p < 0.0001$) (Figure 24) and Hepatitis B infection (RR = 1.16, $p < 0.0014$) (Figure 25). The RR for the demographic distribution of liver cancer incidence by province showed significantly elevated rates in the Northwest Territories, British Columbia, Alberta and Manitoba (Table 5). The lowest liver cancer incidence rate for the country was observed in Newfoundland.

Due to the lack of a complete dataset for cyanobacteria and/or MC occurrence within Canada, upstream predicting variables for potential cyanobacterial contamination were utilized. For the overall analysis (as shown in Table 5), the only up-stream cyanobacterial contamination variables that predicted increased RR for liver cancer was high cattle density (Figure 26 and Table 5) (RR = 1.27, $p < 0.01$). It was also observed that the RR for liver cancer incidence was highest in urban residences compared to rural (Table 5).

Table 5. Univariate analysis of combined urban and rural socio-demographic and landscape factors to predict relative risk of primary liver cancer (PLC) in Canada.

Variable	Range	Overall RR (95% CI-RR)
Hepatitis B (<i>Cases/100 000</i>), 1989-2004	< 3.55 3.55 – 5.24 >5.24	1.00 0.72** (0.61-0.65) 1.16** (1.06-1.27)
Hepatitis C (<i>Cases/100 000</i>), 1989-2004	<36.21 36.21-56.49 >56.49	1.00 0.94 (0.85-1.05) 0.99 (0.87-1.27)
Recent Immigration (%), 2001	<1.28 1.28-4.42 >4.42	1.00 1.18** (1.05-1.33) 1.89** (1.58-2.27)
Avg Family Income (\$), 2001	<52,179 52,179-64,006 >64,006	1.00 0.93 (0.82-1.05) 1.15 (0.99-1.33)
Binge Drinkers (%), 40- 64 yrs, 2007	<13.5 13.5-18.76 >18.76	1.00 1.00 (0.89-1.13) 0.84* (0.72-0.99)
Province	10 Newfoundland 24 Quebec 45 Manitoba 48 Alberta 59 British Columbia 63 Nunavut	1.00 n/s 1.73** (1.29-2.50) 1.60** (1.31-2.49) 1.83** (1.33-2.57) 2.22* (1.02-4.62)
Swine Density Animals/1000 Km ² , 2006	<33.67 33.67-142,937 >142,937	1.00 0.93 (0.82-1.05) 1.00 (0.82- 1.23)
Cattle Density Animals/1000 Km ² , 2006	<62,460 62,460-262,169 >262,169	1.00 0.96 (0.74-1.01) 1.27 (0.95-1.70)
Agriculture (%), 2006	>62.68 23.66-62.68 < 23.66	1.00 1.05 (0.92-1.20) 1.11 (0.98-1.25)
Urban/Rural	Rural Urban	1.00 1.25** (1.12-1.40)

Note: Each variable is controlled for by age and gender. RR = relative risk

P < 0.05 *

P < 0.01 **

Figure 23. Primarily urban populations (Census divisions with cities > 50,000 population) in Canada, 2001.

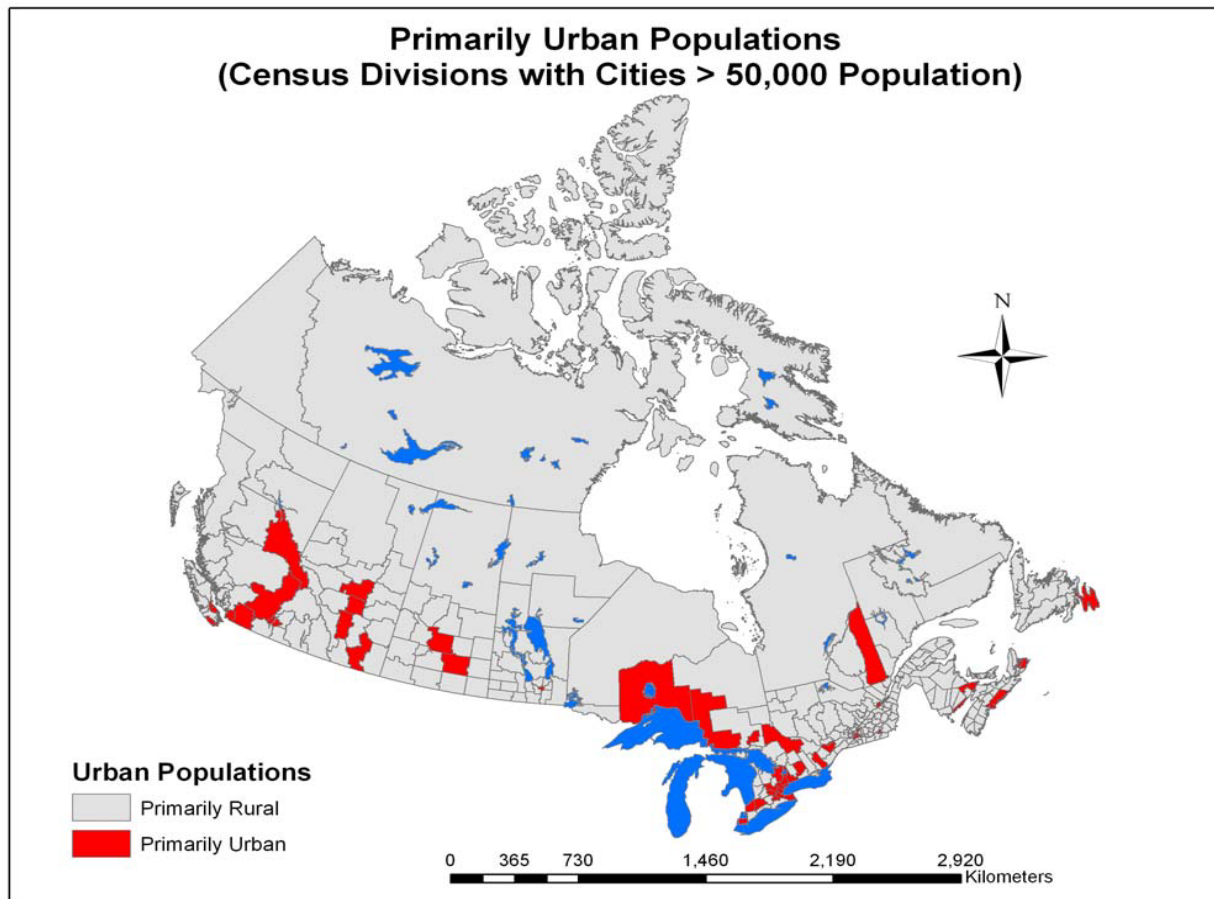


Figure 24.Percent recent immigrants to Canada, 2001.

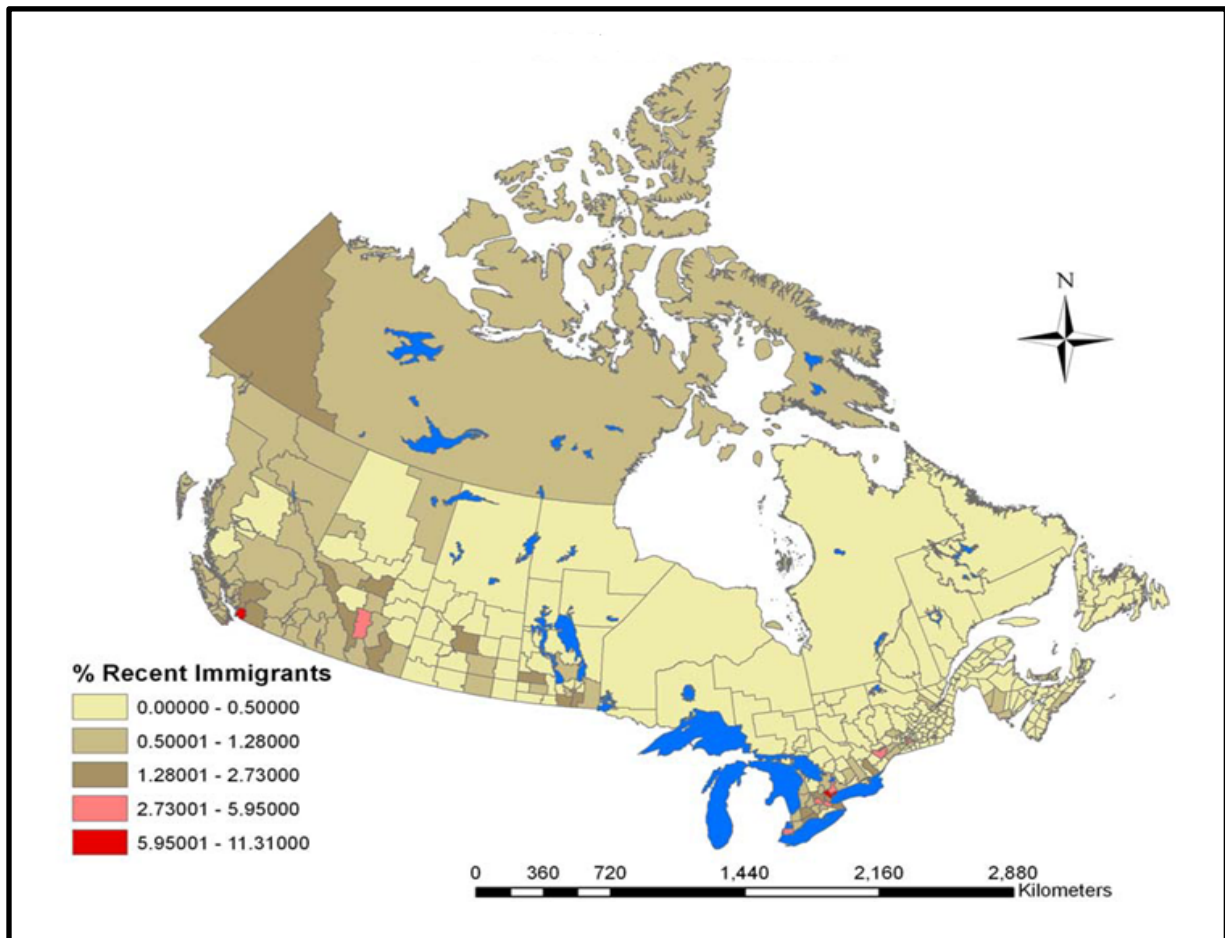


Figure 25. Hepatitis B, cases/100,000 in Canada 1989 to 2004.

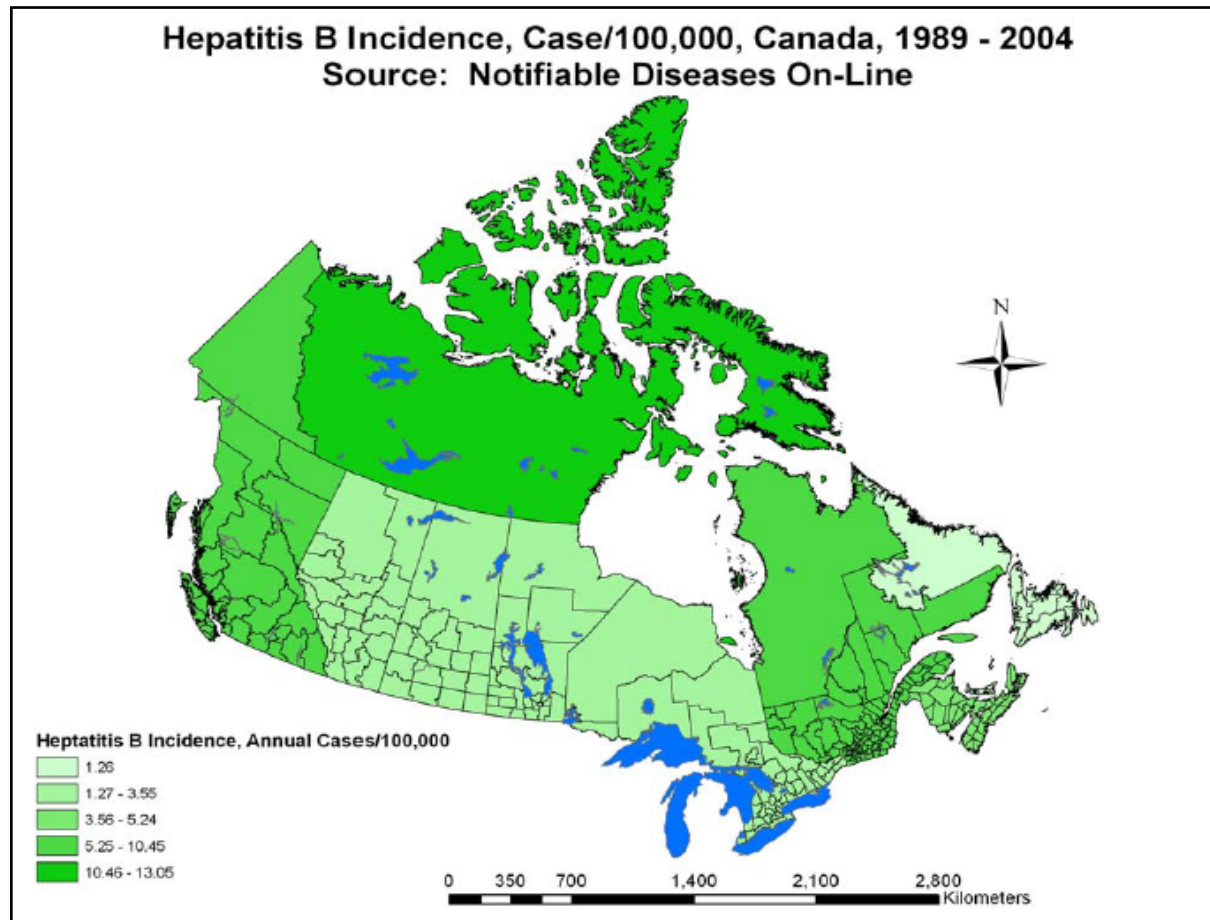
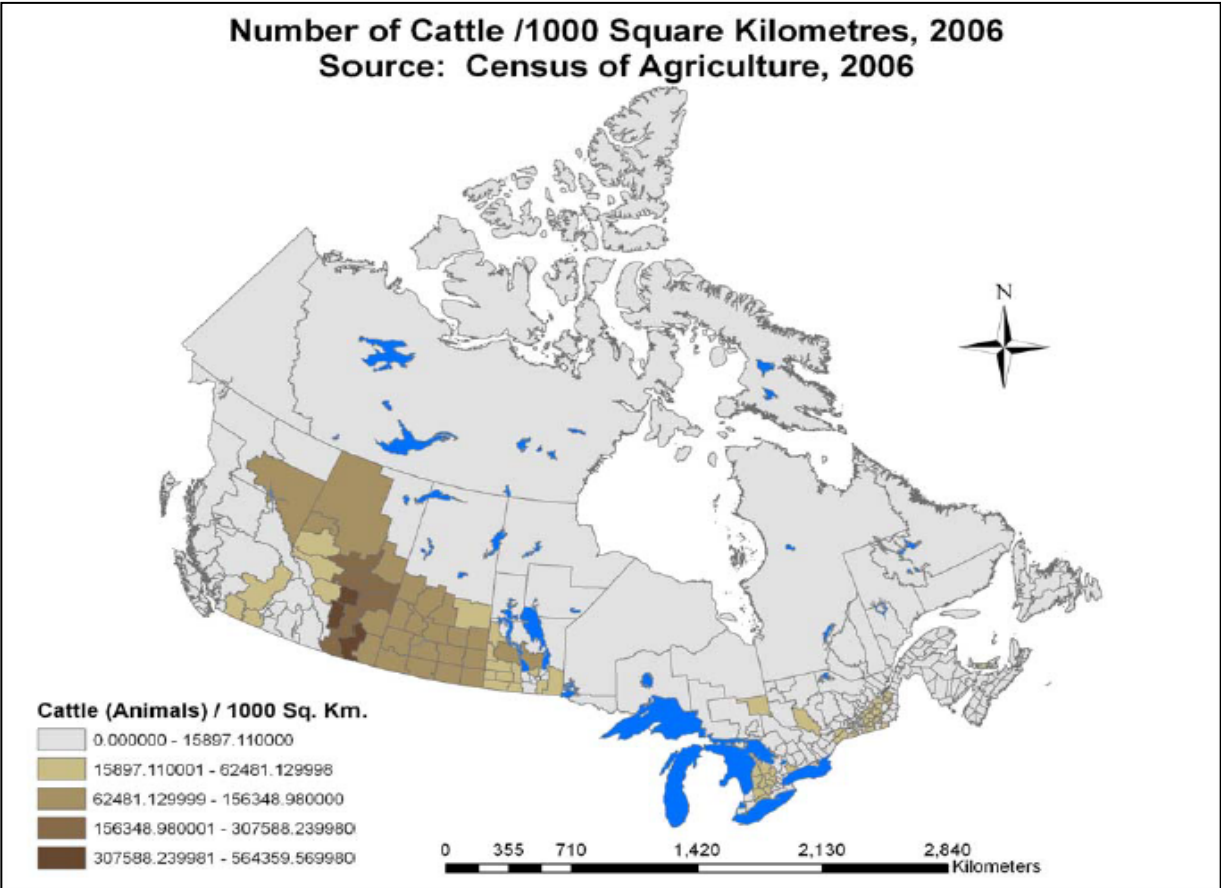


Figure 26. Number of cattle/1000 square kilometers in Canada, 2006.



6.6. *Provincial Epidemiological Study*

In Manitoba, similar to other regions of the country, the incidence rates of liver cancer have been steadily increasing over the past two decades. Cyanobacterial contamination of Manitoba fresh water lakes is a persistent problem, rendering many areas inaccessible due to potential MCLR toxicity. When MCLR is detected, drinking water sources are treated to remove MCLR, however in many rural areas low-dose concentrations of MCLR may still be present in drinking water sources. High dose MCLR exposure through drinking water is associated with increased liver cancer incidence in various countries. It is unclear whether chronic low-dose exposure to MCLR in drinking water is associated with increasing cancer incidence in Manitoba.

The Canadian epidemiological study, although thorough, was unable to assess the association between measured MCLR concentrations in fresh water sources (surrogate upstream markers had to be utilized) and liver cancer incidence. Since 2007, the Manitoba Water Stewardship has been collecting MCLR data across the province, from lakes with reported toxic cyanobacterial blooms. Although the MCLR data does not provide direct information regarding MCLR concentrations in drinking water sources, it does provide an opportunity to determine whether an indirect association exists between liver cancer incidence and MCLR concentrations in fresh water sources. This study sought to address some of the limitations encountered with the Canadian epidemiological study, in order to provide greater insight into whether MCLR contamination is posing a serious public health risk.

6.6.1. Distribution of Liver Cancer

Within Manitoba, liver cancer was analyzed for changes in incidence rates, and whether clustering was occurring within regions of the province (Figure 27). Over the 22 year observation period, 751 cases of invasive liver cancer have occurred in the Manitoban population, having an annual crude rate of 2.69 cases per 100,000 persons- years of exposure. The annual age-standardized rates for liver cancer in Manitoba by each 3-5 year period are 2.1 ± 0.7 (1985 - 89), 2.3 ± 0.4 (1990 - 94), 2.7 ± 0.7 (1995 - 99), 3.3 ± 0.4 (2000 - 04) and 4.3 ± 0.5 (2005 - 07). For liver cancers in the age-standardized population, regions that showed a >2-fold increase change in incidence rate between the years of 1985 to 2007 were; North Eastman, Central, Norman, Churchill and Winnipeg regions; St. Boniface, Inkster, Point Douglas, River Heights and Downtown) (Winnipeg as a whole, did not experience a > 2 fold increase in incidence rate) (Table 6).

Figure 27. Liver cancer rates/100,000 in Manitoba from 1985 to 2007.

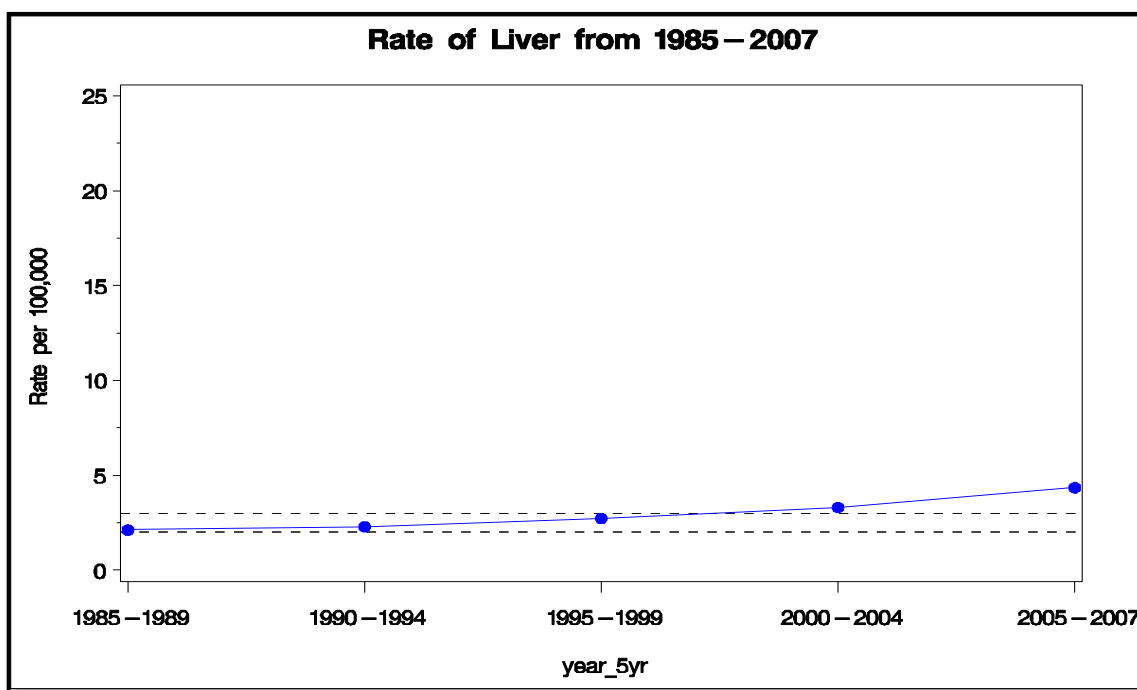


Table 6. Age-standardized liver cancer incidence rates/100,000 according to regional health authority (RHA) in Manitoba from 1985 to 2007.

RHA	1985-1989	1990-1994	1995-1999	2000-2004	2005-2007	Real Change ₺	Proportional Change €
Winnipeg	2.45	2.34	2.65	3.33	4.01	1.56	63.67
St. James	0.89	1.95	2.65	4.98	2.73	1.83	205.49
Assiniboine S.	2.67	2.25	1.27	3.08	0.60	-2.07	77.60
Fort Garry	2.82	0.79	2.46	2.40	3.95	1.13	39.87
St. Vital	2.59	1.45	1.70	2.39	2.47	-0.12	4.74
St. Boniface	3.43	0.73	0.76	3.46	5.91	2.48	72.36
Transcona	0.00	1.49	3.55	4.16	1.89	1.89	188.70
River E.	4.08	1.59	3.17	2.01	3.22	-0.86	21.08
Seven Oaks	2.32	2.70	3.75	1.98	3.68	1.36	58.81
Inkster	3.05	1.71	3.65	2.93	5.92	2.87	94.13
Pt. Douglas	2.22	4.60	3.72	4.98	9.41	7.19	324.49
Downtown	2.75	4.88	3.85	5.14	7.23	4.48	163.32
River Heights	1.18	2.69	1.46	3.37	3.81	2.63	223.34
Brandon	1.99	1.08	2.00	2.00	1.96	-0.04	1.81
North Eastman	1.83	3.26	1.44	2.05	6.60	4.77	260.40
South Eastman	1.50	3.21	3.49	0.32	2.37	0.87	57.88
Interlake	1.94	1.50	2.61	3.81	1.31	-0.62	32.18
Central	1.07	1.48	1.93	2.48	3.46	2.39	223.97
Assiniboine S.	2.08	1.99	1.53	1.46	2.76	0.68	32.42
Parkland	1.35	1.34	1.70	2.95	2.93	1.58	116.95
Norman	2.42	1.16	2.01	4.73	4.64	2.22	91.86
Churchill	0.29	3.33	2.98	0.94	6.36	6.08	2124.48

₺ Real change refers to the change calculated from the 2005-2007 rate per 100 000 person-years minus 1985 – 1989 rate per 100 000 person-years.

€ Proportional change refers to [(absolute change)/1985 – 1989 rate per 100 000 person-years]] x 100.

6.6.2. *Microcystin-LR Distribution and Cyanobacteria Geneses in Manitoba*

For locations that had persistent cyanobloom occurrence, repeated MCLR measures were taken throughout a single year, and from year to year. The cut-off limit of detection was 0.2 µg/L of MCLR and the highest recorded MCLR was 205 µg/L. Areas that had persistent and highest recorded MCLR values were; Killarney Lake Beach (Highest: 205 µg/L of MCLR), Pelican Lake Beach (High: 25.6 µg/L), Rock Lake Friesens Beach (High: 57 µg/L) and Meditation Lake (High: 7.3 µg/L). Lake Winnipeg was also repeatedly sampled and the MCLR concentration levels ranged between a high of 2.1 µg/L to 0.2 µg/L.

The types of cyanobacterial geneses identified in the various freshwater locations included; *Aphanizomenon*, *Pseudoanabaena*, *Microcystis*, *Anabaena*, *Merismopedia*, *Planktolnygba*, *Lyngbya*, *Gomphosphaeria*, *Aphanocapsa*, *Planktothrix* and *Aphanothece*. The cyanobacterial community profile at each location varied considerably, each having only 3-4 dominant cyanobacteria geneses. The most common cyanobacteria identified were *Aphanizomenon*, *Microcystis* and *Anabaena*. Cyanobacterial species were not identified in the data provided.

6.6.3. *Liver Cancer Incidence Rates and Relative Risk*

To determine whether there was an association between increasing liver cancer incidence and MCLR exposure in Manitoba, we examined the following relationships; 1) percentage of liver cancer cases occurring within MCLR buffer zones/rings and outside of MCLR exposure areas, 2) the incidence rates of liver cancers within the MCLR buffer zones/rings and out MCLR exposure areas, 3) logarithmic distance in meters of liver cancer incidence to MCLR buffer zones/rings and 4) relative risk of developing liver cancer within the MCLR buffer zones/rings and outside the exposure areas. The percentage of cancer

cases of increasing incidence falling within the identified MCLR buffer zones/rings and outside exposure areas was calculated, in order to observe any changes in the longitudinal temporal or spatial distribution of cancer incidence in relation to MCLR contamination.

The average percentage of liver cancer cases occurring within the buffer zones, rings and outside the exposure areas over the 22 year period are presented in Table 7 and Figure 28.

Table 7. Percentage of liver cancer cases falling within the microcystin-LR buffer zone, buffer ring and outside the buffer areas in Manitoba between 1985 to 2007.

Years	Percent of cases within buffer zone	Percent of cases within buffer ring	Percent of cases outside buffer ring
1985-1989	4.20%	84.87%	10.93%
1990-1994	6.92%	80.00%	13.08%
1995-1999	3.85%	80.13%	16.02%
2000-2004	3.33%	81.77%	14.90%
2005-2007	3.25%	82.47%	14.28%

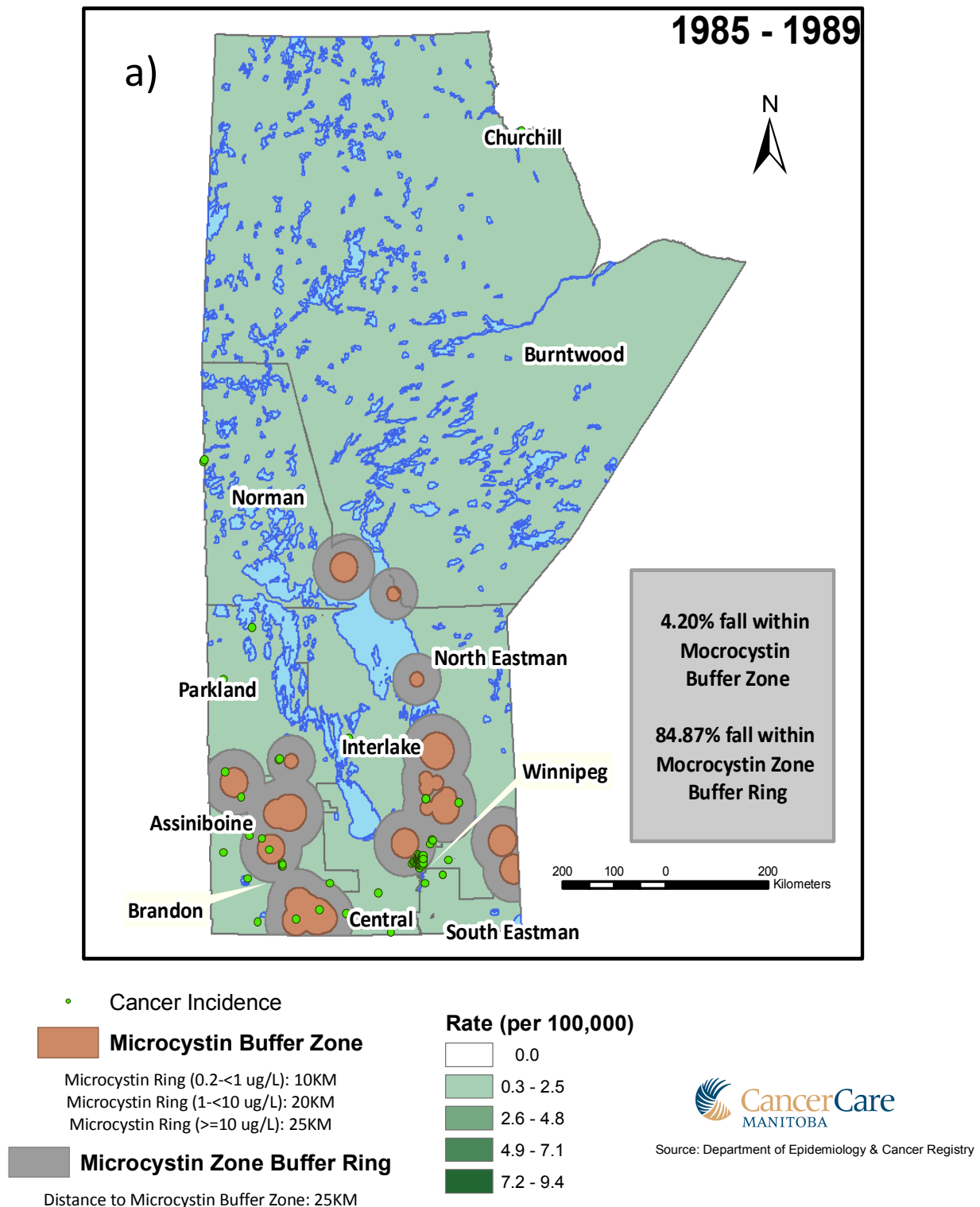
Cancer incidence rates were determined within the MCLR buffer zones, rings and outside the buffer areas, in order to calculate whether significant differences exist between the 1985 – 1989 reference period and remaining observation periods. The results indicated there was no significant difference in cancer incidence within the MCLR buffer zones, rings or outside the exposure areas compared to the corresponding areas of the 1985 – 1989 reference period (Table 8).

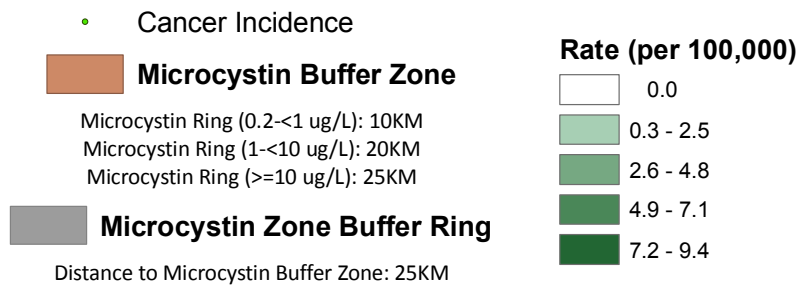
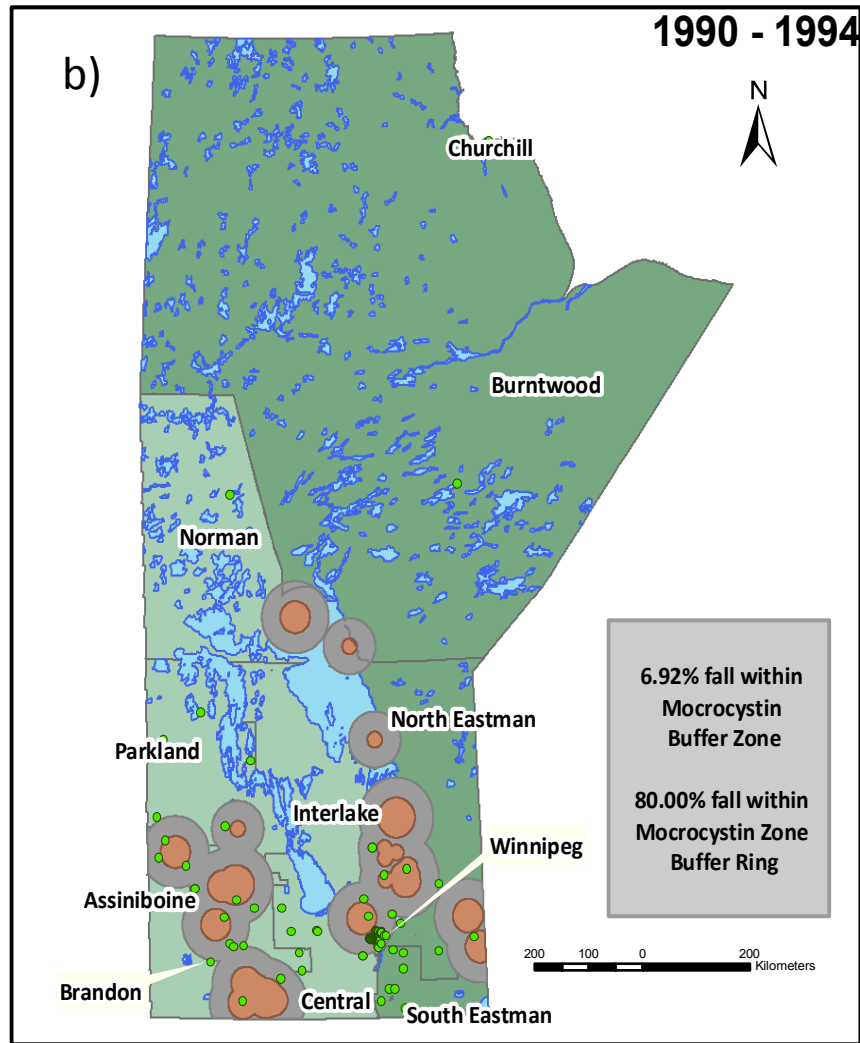
Table 8. Incidence rates of liver cancer within the microcystin-LR buffer zone, ring and outside the buffer areas in Manitoba between 1985 to 2007.

Years	MC-LR Buffer Zone	MC-LR Buffer Ring	Outside MC-LR Buffer Zone and Ring (outside exposure area)
1985-1989	4.2 (0.60 - 7.81)	84.87 (78.44 - 91.31)	10.92 (5.32-16.53)
1990-1994	6.92 (2.56 - 11.29)	80 (73.12 - 86.88)	13.08 (7.28-18.87)
1995-1999	3.85 (0.83 - 6.86)	80.13 (73.87 - 86.39)	16.03 (10.27-21.78)
2000-2004	3.13 (0.66 - 5.59)	81.77 (76.31 - 87.23)	15.10 (10.04-20.17)
2005-2007	3.25 (0.45 - 6.05)	82.47 (76.46 - 88.47)	14.29 (8.76-19.81)

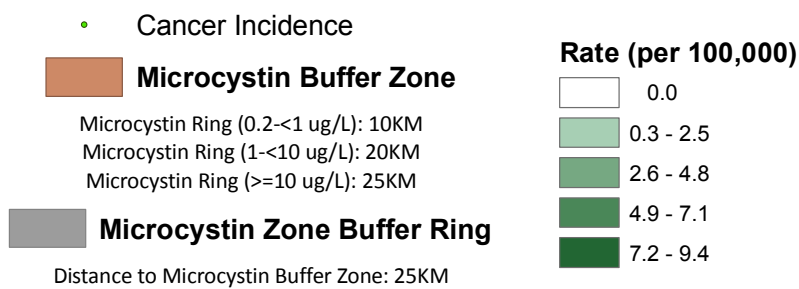
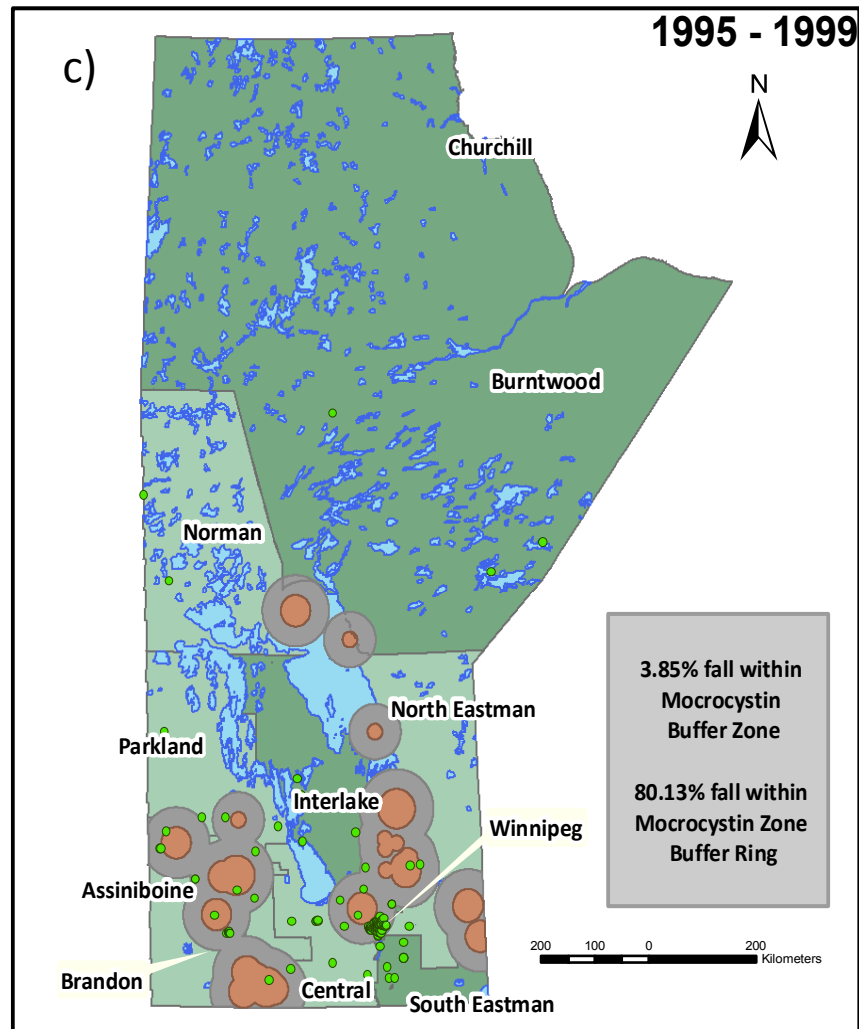
Note: 1985 -1989 used as control group for comparison calculations
P < 0.05 *, P < 0.01 ** and P < 0.001 ***

Figure 28. Percentage of liver cancer incidence occurring within and outside of microcystin-LR contamination areas (buffer zones and buffer rings) within Manitoba from 1985 to 2007: a) Liver cancer incidence from 1985 – 1989, b) 1990 – 1994, c) 1995 – 1999, d) 2000 – 2004 and e) 2005 – 2007.

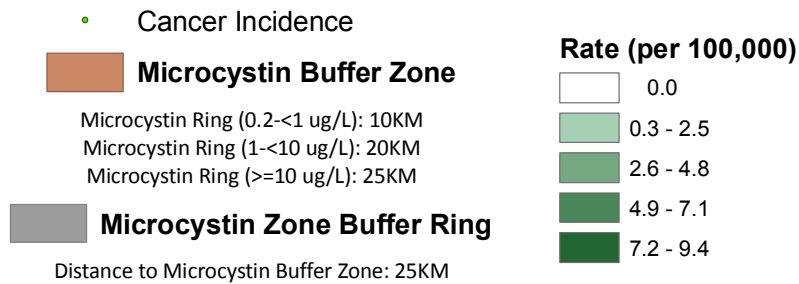
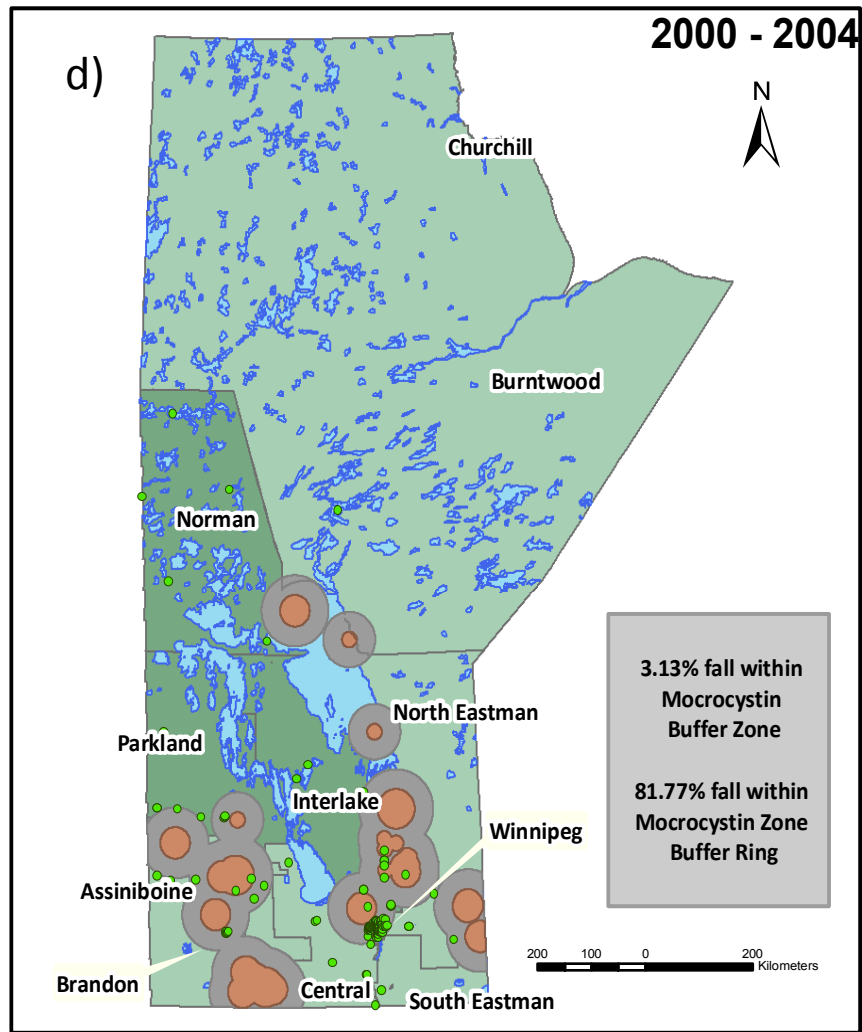




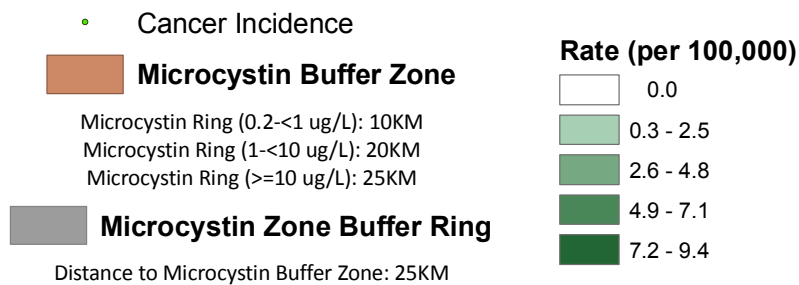
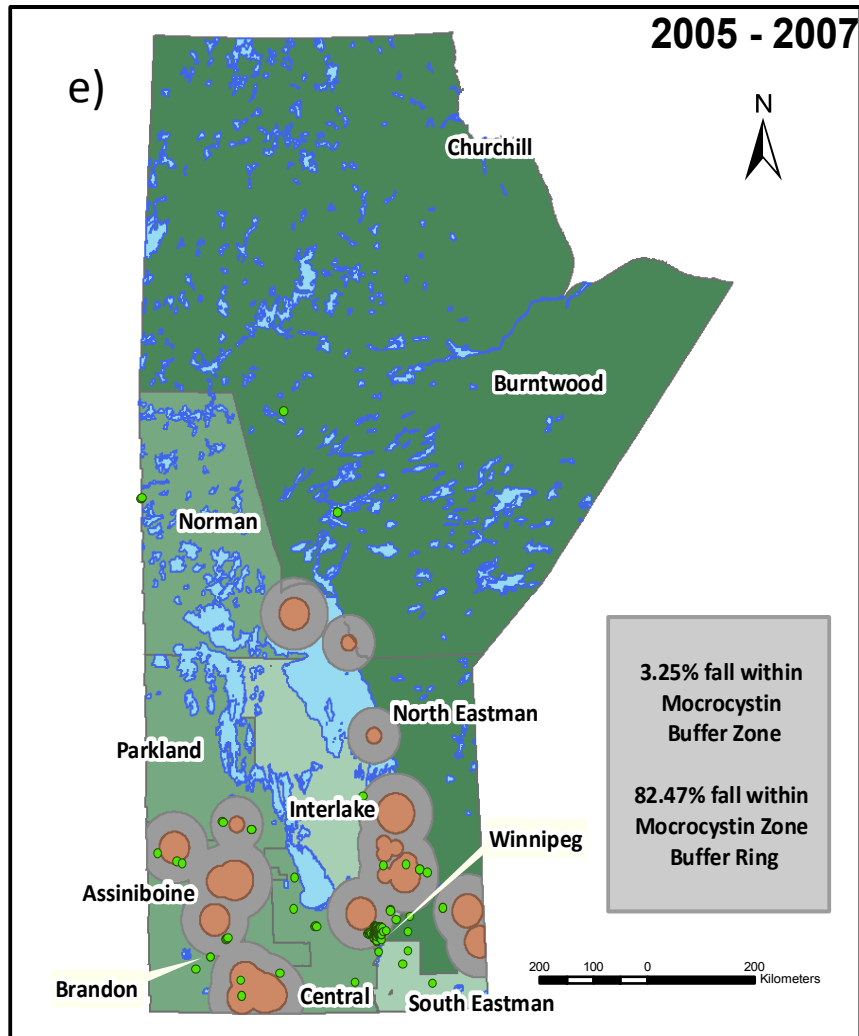
Source: Department of Epidemiology & Cancer Registry



Source: Department of Epidemiology & Cancer Registry



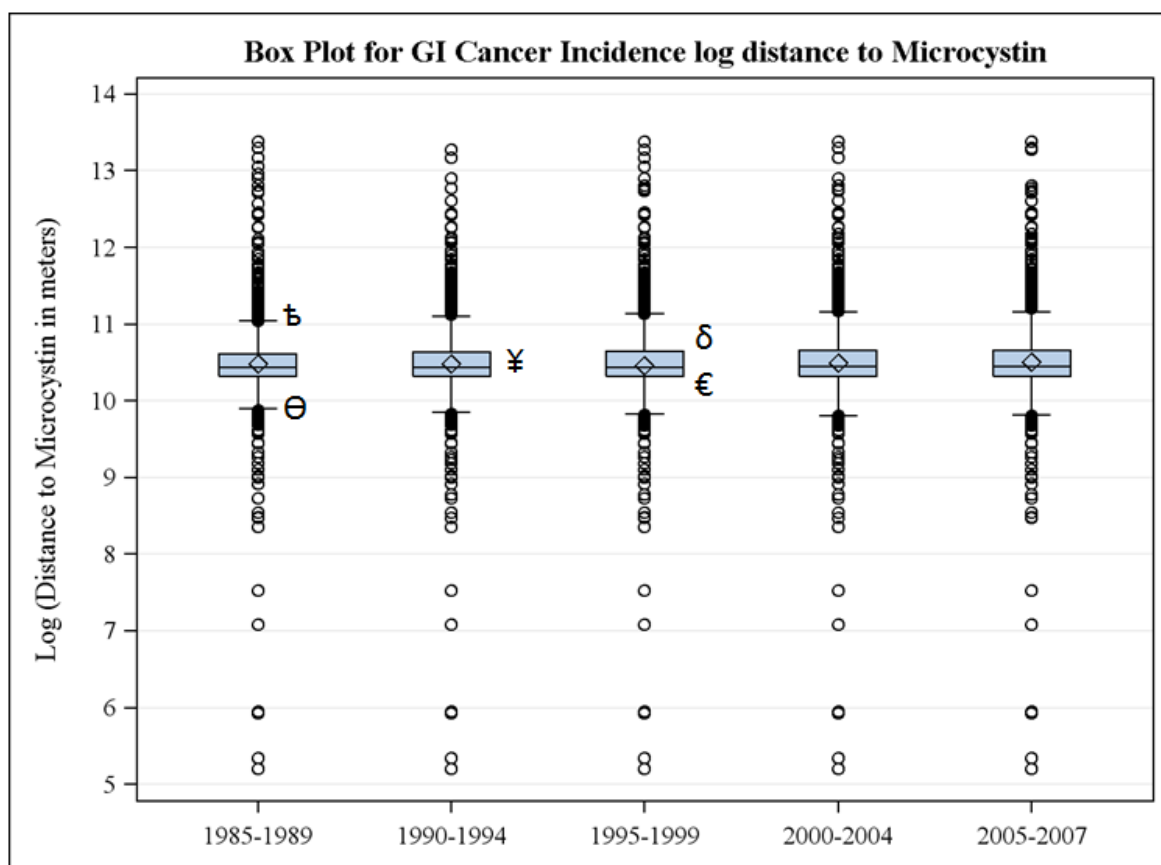
Source: Department of Epidemiology & Cancer Registry



Source: Department of Epidemiology & Cancer Registry

To assess whether there were any subtle changes in the distance in meters of cancer incidence to MCLR contamination throughout the 22 year observation period, the logarithmic distance of cancer incidence to the closest MC-LR contamination site was calculated. The mean, median, minimum, maximum, Q1 and Q3 values are presented, showing that across each five year period, the average distance of cancer incidence to MCLR contamination was relatively constant and did not significantly differ compared to the 1985-1989 reference group (Figure 29 and Table 9).

Figure 29. Logarithmic distance in meters of liver cancer incidence to microcystin-LR contamination sites in Manitoba during each five year period from 1985 to 2007.



◇ = Mean , δ = Q1, € = Q3, ¥ = median, Θ = min and Ⓢ = max

Table 9. The mean, median, minimum and maximum logarithmic distance in meters of liver cancer incidence to microcystin-LR contamination sites in Manitoba from 1985 to 2007.

Year Group	N	Mean	Min	Q1	Median	Q3	Max
1985-1989	119	10.44	5.34	10.32	10.42	10.57	13.38
1990-1994	130	10.46	5.95	10.33	10.40	10.60	13.38
1995-1999	156	10.52	5.34	10.32	10.41	10.61	12.57
2000-2004	192	10.51	7.53	10.32	10.42	10.63	12.61
2005-2007	154	10.47	5.95	10.31	10.41	10.60	12.45

Note: Significant difference of mean compared to 1985 -1989 control group

P < 0.05 *, P < 0.01 ** and P < 0.001 ***

N = number of cancer cases

Min = minimum distance

Max = maximum distance

Q1 = 25% quartile

Q3 = 75% quartile

Finally the relative risk of developing liver cancer, compared to the 1985 – 1989 reference year, was calculated to determine whether or not close proximity to MCLR contaminated sources increases the risk of developing liver cancer. The results indicate that there were no significant increases in the relative risk of developing liver cancer compared to the 1985 – 1989 reference year (Table 10).

Table 10. Relative risk of liver cancer within the microcystin-LR buffer zone, ring and outside the buffer areas in Manitoba between 1985 to 2007.

Year	MC-LR Buffer Zone	MC-LR Buffer Ring	Outside MC-LR Buffer Ring
1985-1989	NA	NA	NA
1990-1994	1.65 (0.57 - 4.78)	0.97 (0.88 - 1.07)	1.20 (0.61 – 2.36)
1995-1999	0.92 (0.27 - 2.93)	0.94 (0.85 - 1.04)	1.47 (0.78 – 2.74)
2000-2004	0.74 (0.23 - 2.38)	0.95 (0.87 - 1.04)	1.38 (0.75 – 2.55)
2005-2007	0.77 (0.23 - 2.61)	0.96 (0.88 - 1.06)	1.31 (0.69 – 2.49)

Note: 1985 -1989 used as control group for relative risk calculations
P < 0.05 *, P < 0.01 ** and P < 0.001 ***

7. Discussion

Over the past decade, fresh water contamination by toxigenic cyanobacterial blooms has been increasing in extent and severity across Canada, and noticeably in Manitoba (Dyble, Fahnenstiel et al. 2008; Bunting 2011; Manitoba 2011; Orihel, Bird et al. 2012). MCLR is a potent cyclic heptapeptide toxin released by toxigenic cyanobacteria under varied environmental conditions (Runnegar, Andrews et al. 1987; Falconer and Buckley 1989). Determinants of cyanobloom formation and release of MCLR are thought to arise from a variety of factors including eutrophication, altered nutrient ratios and increased temperatures (Downing, Sember et al. 2005; Rahman, Al Bakri et al. 2005; Vasconcelos 2006; Orihel, Bird et al. 2012). Anthropogenic activity such as; farming, agriculture, urban run-off, deforestation, etc, are linked to increased eutrophication of fresh water resources (Piehler 2008). For example in Manitoba, Lake Winnipeg has been under intense observation as it is regarded as highly polluted, having recurrent toxigenic bloom formation to the extent of being a public health concern (Bunting 2011; Manitoba 2011).

Monitoring for cyanobloom formation and toxin release in Canada is controlled at the provincial and municipal levels, and therefore no national monitoring program or standards are in place, aside from the WHO 1 µg/L MCLR allowable concentration in drinking water (WHO 1998; WHO 2003). Treated drinking water in large urban centers is routinely monitored for the presence of MCLR, with a detection limit of 0.03 µg/L of MCLR. Although rural drinking water sources should follow provincial drinking water guidelines, there is little information regarding the presence of MCLR in non-treated or rural sources.

MCLR is water soluble, possessing poor cell membrane permeability (Meriluoto, Eriksson et al. 1990; Chong, Gu et al. 2000; Song, Gao et al. 2011). Selective uptake of MCLR into tissue is facilitated by organic anion transporter polypeptides (OATP) 1B1, 1B3 and 1A2, which are expressed predominantly in the liver, kidneys, gastrointestinal tract and blood brain barrier (Fischer, Altheimer et al. 2005; Monks, Liu et al. 2007; Lu, Choudhuri et al. 2008; Wang, Xie et al. 2008). Acute toxic exposure to MCLR is thought to be responsible for severe hepatotoxicity, gastrointestinal upset and renal dysfunction in humans and animals (Ashworth and Mason 1946; Falconer, Beresford et al. 1983; Jackson, McInnes et al. 1984; Falconer, Smith et al. 1988; Hooser 2000). Chronic exposure to sub-lethal MCLR concentrations in drinking water has been associated with an increased incidence of HCC (Harada, Oshikata et al. 1996; Yu, Zhao et al. 2001). Evidence supports the potential of MCLR to promote other chronic diseases such as hepatic fibrosis, colorectal cancer and renal dysfunction, however more investigation is required (Elleman, Falconer et al. 1978; Milutinovic, Sedmak et al. 2002; Zhou, Yu et al. 2002; La-Salette, Oliveira et al. 2008).

MCLR induced hepatotoxicity is complex, resulting primarily from oxidative stress, serine/threonine phosphatase inhibition and DNA damage (Ariza, Keyse et al. 1996; Douglas, Moorhead et al. 2001; Lankoff, Bialczyk et al. 2006; Campos and Vasconcelos 2010). If the association is causative, the underlying mechanism of tumor promotion by MCLR remains unclear, due to the varied toxicological profile of the toxin. Although not investigated, the possibility exists that there is no single tumor promoting MCLR mechanism, but rather a variety of mechanisms work synergistically with a pre-existing chronic disease to promote carcinogenicity.

In western countries where drinking water is treated and of high quality, acute exposure to concentrations of MCLR exceeding 1 µg/L on a routine basis is unlikely. Therefore, investigation into the association between chronic low-dose MCLR exposure and chronic liver disease is required. This study sought to explore the relationship between chronic low-dose exposure to MCLR and HCC at the cellular, systemic and epidemiological levels.

7.1. *Cellular Exposure to MCLR*

In this study we examined the effects of MCLR on various hepatocyte cell types (WB-F344, PLC and CFSC-2G cell lines): those relevant to the various stages of chronic liver disease and HCC progression. WB-F344 cells were utilized in order to observe the effect of MCLR on non-malignant hepatic stem cells, and determine whether chronic low-dose exposure to MCLR could induce malignant transformation. PLC cells were selected in order to observe how MCLR would impact the growth characteristics of established malignant hepatocytes. CFSC-2G were selected in order to observe whether low-dose MCLR could activate stellate cells to form proliferative myofibroblasts able to secrete collagen and initiate the process of fibrogenesis and cirrhosis, environments conducive to HCC development and growth.

Within all three cell lines, it was necessary to determine whether MCLR at low doses was able to penetrate the cell membrane of cells expressing low amounts or absent of oatp/OATP transporters. Tagging of MCLR was initially employed to document cellular uptake of MCLR, however it was abandoned as the process significantly alters the size and activity of MCLR, thereby impacting on cellular import by oatp transporters and cellular

toxicity. Thus, two bioassays which also had the potential of providing mechanistic data were employed; PP2A inhibition and oxidative stress assays. In addition, Endo-porter was utilized for all *in vitro* assays, which facilitated the import of MCLR into target cells using an endocytosis-mediated process.

The ability of MCLR to induce oxidative stress and PP2A inhibition have been well documented in primary rat hepatocytes, HepG2, Hek 293 and HepG3 cells. Thus oxidative stress and PP2A inhibition were measured as functional assays to assess MCLR uptake within our cells of interest. For this study, ROS was directly measured using a fluorescent dye that exhibits a strong fluorescent signal upon excitation by ROS. Our results for all three cell lines showed the same trend following 24 hrs of exposure to varying concentrations of MCLR (0.1 – 1000, 5000 and 10000 µg/L). At the highest concentrations (5000 and 10000 µg/L) there was a dose-dependent increase in oxidative stress, whereas there was no significant increase in oxidative stress at the lower concentrations. A similar trend was observed for PP2A inhibition, wherein the lower concentrations (0.1-1000 µg/L) of MCLR did not alter PP2A activity compared to controls, but the higher doses (5000 and 10000 µg/L) had an inhibitory effect.

Our results differ significantly from other studies reporting an increase in oxidative stress in primary hepatocytes and HepG2 cells (transfected and expressing OATP 1B1 and 1B3 transporters) in response to similar concentrations of MCLR. There are a few possible reasons to explain why we observed changes in oxidative stress and PP2A inhibition only at the 5000 and 10000 µg/L for MCLR. Our studies were conducted following 24 hrs of exposure to MCLR.

Although the reason for the discrepancy is unclear, it should be noted that primary hepatocytes and HepG2 cells express the required OATP1B1 and 1B3 transporters (naturally or artificially). In our study, we were unable to characterize the expression of murine oatp 1b1 and 1b3 in WB-F344, PLC or CFSC-2G cells, despite repeated attempts (data not shown). Thus, the possibility exists that MCLR was not entering cells in sufficient concentrations at the lower concentrations, required to elicit measurable oxidative stress PP2A inhibition at MCLR concentrations of 0.1 – 1000 µg/L.

Although the widely accepted explanation for MCLR cellular uptake is through the use of specific OATP transporters OATP1B1 and 1B3, additional evidence suggests that other mechanisms may assist with cellular uptake independent of the presence of specific transporters. One study investigated the absorption properties of MCLR in zebra fish embryos, using various absorption models to describe the mechanism by which MCLR can be absorbed through membranes in the absence of oatp transporters. Several studies have investigated the relationship between membrane interaction and the mechanism of toxicity (Donato, Jurado et al. 1997; Lopes, Antunes-Madeira et al. 1997; Donato, Jurado et al. 2000).

The absence of data supporting MCLR uptake by our cells does not preclude MCLR's ability to enter the cell to induce injury via oxidative stress and PP2A inhibition. MCLR cellular uptake is facilitated by the presence of OATP1B1, 1B3 and 1B2, however it has also been postulated that MCLR dose may impact cellular uptake. MCLR has a complex structure, containing both hydrophobic and hydrophilic groups. Due to the unique structure of MCLR, the compound is able to alter surface membrane structure by affecting phospholipid flow/rotation, transport protein function and import of necessary substances.

In previous studies, it was observed that depending on the concentration used, MCLR employed various absorption models to gain access to the embryonic cytosol. Using scanning electron microscopy (SEM), fluorescence microscopy and fragmentation of zebra fish embryos, MCLR membrane transport was observed to alter its absorption potential based on toxin concentration. At concentrations < 0.04 mmol/L, according to the Langmuir isotherm model of absorption, MCLR localized primarily on the membrane surface of embryos due to electrostatic attraction, hydrogen bonding and van der Waals forces. At concentrations between the range of 0.04 and 0.5 mmol/L, MCLR displayed absorption characteristics following that of the Freundlich isotherm model. The toxin is able to enter into the phospholipid bilayer, bound by hydrostatic forces and migrating into the cytosol. At concentrations of >0.5 mmol/L, thinning and disruption of cellular membranes was observed via SEM and fluorescence microscopy, showing MCLR entering directly into the cytoplasm by a mechanism described by the water-lipid partition law. It was concluded that MCLR has the potential to be absorbed through lipid membranes, and its absorption characteristics differ depending on toxin concentration within the surrounding environment (Song, Gao et al. 2011).

7.1.1. MCLR and Hepatic Stem Cells

In the context of chronic liver disease, adult liver stem cells function to regenerate liver tissue. In the process of proliferation under conditions of chronic inflammation and remodeling, stem cells can accumulate mutations and lose control of the self-renewal process, transforming themselves into cancer stem cells (CSCs). CSCs are now considered an important component of hepatic carcinogenesis. Specifically, small populations of CSCs, (often $<5\%$ of the entire tumor cell population) have been observed as the ultimate source

of malignant hepatocytes, generating mature malignant hepatocytes that comprise the bulk of solid liver tumors. They are also largely responsible for tumor recurrences and distal metastases. Consequently, it has been proposed that HCC is a disease of adult CSCs (Avril, Pichard et al. 2004).

Models of experimental hepatocarcinogenesis have observed that hepatic adult stem cells possess characteristics that enable tumorigenesis; life span > 1yr, progenitor cell population and able to undergo neoplastic transformation. The purpose of using WB-F344 hepatic stem cells was to observe the effects of chronic low-dose MCLR exposure on cellular characteristics pertaining to carcinogenesis and tumor promotion within non-malignant hepatic stem cells. Research supports the theory that MCLR carcinogenicity arises from its ability to induce oxidative stress, causing genotoxicity and altered cellular proliferation respectively. Numerous studies have examined the genotoxic potential of MCLR in a variety of cell types, experimental concentrations and exposure periods. In hepatic cell lines such as; HepG2 and isolated primary hepatocytes, ROS formation was observed, causing DNA damage at nano-molar concentrations. In our study, WB-F344 exposure to varying concentrations of MCLR did not result in an increase in the levels of oxidative stress observed at lower MCLR concentrations (0.1-1000 µg/L). However, at MCLR concentrations of 5000 and 10000 µg/L, a significant increase in oxidative stress occurred. These findings do not support the possibility that low-dose exposure to MCLR induces HCC through oxidative stress.

The other possibility that needed to be addressed was whether low-dose exposure to MCLR might induce HCC by inhibiting PP1 and PP2A activity, leading to hyperphosphorylation of cellular proteins and alteration of cell cycle control. Thus we

examined the effects of MCLR on WB-F344 PP2A activity (PP2A and PP1 act in concert). Once again, at the lower concentrations, MCLR did not alter PP2A compared to negative controls, however at higher concentrations (5000 and 10000 $\mu\text{g/L}$) a significant decrease in PP2A activity was observed.

MCLR has been shown to elicit cellular effects in a dose and time dependent manner, whereby increasing dose and exposure period, enhances cellular toxicity. In our study, oxidative stress and PP2A inhibitory activity was only enhanced in cells at concentrations of 5000 and 10000 $\mu\text{g/L}$ of MCLR. Bouaicha et al (2004) observed increased cellular oxidative stress in response to low-concentrations of MCLR (2 and 10 ng/ml) in primary hepatocytes following 1 hr of exposure (Bouaicha and Maatouk 2004). Our results differ from these findings, as we did not observe increased oxidative stress at MCLR doses comparable to 2 and 10 ng/ml (0.1 – 1000 $\mu\text{g/L}$). Various factors may have influenced our results such as exposure period and cell type; immortalized rat hepatic stem cells versus primary mouse hepatocytes, thereby generating results that differ from the published literature.

As previously discussed, our results from the PP2A inhibitory assay revealed that MCLR inhibits PP2A activity at doses of 5000 and 10000 $\mu\text{g/L}$. Similar results were reported by Li et al (2011), who described increased PP2A activity in HEK293 cells following exposure, but decreased activity in response to 10 μM of MCLR after 24 hrs exposure (Li, Huang et al. 2011).

Perhaps unrelated to oxidative stress and PP1/PP2A inhibition is the possibility that low-dose MCLR exposure directly alters key regulatory signaling pathways involved in

carcinogenesis and proliferative activity. In a study by Dias et al (2010), MCLR (1-10 nM) was reported to enhance ERK 1/2 and the proliferative activity of Vero-E6 kidney cells. It has also been observed through the BrdU incorporation assay, that lower concentrations of MCLR (5, 50, 500 nM) activate the ERK1/2 pathway and stimulate cell cycle progression from the G1 to S phase (Dias, Matos et al. 2010). In addition to ERK1/2, activation of downstream transcription factors associated with MAPK activity have been implicated in stimulating toxin induced cellular proliferation. Of note, MAPK signaling is activated in response to a variety of stimuli, including oxidative stress (McCubrey, Lahair et al. 2006).

NF-E2 related factor activation (Nrf-2), is a transcription factor that regulates transcription of endogenous antioxidants and phase II redox enzymes for detoxification of environmental toxins. Under oxidative conditions, Nrf-2 is up-regulated at the protein level, through modification of cysteine residues which prevent targeted ubiquitin-dependent degradation, allowing for nuclear accumulation (Sun, Huang et al. 2009). Constitutive activation of Nrf-2 has been observed in various malignant phenotypes and cancer cell types. Gan et al (2010) exposed HepG2 and G3 cells to MCLR, resulting in up-regulation of Nrf-2 and down-stream genes; HO-1 and NQO-1 and an increase in cellular proliferation. Exposure of Nrf-2 knock-down cells to MCLR did not result in an increase in cellular proliferative activity (Gan, Sun et al. 2010). Our results did not show increased proliferative activity of WB-F344 hepatic stem cells in response to low-concentrations of MCLR. There is a possibility that stimulation of hepatic stem cell proliferation may require additional pro-inflammatory signals from other hepatic cell types such as Kupffer cells or activated HSCs, in order to simulate the setting of chronic inflammation and hepatic remodeling.

The most telling data that argues against chronic low-dose exposure to MCLR causing malignant transformation of non-malignant hepatic stem cells are those of experiments documenting EpCAM and Cyclin-D1 expression and colony formation in soft agar gels. Here, EpCAM and Cyclin-D1 protein expression remained unaltered and no colonies developed in soft agar gels following prolonged MCLR exposure. As discussed previously, factors such as cell type, oatp expression, length of exposure and MCLR concentration may explain why we did not observe altered protein expression of EpCAM and Cyclin-D1 proteins.

Overall, this is the first study examining the relationship between MCLR exposure and hepatic stem cell malignant transformation. MCLR is known to cause hepatotoxicity through induction of oxidative stress and PP1 and PP2A inhibition, which may influence cellular characteristics associated with carcinogenesis; increased proliferation, expression of HCC protein markers and malignant transformation. The results suggest that non-malignant hepatic stem cells do not experience increased oxidative stress, PP2A inhibition, cellular proliferation, protein expression or malignant transformation following exposure to chronic low-dose concentrations of MCLR.

There were a variety of limitations encountered with our studies which need to be addressed for future consideration. The most obvious limitation with our *in vitro* studies, arose from our inability to identify and characterize the oatp profile within our target cells; WB-F344, PLC and CFSC-2G. To overcome this obstacle, a peptide delivery system was used (Endo-Porter), which allowed for the transport of peptides across the cell membrane using an endocytosis-mediated process, without causing cell damage. In addition, the use of a single rat hepatic stem cell line (rather than primary human hepatic stem cells), and

exposing cells to MCLR for relatively limited periods of time, further contributed to the limitations surrounding MCLR import. However, the ability to isolate human hepatic stem cells from healthy human livers and maintain them in culture for sufficient lengths of time and numbers to perform the experiments outlined, has yet to be developed. Yet another limitation was the failure to demonstrate a lack of malignant transformation by injections of MCLR exposed WB-F344 cells into immune deficient mice. The absence of any signal that these cells had developed malignant features (no effect on EpCAM or Cyclin-D1 protein expression or colony formation in soft agar gels) was considered sufficient to render such long-term exposure experiments futile.

7.1.2. MCLR and Malignant Hepatocytes

Enhanced proliferation and invasion are key features of HCC progression (Park, Chae et al. 2001). MCLR has been shown to promote cellular proliferation of cells through alteration of various signaling pathways including; MAPK, Akt and Wnt (Nishiwaki-Matsushima, Ohta et al. 1992; Komatsu, Furukawa et al. 2007; Gan, Sun et al. 2010). MCLR also stabilizes β -catenin by inhibiting activity of glycogen synthase kinase-3 β , which phosphorylates β -catenin for targeted proteasomal degradation. β -catenin is a multifunctional protein that assists in the transduction of wingless (Wnt) protein signals and promotes accumulation of proto-oncogenes cyclin D1 and c-myc. Enhanced Wnt signaling and activation of proto-oncogenes plays a significant role in tumor promotion and hyperplasia (Li, Yost et al. 2001; Wang, Chien et al. 2005). Takumi et al (2010) reported that in addition to stabilizing β -catenin, low doses of MCLR under conditions of p53 inactivation, stimulates cellular proliferation through activation of the Akt signaling pathway (Takumi, Komatsu et al. 2010). However a delicate balance exists between MCLR

tumorigenesis and cellular apoptosis. In response to oxidative stress, activation of MAPK is able to phosphorylate p53, triggering cellular apoptosis (Guan, Huang et al. 2008; Karunakaran, Saeed et al. 2008; Liu, Cheng et al. 2009). This balance between proliferation and apoptosis may explain our findings in PLC cells, wherein proliferative activity was only enhanced at 96 hours and only at high concentrations of MCLR (1000 µg/L).

Cell migration and invasiveness are characteristics of highly malignant tumors and are associated with poor clinical outcome. Cancer metastasis is characterized by the ability of cancer cells to migrate away from the primary tumor, and form neoplastic tissue at locations distant to the original site of the tumor (Yamanaka, Okamoto et al. 1990). Important steps during the process of metastasis include; cell adhesion, migration and proteolysis of extracellular matrix (ECM) proteins (Murphy and Gavrilovic 1999). As a consequence of cirrhosis, HCC cells grow in an environment densely surrounded by ECM proteins including; collagen, fibronectin, vitronectin and laminin (Bianchi, Biagini et al. 1984). Invasion of cancer cells, involves altered interaction and breakdown of ECM proteins. These features are associated with activation of Akt and Wnt signaling pathways, as well as enhanced expression of matrix metalloproteinase proteins (MMPs) (Zhu, Zhong et al. 2005; Zhang, Zhang et al. 2010). MMPs are a group of proteolytic enzymes that facilitate enhanced degradation of ECM proteins, facilitating increased cancer cell growth, migration across structural barriers and invasion into surrounding tissue (Kohn and LioTAA 1995; Coussens and Werb 1996; Koshikawa, Giannelli et al. 2000). Enhanced expression of MMP2 and 9 have been observed in a variety of malignant cancer types (Itoh and Nagase 2002). In a study by Zhang et al (2012) chronic low-dose exposure of MCLR was associated with increased expression of MMP2 and 9. Within cultured breast cancer

cells, cell migration was enhanced in a dose and time dependent manner, observed by measuring wound healing, and was also associated with increased expression of MMP2 and 9 (Zhang, Zhang et al. 2012). Within HCC cells, it has been hypothesized that increased expression of MMPs, results from paracrine stimulation by inflammatory, stromal or tumor cells interacting and releasing growth factors and cytokines (Basset, Okada et al. 1997; Uría, Stahle-Bäckdahl et al. 1997; Westermarck and Kahari 1999).

The wound healing assay is commonly employed to measure the cell migration activity of HCC cell lines (Ren, Tsui et al. 2003). In our study, it was observed that exposure to low-doses of MCLR did not enhance the cell migratory activity of PLC/PRF/5 cells over 96 hours of exposure, and instead resulted in an inhibition of migratory activity at 96 hours of exposure. The observed inhibition may have been the result of cellular apoptosis, as opposed to inhibition of migration. Zhang et al (2012) have observed increased invasion of melanoma cells following MCLR exposure, through increased expression of MMP -2/-9 through activation of nuclear factor kappa beta (NF- κ B). In this experiment, concentrations of >12.5 nM of MCLR were able to enhance metastatic activity of melanoma cells (Zhang et al 2012). Although our results do not conform with these findings, future studies may require longer exposure times and determinations of MMP expression/activity.

Overall, our findings reveal that long-term exposure to low-concentrations of MCLR does not consistently increase malignant hepatocyte proliferative activity or wound healing. Similar to the WB-F344 study, the PLC/PRF/5 cell line study was also designed to be broad in scope, allowing for general observations to be made regarding the impact of MCLR on tumor promotion *in vitro*. A discussion of study limitations is therefore required to better instruct future investigation. Aside from altering MCLR exposure time and dose,

additional molecular studies are required to investigate alterations in gene expression and signaling pathways (Akt and Wnt signaling pathways) associated with tumor promotion. It would also be interesting to determine whether similar results would be obtained with other malignant hepatocyte cell lines and/or primary cultures developed from human HCC tissues.

7.1.3. *MCLR and Hepatic Stellate Cell Activation*

Hepatic fibrogenesis is a mechanism of wound healing and repair, occurring in response to a variety of stimuli causing hepatic injury and inflammation. Fibrogenesis is characterized by excessive deposition of extracellular membrane (ECM) proteins, particularly type-1 collagen, leading to scar formation, alteration of hepatic architecture and formation of regenerative nodules. HSCs are cells found within the liver's space of Disse and remain quiescent until activated in response to liver injury. Once activated, HSCs mediate the process of hepatic fibrogenesis by two methods; 1) secretion of ECM proteins, mainly collagen and 2) becoming proliferative and further stimulating the fibrogenic response (Amann, Bataille et al. 2009).

Activation of HSCs occurs as a result of hepatic injury and inflammation, and can occur due to prolonged oxidative stress (Guzman and Solter 1999). Oxidative stress activation of HSCs is reported to occur through a variety of mechanisms including the induction of c-myc and NF-K β (Lee, Buck et al. 1995; Lee, Lee et al. 2001). Once activated HSCs become responsive to fibrogenic (transforming growth factor beta: TGF- β) and proliferative (platelet derived growth factor: PDGF) cytokines: wherein stimulation of p38-MAPK and 3-kinase-Akt-p70 S6 kinase signaling pathways, regulates HSC proliferation and collagen gene transcription (Parsons, Takashima et al. 2007). In a study by Galli et al (2005), it was

observed that the mitogenic and proinvasive characteristics of HSCs require increased expression of MMP-2. The authors concluded that the ERK1/2 and P13K signaling pathways mediate oxidatively induced MMP-2 expression in HSCs (Galli, Svegliati-Baroni et al. 2005). HSC activation is also associated with promoting HCC tumorigenicity, by enhancing HCC cell migration and proliferation *in vitro* and *in vivo* through activation of NF- κ B and ERK signaling (Amann, Bataille et al. 2009).

Previous studies investigating the relationship between sub-lethal MCLR exposure and fibrosis, have reported a positive association between MCLR exposure and fibrosis in liver and heart tissue of treated animals. In an early study investigating the toxic effects of MCLR *in vivo*, repeated sub-lethal doses of MCLR in mice produced hepatic degeneration, necrosis and fine hepatic fibrosis (Elleman, Falconer et al. 1978). Within cardiac tissue, after 8 months of exposure to sub-lethal concentrations of MCLR, fibrosis and mononuclear infiltration were observed in the hearts of male Wistar rats (Milutinovic, Zorc-Pleskovic et al. 2006). Similar results were reported in another study investigating the toxic effects of sub-lethal MCYR on the hearts of exposed male Wistar rats following 8 months of exposure (Suput, Zorc-Pleskovic et al. 2010).

In our study, we investigated the effect of exposure of low-concentrations of MCLR on CFSC-2G HSC activation by measuring cellular proliferation and smooth muscle alpha actin expression (SMAA) (marker of collagen secretion). Our results showed there was no increase in proliferative activity over the course of the experiment, other than at 96 hrs of exposure, when there was inhibition. Similarly, SMAA expression did not increase compared to controls following concentration dependent exposure to MCLR. Thus, our results do not support the concern that exposure to low-concentrations of MCLR will lead

to hepatic fibrogenesis and thereby increased risk of HCC development. However it remains to be determined whether CFSC-2G cells possess the specific oatp transporters required to facilitate adequate uptake of MCLR. To date, the oatp expression profile of HSCs has not been characterized.

The question remains as to whether *in vitro* HSC activation models are an accurate measure of *in vivo* fibrogenesis. Comparison of HSC activation *in vitro* and *in vivo* has revealed, that the *in vitro* HSC activation gene expression profile only partially overlaps with that observed in two well-known *in vivo* fibrosis models; carbon tetrachloride and bile duct ligation (Dashti, Jeppsson et al 2008; Hajovsky, Hu et al 2012). *In vivo* regulation of HSC activation uses a different gene set than that of cultured HSCs, resulting in expression of phenotypic characteristics that do not resemble those found in human cirrhotic liver (Sancho-Bru, Bataller et al. 2005; De Minicis, Seki et al. 2007). When cultured on plastic, HSCs undergo transdifferentiation or activation in the absence of stimulating factors, to produce a homogeneous culture of myofibroblasts like cells, mimicking the general process observed *in vivo* (Friedman 1993; Friedman 1999). Moreover, it is speculated that many cultured HSC cell lines are contaminated with other hepatic cell lines, possibly leading to misinterpretation of results. Co-culturing of HSCs with Kupffer cells has been reported to overcome some of the culture limitations experienced with HSCs, resulting in a more physiologically relevant HSC activation phenotype (Sancho-Bru, Bataller et al. 2005). Finally, evidence is emerging to suggest that various cell types are involved in fibrogenesis, and that HSCs are not solely responsible for mediating hepatic fibrosis. Within the liver, various distinct fibroblastic cell types have been described, each having a unique localization and immunohistochemical characteristics (Cassiman and Roskams 2002).

Therefore, given the complexity of HSC activation and mechanisms underlying hepatic fibrosis, *in vivo* analyses are deemed important in elucidating the role MCLR might play in hepatic fibrogenesis.

7.2. *In Vivo Analysis*

Our *in vivo* study served to support our *in vitro* findings by investigating the relationship between chronic low-dose oral exposure to MCLR in drinking water and HCC initiation, promotion and fibrosis. The dose and route of exposure were selected specifically to mimic exposure parameters encountered in Canada; the WHO stipulated maximal concentration allowed is 1 µg/L of MCLR within drinking water.

7.2.1. *Tumor Initiation*

In this aspect of the study we investigated whether chronic oral exposure to 1 µg/L of MCLR, initiates tumor development or altered gene expression associated with HCC *in vivo*. Tumor formation in animals following MCLR exposure, in the absence of other carcinogenic compounds, has not been observed *in vivo* to date. Nishiwaki-Matsushima et al (1992) observed that in the absence of a potent tumor initiator such as diethylnitrosamine (DEN), tumor formation in the liver did not occur, even in the presence of high concentrations of MCLR injected i.p. A second study was conducted using a different methodology, whereby tumors were first initiated by DEN and promoted through a combination of MCLR i.p injection pre and post partial hepatectomy. The group lacking DEN, failed to develop liver tumors even though animals were subject to MCLR i.p injection pre and post partial hepatectomy (Nishiwaki-Matsushima, Ohta et al. 1992).

The potential of MCLR to act as a carcinogen and initiate tumor development at low-doses is subject to debate (Svirčev, Baltić et al. 2010). At the molecular level, sub-lethal/low doses of MCLR have been observed to cause DNA damage and inhibit DNA repair mechanisms (Lankoff, Bialczyk et al. 2006; Shi, Guo et al. 2011). In colorectal crypt cells, MCLR was reported to malignantly transform immortalized cells and increase the proliferative activity of transformed cells by stimulating mitogen activated protein kinase (MAPK) pathways; Atk, p38 and JNK .

Our results revealed no formation of tumors, change in coloration or texture of the liver by visual inspection with the MCLR treated group. Histological examination of H&E stained MCLR treated liver sections revealed similar hepatic architecture characteristics, and no indication of tumor formation. These findings are keeping with our *in vitro* studies using WB-F344 hepatic stem cells. Together they suggest that chronic low-dose exposure to MCLR does not initiate tumor formation at the cellular or organ level.

Further evidence against chronic MCLR exposure playing an important carcinogenic role in tumor development were revealed through gene array analysis. To date the HCC gene expression profile of liver derived from animals chronically exposed to low-dose (1 µg/L) MCLR in drinking water has not been described. In this study, livers of mice from the MCLR treatment group had a total of 12 genes with > 2 fold altered regulation. Of those genes with altered regulation, signal transducer and activator of transcription (STAT3), epithelial growth factor receptor (Egfr), cyclin-dependent kinase inhibitor 1 (Cdkn1a) and Rassf1 were significantly up-regulated compared to the water alone exposed control. Comparison of gene expression to the positive control TAA group, revealed that only two

genes were up-regulated; Cdkn2a and Wt1, however neither change in expression was statistically significant.

Constitutive activation and up-regulation of STAT3 is associated with a variety of malignancies including HCC. STAT3 is transcription factor encoded by the STAT3 gene. Samples of 55 different HCC samples revealed that phosphorylation of the JNK/STAT3 cascade was associated with malignant transformation of hepatocytes, indicating that activation of STAT3 proteins may be an early event in HCC development (Feng, Zheng et al. 2001; Zhu, Zhong et al. 2005). Through the use of a STAT3-decoy to block over-activated STAT3 in HCC cells, it was observed that HCC cell proliferation was significantly suppressed, promoting cell cycle arrest at the G0/G1 to S phase and promoting apoptosis. It was also demonstrated that STAT3 regulated genes associated with cell cycle progression and apoptosis (bcl-x1, cyclin-D1 and c-myc), were down-regulated at the level of transcription and translation (Sun, Zhang et al. 2008). Within our positive control TAA treated group, STAT3 was not up-regulated.

Elevated Egfr expression has been well documented in HCC tissues. Egfr is a transmembrane glycoprotein receptor present on the surface of hepatocytes, playing a role in liver regeneration following injury (Taub 2004). Stimulation of Egfr by its ligands, tumor growth factor- α (TGF- α) and epidermal growth factor (EGF), contributes to hepatocarcinogenesis and HCC cell proliferation by activating signaling pathways RAS/RAF/MEK/ERK and P13K/AGT/mTOR (Harada, Shiota et al. 1999; Hisaka, Yano et al. 1999; Villanueva, Newell et al. 2007). In a study by Kira et al (1997) it was observed that Egfr was significantly up-regulated in poorly differentiated HCC samples (Kira, Nakanishi et al. 1997). Similar findings were also observed by Zhao et al (2004), where egfr expression

in HCC tissue was significantly elevated compared to surrounding liver tissue. The authors concluded that Egfr expression could be used as a predictive marker of HCC metastasis and recurrence (Zhao, Cao et al. 2004). Of note, Egfr is modulated by serine/threonine phosphorylation. Okadaic acid, a well characterized serine/threonine phosphatase inhibitor functions similar to MCs, assists with hyperphosphorylating Egfr, activating down-stream signal cascades to promote cellular proliferation (Hernandez-Sotomayor, Mumby et al. 1991). Although there has been no investigation into the relationship between MCLR exposure and Egfr phosphorylation, *in vitro* evidence supports the hypothesis that MCLR is able to modulate Egfr activity.

Cdkn1a gene expression is down regulated in HCC (Gramantieri, Chieco et al. 2005). Cdkn1a is a gene that encodes for cyclin-dependent kinase inhibitor 1, otherwise referred to as p21 or CDK interacting protein 1. When transcribed, p21 binds to cyclin-dependent kinases, and regulates cell cycle progression at the G1 phase (Gartel, Najmabadi et al. 2000). P21 is known to facilitate hypophosphorylation of retinoblastoma (Rb) proteins, through inhibition of phosphorylation. Rbs are important tumor suppressor proteins: controlling cell cycle progression by repressing activity of E2F transcription factors. Cyclin-dependent kinases phosphorylate Rbs, activating the proteins and facilitating G1/S cell cycle progression. Inhibition of p21 contributes to hepatocarcinogenesis through kinase-mediated phosphorylation of Rbs, thereby contributing to cell proliferation (Hsia, di Bisceglie et al. 1994; Weinberg 1995; Löhr, Möritz et al. 2003). In our study, cdkn1a expression was up-regulated in both MCLR alone, TAA alone and MCLR & TAA exposed animals. However liver tumor formation was only observed in TAA alone and MCLR & TAA

treated animals. This may explain why despite elevation of a variety of HCC specific genes, there was no liver tumor development.

The Rassf1 gene encodes for the ras association domain protein 1, and is associated with cell cycle regulation, microtubule stability and apoptosis. It is categorized as a potent tumor suppressor gene and is silenced in a variety of cancer types including; breast, lung, cervical, prostate, gastric, brain and colorectal (Schagdarsurengin, Wilkens et al. 2002; Agathangelou, Cooper et al. 2005; Li, Fu et al. 2008). Rassf1 has been shown to associate with Rb and p53, two well known tumor suppressors, through transcription factor p120 (Fenton, Dallol et al. 2004). In vitro and in vivo studies have shown that tumorigenicity is inhibited in various cancer cell lines following re-insertion of Rassf1 (Dammann, Li et al. 2000; Burbee, Forgacs et al. 2001). Within HepG2 and HepB3 HCC cell lines, hypermethylation of the Rassf1 promoter region inactivated Rassf1 transcription. Transcription of Rassf1 was reactivated following treatment with a DNA methylation inhibitor. The state of Rassf1 methylation was investigated within 26 primary liver tissues including; fibrosis, cirrhosis, HCC and hepatic adenoma. Rassf1 methylation was found in the following tissues; 93% (14 out of 15) of the HCC tissue samples, 100% (2 out of 2) of the fibrosis samples and 75% (3 out of 4) of the cirrhosis samples (Schagdarsurengin, Wilkens et al. 2003). It is suspected that histone inactivation in epithelial cells, precedes Rassf1 promoter methylation and is triggered by de novo DNA methylation (Strunnikova, Schagdarsurengin et al. 2005).

Although Rassf1 hypermethylation and resulting inactivation is associated with HCC, we report different findings. Up-regulation of Rassf1 gene expression was observed in both our MCLR and TAA treated groups (> 8 fold increase). Enhanced Rassf1 expression is

associated with promoting cellular apoptosis, and inhibiting cell cycle progression by engaging the Rb family cell cycle check-point and inhibiting cyclin-D1 accumulation (Shivakumar, Minna et al. 2002). It may stand to reason that in tissues exposed to environmental carcinogens, Rassf1 may initially be up-regulated in order to suppress the onset of tumorigenesis.

Rassf1 is also associated with activation of the Bcl2-associated X protein (Bax), which is known to be activated in response to DNA damage, triggering apoptosis (Vos, Dallol et al. 2006). As observed in the TAA treatment group, Rassf1 was up-regulated by > 8 fold, yet despite the marked increase in the tumor suppressor gene, hepatic tumorigenesis occurred. Therefore, Rassf1 may be potentially up-regulated to mitigate MCLR induced DNA damage, and prevent tumor initiation. Whether the degree to which Rassf1 is elevated, could serve as an indicator of the level of DNA damage, and carcinogenic potential of a compound remains to be determined.

Overall, it has been observed *in vivo*, that exposure to environmental carcinogens elicits a dual response within hepatocytes: activating both tumor initiating (Stat3 and Egfr) and tumor suppressor genes (Cdkn1a and Rassf1). In the MCLR and TAA exposed groups, there was an up-regulation of tumor initiating factors and potent tumor suppressors. The TAA exposed animals had tumor development in approximately 20 - 25% of animals. Whereas MCLR treated animals experienced no tumor development. The expression of tumor initiator and suppressor genes were more pronounced within the TAA alone exposed groups, compared to the MCLR alone treated group. Therefore, perhaps within the context of chronic liver disease, tumorigenesis occurs when the balance between tumor

suppression (Cdkn1a and Rassf1) and initiation (STAT3 and Egfr) is altered in support of the later, particularly in the presence of a potent carcinogen.

Despite altered gene expression in MCLR alone treated mice, HCC development was not observed. Somatic mutation theory suggests that cells undergo malignant transformation following accumulation of multiple genetic mutations that affect cellular regulatory controls (Friedberg, McDaniel et al. 2004; Rosenfeld 2013). In our study, only 4 genes were significantly altered within the MCLR treatment group, a number which may not have been sufficient to initiate carcinogenesis and overcome cellular control redundancies (Friedberg, McDaniel et al. 2004). In this instance, our study findings support the literature in refuting that chronic low-dose MCLR exposure functions as a tumor initiator.

7.2.2. Tumor Promotion

In this portion of the *in vivo* study, we sought to examine the ability of chronic low-dose MCLR to promote tumor growth in the context of pre-existing liver disease and tumors. To address this question, MCLR was administered to mice in conjunction with 0.03% TAA (TAA & MCLR) in drinking water over a period of 7 months. As previously discussed, after being metabolized and activated within the liver, TAA induces chronic liver disease similar to that of humans who go on to develop HCC. Under chronic liver disease conditions, the ability of MCLR to promote tumor growth should be evident.

The ability of MCLR to promote HCC progression has been explored using different models of HCC initiation and promotion *in vivo*. Using a two stage *in vivo* carcinogenesis model, Nishiwaki-Matsushima et al (1992) demonstrated that in conjunction with a tumor initiator such as DEN, MCLR could increase the number of GST-P positive liver foci by

roughly two fold when injected at high concentrations (10 ug/kg). In that same study, higher concentrations of MCLR further increased the number of GST-P positive foci within the liver, when administered following DEN initiation and partial hepatectomy.

The results of the present study revealed that the size, number and phenotype of tumors identified within the TAA alone and TAA & MCLR treated groups were similar. Thus, there was no evidence to support MCLR's role in promoting HCC development in TAA treated mice.

HCC gene analysis comparing TAA and TAA & MCLR treated groups revealed a significant down-regulation (by 2 fold) of the prostaglandin endoperoxidase synthase-2 (Ptgs2) gene. Ptgs2 is the inducible isoform of cyclooxygenase (COX); COX-2, and plays a key role in prostaglandin synthesis, a pro-inflammatory mediator (Kosaka, Miyata et al. 1994). COX-2 inhibitors are being investigated for their ability to suppress HCC progression during the early stages of disease, particularly when administered in combination with other anti-cancer agents. It is unknown what role down-regulation of COX-2 plays in TAA mediated tumor development, and further investigation is required (Koga 2003).

Our overall observations regarding MCLR's inability to induce tumor formation or promotion may be explained by the fact that the dose of MCLR used within these experiments, was too low to induce a significant degree of toxicity associated with tumor promotion. As discussed previously, higher doses of MCLR introduced through i.p injection have been demonstrated to have a stronger tumor promoting effect than low dose oral exposure (Nishiwaki-Matsushima, Ohta et al. 1992). However, given that the Canadian

population dose not routinely inject high concentrations MCLR, the i.p route of exposure was not a suitable delivery method for this particular experiment.

7.2.3. Fibrogenesis

Previous studies investigating the histological and plasma chemistry changes in livers associated with exposure to MCLR, were conducted using lethal or highly toxic doses of toxin. At these doses, MCLR can cause a variety of pathological changes in normal hepatic architecture of mammals including rounding of hepatocytes, loss of normal hepatic architecture, necrosis, increased liver weight and fulminant intrahepatic hemorrhage (Fischer, Hitzfeld et al. 2000). Livers of mice exposed to lethal doses of purified MCLR extracts from *M. aeruginosa* experienced perlobular distribution, hepatocyte necrosis, progressive destruction of parenchymal structure, loss of sinusoidal endothelium and accumulation of erythrocytes (i.e. hemorrhage) within 45 minutes of exposure (R Falconer, Jackson et al. 1981). Sheep poisoned by toxic *M. aeruginosa* extracts, experienced hepatocyte necrosis, alterations in liver parenchymal structure and an increase in serum concentrations of bilirubin, blood urea nitrogen and inorganic phosphorous (Jackson, McInnes et al. 1984). The histological impact of MCLR on liver architecture has also been investigated in rainbow trout, common carp and broiler chicken, revealing a similar trend of hepatic injury (Jackson, Runnegar et al. 1986; Råbergh, Bylund et al. 1991; Fischer, Hitzfeld et al. 2000).

The association between MCLR exposure and fibrosis has been discussed thoroughly within the CFSC-2G cell line analysis portion of this document. The mechanisms by which MCLR induces hepatotoxicity; oxidative stress, DNA damage, necrosis, apoptosis, etc, are similar to those required to stimulate HSC activation (Lee, Buck et al. 1995; Guzman and

Solter 1999; Guo and Friedman 2007). *In vivo* studies have reported fibrosis in cardiac tissue of rats exposed to low-doses of MCLR (Suput, Zorc-Pleskovic et al. 2010). In this portion of the study, the initiation and promotion of hepatic fibrogenesis was investigated *in vivo* by comparing the fibrosis scores in the MCLR and TAA & MCLR treated groups to the negative and positive controls.

In the present study, our results revealed that MCLR did not initiate or promote fibrosis within healthy animals or animal model of chronic liver disease respectfully. Comparison of MCLR alone to the water alone treated controls, revealed no significant difference in fibrosis grade, a trend that was also observed when comparing the TAA alone and TAA & MCLR treated groups. Following liver resection, livers were weighed and the percent liver weight of body weight (LW% of BW) was calculated. The general liver weight and LW% of BW of the MCLR treated group was slightly less compared to the water alone controls, however not to a significant degree. Histological examination revealed that hepatic lobules maintained an organized hexagonal arrangement, with little or no evidence of inflammation or fibrosis around the central or portal veins. Plasma chemistries of ALT levels and total bilirubin, used to assess for inflammatory activity and abnormal liver function respectively, were similar in the MCLR alone and water alone treated groups. All physical, histological and plasma chemistry results were in keeping with the conclusions that chronic oral exposure to 1 µg/L of MCLR is not associated with fibrosis or cirrhosis that favors HCC formation.

The limitations of this study involved a fixed duration of exposure and concentration of MCLR. It is conceivable that extending the duration of the study and using higher

concentrations of MCLR, might have provided different results, but were beyond the principle objective and scope of this study.

7.3. *Canadian Epidemiological Analysis*

The results of this aspect of the study revealed that liver cancer rates are not higher in regions of the country where cyanobacterial contamination of the lakes and drinking water would be expected to be most problematic; as reflected by surrogate markers for cyanotoxin exposure (agriculture, cattle and swine density). The results also indicate that as in other parts of the world, liver cancers in Canada tend to be more common in those individuals with chronic hepatitis B infections, areas where there has been significant immigration and large urban centers.

Further supporting the finding that cyanobacterial contamination of lakes and drinking water does not represent a significant risk for liver cancer development in Canada, are the results of the Gini coefficient analysis. The relatively low coefficient suggests that liver cancer cases are not highly concentrated in a small number of geographic areas of the country (as might be expected with cyanobacterial contamination of drinking water), but rather, are distributed across all population groupings. This suggests that single point sources of exposure leading to liver cancer are unlikely in discrete geographic populations, and the causative conditions are more widely distributed.

Overall, the findings of this study differ from what was described by investigators in China, Serbia and the United States. Specifically in China, the incidence of liver cancer was documented to be highest in rural populations receiving drinking water from cyanobacterial contaminated pond and ditch sources, compared to those whose drinking

water was derived from cyanobacterial-free deep wells or rivers (Yu and Chen 1994; Yu 1995). Similarly, in Serbia, Scircev et al (2009) reported 1.5 - 4.4 fold increases in primary liver cancer (PLC) mortality rates in regions of Central Serbia receiving their drinking water from cyanobacterial contaminated surface water reservoirs, compared to regions receiving their drinking water from non- cyanobacterial contaminated underground sources. Finally, in the American state of Florida, Fleming et al (2002) documented a 1.38 fold increase risk of liver cancer in communities receiving drinking water from cyanobacterial contaminated surface water reservoirs, compared to the non-exposed contiguous matched control zones.

There are a number of potential explanations for why our results differ from those discussed above. First, our study population consisted of the entire national Canadian population rather than a smaller regional or state sub-population. Thus, relevant local level relationships are more likely to have gone undetected in our study. Second, our limited demographic data meant that age and gender could only be controlled for at the ecological level. To partially overcome this barrier, analyses were restricted to the 40 plus population in whom the majority of liver cancer cases occur. Third, liver cancer case data received from the National Cancer Registry were highly suppressed (92 CDs with suppressed data) and as a result, the geographic trends observed may represent an artifact of the data imputation method used to overcome data suppression. To minimize the impact of this limitation, spatial smoothing and cluster identification techniques were employed.

Fourth, the liver cancer case data received from the National Cancer Registry was aggregated to Census Divisions (CDs). CDs are highly variable in size, with some containing populations in excess of 2.5 million people. As a result, an examination of the variation in liver cancer rates within large urban centers was not feasible, resulting in a likely truncation of observed liver cancer variability across Canada. Furthermore, the ecological regression models used in the study were often imprecise because they had to classify all individuals living in a geographic area with the same ecological predictor value. This may not have been a problem for ecological modeling of predictors which homogeneously affected the whole population in a CD (e.g. urban residence), but was likely a significant problem for predictors where there may have been significant heterogeneity within geographic areas (e.g. family income in large urban CDs). Thus, the modeling results may have been biased in some cases, with the observed relative risk being either muted or possibly in a direction opposite to what one would have observed if modeling could have occurred on a finer scale.

Fifth, for a number of predictor variables, the data was not available at the CD level and had to be spatially interpolated from another scale. For example, binge drinking had to be interpolated from regional health authority area, and hepatitis B and C infections were interpolated from scale of the province. In the case of hepatitis B and C, it is unlikely that all CDs in a province had the same rates of these diseases. In addition, the definition of urban/rural used in the study was potentially problematic in that many CDs are of sufficient size that they often contain both significant urban and rural populations.

Finally, we utilized surrogate markers of cyanobacterial contamination to document sources of contaminated drinking water, rather than the direct cyanotoxin or cyanobacterial quantification used by Chinese, Serbian and American investigators. The lack of Canadian wide cyanobloom or MC toxin concentration data and inability to identify more sensitive upstream proxies of exposure may well have compromised our results. However, since we utilized upstream predicting factors for cyanobacterial contamination, it addressed the possibility that various hepatotoxigenic strains of cyanobacteria may be present, releasing MC congeners aside from MCLR.

7.4. *Manitoba Epidemiological Analysis*

Within Manitoba, monitoring for the presence of MCLR in surface waters of recreational use lakes is not performed on a consistent basis. Regulations are in place to limit MCLR content in drinking water sources, measuring for cyanobacterial levels and toxin loads in areas reported to be problematic. The purpose of the Manitoba epidemiological study was to further investigate the relationship between liver cancer incidence and exposure to MCLR through contaminated drinking water or recreational sources at the provincial level: addressing many of the limitations of the Canadian epidemiological study. Eutrophication of freshwater resources in Manitoba is a major problem, leading to excessive cyanobacterial growth in many prominent lakes such as Killarney Lake, Pelican Lake and Lake Winnipeg.

Age-standardized analysis of liver cancer rates revealed increased incidences over the 22 year study period from 1985 to 2007 in Manitoba. The potential hepatocarcinogenic effects of MCLR have been discussed in detail at the cellular and Canadian epidemiological

levels. However, the relationship between MCLR exposure and liver cancer at the provincial level has not been explored.

7.4.1. *Microcystin-LR Data*

The MCLR data used for this study was gathered throughout a four year (2007-2011) period by Manitoba Conservation and Water Stewardship, analyzing for the presence of MCLR and cyanobacteria composition: Data collected was high quality, yet variable. The MCLR concentration data collected at each location throughout the four year period was averaged to provide general toxin load data for each location. A set of assumptions were applied to the data set in order to allow for temporal and geographic analysis of suspected MCLR concentrations with known cancer incidence. Three assumptions were used regarding the nature of the MCLR data collected; 1) the average MCLR values calculated would be representative of past MCLR concentration levels for their respective locations from 2007 back to 1985, 2) the communities located near MCLR contamination sites would be consistently exposed to those toxins; drinking water, ingestion of contaminated fish or dermal exposure, and 3) that MCLR (the only cyanobacterial toxin measured) was the predominant toxin present within contamination sites. Although the methodology employed was not ideal, given that a complete toxin data set for all water sources in Manitoba was not available, it does allow for general observations to be made.

Cyanotoxin production is under complex regulation, influenced by a combination of limnological factors that control cellular proliferation and toxin synthesis; nutrient loading and stoichiometric ratios of N and P, trace metals, genotypic composition and abundance of cyanobacteria community, water pH and temperature, light, oxygen saturation and interaction with biota (bacteria, viruses and grazers) (Wicks and Thiel 1990; Kotak, Lam et

al. 2000; Cataldo, Vinocur et al. 2012; Paerl and Otten 2013). Given the variable conditions under which cyanotoxins are produced, it becomes difficult to predict long-term trends in cyanotoxin loading and population exposure. Therefore the base set of assumptions applied to the MCLR data may have under or over-estimated toxin exposure.

7.4.2. Liver Cancer Incidence and Relative Risk

In Manitoba, liver cancer rates have been steadily increasing from 1985 to 2007 by 2.35 cases/100,000. Liver cancer incidence occurred primarily within southern Manitoba near urban centers, coinciding with high population density. Throughout the 22 (1985 – 2007) year observation period, the number of liver cancer cases occurring within the allotted MCLR buffer zone and correlating buffer ring were relatively constant. Despite a mild increase in liver cancer incidence occurring within the MCLR buffer ring between 1990 to 1994, there was little variation observed throughout the 22 year period. Inspection of the change in distance of liver cancer cases to MCLR contamination sites throughout the observation period showed that the variation in logarithmic distance was insignificant. Similarly, there was no significant increase in the relative risk of liver cancer observed within the MCLR buffer zones and rings as compared to the 1985 – 1989 reference year.

Once again, our results do not comply with previous findings from China, Serbia and Florida (Harada, Oshikata et al. 1996; Ueno, Nagata et al. 1996; Yu, Zhao et al. 2001; Burns 2008; Huang, Zhao et al. 2008; Svircev, Krstic et al. 2009), where high levels of MCLR concentrations in drinking were associated with increased cancer incidence. The Manitoba study did not have the data to directly analyze drinking water sources for MCLR concentrations. Instead, the provincial toxin data was used to provide a preliminary view of environmental contaminants. Furthermore, given that drinking water sources are

routinely monitored for MCLR contamination in Canada, treated drinking water sources would have limited MCLR content ($<0.03 \mu\text{g/L}$) compared to countries with less stringent water quality standards.

The limitations of the study are clear; there is simply a lack of consistent MCLR data available at the provincial level. The toxin data was used to extrapolate past MCLR toxin concentrations within the province relevant for the 22 year cancer incidence observation period. Until a consistent MCLR monitoring program is developed in the province, future epidemiological studies will also be restricted in the same manner.

Additional limitations that could not be overcome due to the nature of the study include; 1) limited and/or seasonal exposure of population to MCLR contaminated water, 2) inconsistent MCLR concentration level exposure, 3) insufficient MCLR exposure time, 4) MCLR contamination sites not consistent with drinking water sources and 5) improved cancer detection. Another limitation of this study is that we only assessed the potential association between liver cancer incidence and one MC variant (MCLR). A single toxigenic cyanobacterial species can synthesize and release various MC congeners into the environment, each having their own unique toxic profile (Oh, Lee et al. 2000; Gupta, Pant et al. 2003; Mbukwa, Msagati et al. 2012). It is possible that additional MC congeners aside from MCLR were present within the water samples taken in Manitoba, and due to testing for MCLR only, the presence of additional congeners was overlooked. It is unknown to what extent MC variants function synergistically to promote hepatotoxicity. However, the possibility exists that a combination of MCs may be more potent than a single variant, and elicit more pronounced hepatotoxicity. To fully address the impact of chronic exposure of MCLR on human health in Canada, a more in-depth long-term study is required, wherein

MC concentrations from drinking water sources are measured and observed along with cancer incidence.

8. Conclusions

The incidence of HCC in Canada has been increasing over the past two decades, without clear evidence to explain the trend. The potential of cyanobacterial hepatotoxins to impact chronic liver disease and HCC development has been explored through our study. The findings from our *in vitro* and *in vivo* experiments revealed limited evidence to support the hypothesis that low-dose exposure to MCLR is associated with HCC initiation, promotion or hepatic fibrogenesis.

Similarly, findings from our Canadian epidemiological study revealed that predicted cyanotoxin exposure does not appear to be a contributing factor to the liver cancer incidence rates at the national level. The Manitoba epidemiological study findings further supported the Canadian epidemiological conclusions, whereby no association was observed between liver cancer incidences and MCLR contamination sites in the province.

In conclusion we report that chronic low-dose exposure to MCLR is not associated with HCC initiation, promotion or hepatic fibrogenesis at the cellular, systemic or epidemiological levels. In Canada, drinking water quality is paramount to maintenance of population health. With changing climate and increased eutrophication of fresh water sources, the public should be aware of the potential harmful effects associated with persistent low dose toxigenic cyanobacterial exposure. However, with respect to an increased risk of HCC development or growth, there does not appear to be cause for concern.

9. Future Directions

Researchers are just beginning to understand the complexity of cyanobacterial toxins, and the role they play in the ecosystem and human health. Research into the synergistic toxic relationship between MC congeners is starting to emerge. To date >80 different types of MCs have been recorded, each having their own unique toxicity profile and potential biological impact. Single toxigenic cyanobacterial species can release a variety of MCs into the environment, varying their concentration and temporal expression. In the environment, fresh water MC congener content varies significantly, and investigation of MCLR alone is highly limited. Further studies into the impact of MC exposure on chronic disease conditions, should be conducted using MC cocktails, as opposed to single toxins: thereby simulating conditions that reflect environmental conditions more accurately.

There are many areas in which MCLR toxicological research could be expanded, making the research more relevant to the population under investigation. As previously alluded to, chronic exposure to sub-lethal concentrations of MCLR would be best studied with subjects either at risk or having a pre-existing chronic health condition. Further exploration into the association between MCLR exposure and neurological degeneration and the epidemic of diabetes in our rural Aboriginal populations, also warrants investigation (MCLR induced neurotoxicity resembles that observed in patients with Alzheimer's Disease and increases to apoptosis of pancreatic islet cells) (Walsh, Klyubin et al. 2002; Belmeguenai and Hansel 2005; Ji 2011). Finally, whether the harmful effects of high concentrations and targeted delivery of cyanobacterial toxins could be harvested for potential use as tumoricidal agents remains to be determined.

10. References

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