LAND USE INFLUENCES ON CYANOTOXIN ABUNDANCE IN SMALL SOUTH-CENTRAL SASKATCHEWAN WATER BODIES

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By

Ashley Katerine James

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ABSTRACT

Blooms of cyanobacteria that produce toxic compounds occur in ponds and wetlands in Saskatchewan, where they pose a risk to domestic livestock and wildlife. Most documented poisonings of animals that are caused by cyanotoxins result from exposure to the microcystins, which cause damage to the liver. The present study was intended to identify whether agricultural land use (arable land vs. permanent pasture), and/or geographic region were associated with the presence and abundance of toxins produced by cyanobacteria in small Saskatchewan water bodies in the Saskatchewan northern prairie ecozone. Cyanobacterial abundance and the risk of toxin production are thought to be associated with nutrient enrichment of water bodies, which might be linked to agricultural practices. Therefore, the study was also intended to determine if concentrations of nutrients in surface waters and/or soils were different for small water bodies and their surrounding drainage basins located within arable land compared with permanent pasture.

Study sites were clustered in three regions of south-central Saskatchewan, including Saskatoon/Aberdeen, Elrose/Rosetown and Swift Current. These regions were located within two of the three main soil zones found within southern Saskatchewan. Each cluster consisted of eight small water bodies (natural ponds or wetlands, or farm dugouts) located within arable crop fields (mostly grain or pulse crops), and eight small water bodies located in permanent pasture land used for cattle grazing. Water was collected twice from all 48 sites, first in July and again in late August/September of 2009. Soil was collected once at all 48 sites. Samples collected included: 1) drainage basin soil cores for nutrient analysis, including available nitrate, ammonium, phosphorus and potassium; 2) integrated surface water samples for total phosphorus, soluble reactive phosphorus, nitrate, nitrite, total kjeldahl nitrogen, ammonia and chlorophyll-a; and 3)

integrated surface water and algal bloom samples for cyanotoxin analyses and possible algal identification.

Saxitoxins, which are neurotoxic cyanotoxins were identified and quantified by use of HPLC-MS/MS, while microcystins, which are hepatotoxins, were identified and quantified by use of both HPLC-MS/MS and a bioassay that measured inhibition of phosphatase activity. Results of the neurotoxin analyses indicated only trace amounts of saxitoxins present in any water bodies at the time of sampling. Although more work is necessary to determine the extent to which saxitoxins occur in small Saskatchewan water bodies, the results of the present study suggest that these neurotoxins pose little risk to humans, domestic animals or wildlife.

Microcystins occurred consistently in lesser levels throughout the summer months in small water bodies in south central Saskatchewan. Although microcystins were detected in water bodies located within areas of both types of land use, the water bodies surrounded by arable land contained significantly greater concentrations of these toxins. This suggests that practices specific to arable land are influencing abundances of microcystins.

Since there were no statistically significant differences in concentrations of microcystins between geographic regions, results were analyzed using data pooled from all areas. No significant differences between land uses were found for any water quality parameters. Concentrations of soluble reactive phosphorus in water and total phosphorus in water and soil were found to be significantly different between land uses, and were positively correlated with concentrations of microcystins in water. Nitrogenous compounds in soil and water were also positively associated with concentrations of microcystins. This result suggests that repeated fertilization with nitrogenous fertilizers is possibly leading to accumulation of nitrogen

compounds in soil, which can be mobilized into local surface water, and potentially promote the growth of cyanobacteria.

The triggers for formation of cyanobacterial blooms and subsequent production of microcystins are multi-factorial and complex, such that predicting formation of blooms at any given location is not currently possible. However, the results of this study of environmental factors and land use on the Canadian prairies generally confirms work done in other systems in that the presence of greater concentrations of nutrients, potentially due to fertilization practices, appears to be the critical factor affected by human activity that can be associated with the likelihood of production of microcystins.

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LIST OF ABBREVIATIONS

µg	microgram
µg/kg	micrograms per kilogram
µg/L	micrograms per liter
µL	microliter
µm	micrometer
µM	micromolar
ANOVA	analysis of variance
AU	arbitrary units
A.L.	arable land
˚C	degrees centigrade
Chl a	chlorophyll-a
cm	centimeter
CO_2	carbon dioxide
dcNEO-b	decarbamoyl neosaxitoxin
dcSTX	decarbamoyl saxitoxin
dmMC-LR	demethylated microcystin-LR
EV	electron volts
g	gram
GC/MS	gas chromatography - mass spectrometry
GIS	geographic information systems
HPLC	high performance liquid chromatography
HPLC-MS/MS	high performance liquid chromatography tandem mass spectrometry
i.p.	intraperitoneal
KCl	potassium chloride
kg	kilogram
km^2	square kilometers
L	liter
m	meter
M	molar
MC-LR	microcystin-LR
MC-RR	microcystin-RR
mg	milligram
mg/L	milligrams per liter
mL	milliliter
mm	millimeter
mM	millimolar
mS/cm	millisiemens per centimeter
m/z	mass-to-charge ratio
n	number of samples
NEO-b	neosaxitoxin
NH_{4+}	ammonium
NO_{2-}	nitrite
NO_{3-}	nitrate

NOAEL	no observed adverse effect level
NOD	nodularin
N:P	total kjeldahl nitrogen to total phosphorus ratio
PFRA	prairie farm rehabilitation administration
P.P.	permanent pasture
ppb	parts per billion
ppt	parts per trillion
PSP	paralytic shellfish poisoning
rpm	revolutions per minute
r^2	coefficient of determination
s	seconds
SD	standard deviation
SRP	soluble reactive phosphorus
STX-e	saxitoxin
TKN	total kjeldahl nitrogen
TP	total phosphorus
WCVM	Western College of Veterinary Medicine
WHO	World Health Organization

CHAPTER 1

1 GENERAL INTRODUCTION

1.1 Cyanobacteria: An introduction

Cyanobacteria, which historically were classified as blue-green algae, are a group of prokaryotic, photosynthetic bacteria. Their common name "blue-green algae", so named because of their appearance, is misleading, because although these organisms can photosynthesize, they are still classified as prokaryotes, not eukaryotes. Cyanobacteria were some of the earliest known organisms, their presence dating back as far as 3.5 billion years in the fossil record (Falconer, 2005). Their development from the most primitive DNA and protein based organisms is thought to have taken approximately ten million years or less (Lazcano and Miller, 1994), and they were most likely among the first organisms to release oxygen into the atmosphere (Chorus and Bartram, 1999).

Cyanobacteria are found in fresh and brackish water, marine systems, and on land (Barco et al., 2005, Florencio et al., 2006). Their ability to colonize environments of extreme salinity and temperature (Chorus and Bartram, 1999) is well documented, as is their potential to produce a variety of lethal toxins. Toxin-producing cyanobacteria have been reported from all over the world (McElhiney and Lawton, 2005), including Australia (Kankaanpää et al., 2005), South America (Irisarri et al., 2001), North America (Carmichael and Li, 2006), Asia (Mohamed and Al Shehri, 2007), Europe (Bláhová et al., 2009), Africa (Nyenje et al., 2010) and even Antarctica (Hitzfeld et al., 2000).

1.2 Cyanobacterial toxins

Some genera of cyanobacteria have the ability to produce lethal toxins termed cyanotoxins. Genera commonly implicated in cases of poisonings include: *Microcystis, Anabaena, Planktothrix, Aphanizomenon, Cylindrospermopsis, Phormidium, Nostoc,*

Anabaenopsis and *Nodularia* (Metcalf and Codd, 2004).Three major groups of cyanotoxins have been identified. These include: lipopolysaccharide endotoxins, neurotoxins and hepatotoxins. Since they are a component of the gram-negative cell wall, the endotoxins are common to all cyanobacteria, (McElhiney and Lawton, 2005). Endotoxins might cause gastroenteritis or dermal or respiratory irritation in animals or humans exposed to cyanobacteria. The hepatotoxins and neurotoxins, which are not produced by all genera of cyanobacteria, have been implicated in the deaths of humans, domestic animals and wildlife worldwide.

1.2.1 Cyanobacterial neurotoxins

There are three distinct types of cyanobacterial neurotoxins: anatoxin-a, anatoxin-a(s) and saxitoxins. Anatoxin-a is an alkaloid that was historically known as VFDF, or "very fast-death factor" (Sawyer et al., 1967). It is produced by various species in the genera *Anabaena*, *Aphanizomenon, Cylindrospermum, Microcystis, Oscillatoria, Planktothrix* and *Raphidiopsis* (Aráoz et al., 2010). Once ingested, anatoxin-a act by binding to post-synaptic acetylcholine receptors at neuromuscular junctions, causing depolarization and continuous stimulation of muscle cells, with eventual death due to respiratory paralysis (Carmichael et al., 1992). The LD_{50} determined by intraperitoneal (i.p.) injection of mice is 200µg kg^{-1} (Carmichael et al., 1992). The USEPA (2006) suggested a NOAEL of 2.5 mg/kg-day for anatoxin-a, but this information comes from a draft report, in which it is suggested that more work is needed. Recent research by Alberta Environment has detected the presence of anatoxin-a in a number of water bodies, albeit infrequently, including one in Saskatchewan (Government of Alberta, 2010).

Anatoxin-a(s) is another neurotoxin produced by a number of species in the genus *Anabaena* (Aráoz et al., 2010, Becker et al., 2010). The "s" stands for salivation, a clinical sign indicative of the parasympathomimetic effects associated with anatoxin-a(s). Little information

is available on the presence of this toxin in Saskatchewan. Anatoxin-a(s) binds to acetylcholinesterase in cholinergic synapses in the peripheral nervous system, which prevents the enzyme from breaking down acetylcholine bound to post-synaptic receptors at neuromuscular junctions (Becker et al., 2010). As with anatoxin-a, death occurs due to respiratory paralysis via fatigue of muscle cells. The LD_{50} determined by i.p. injection of mice is 20µg kg^{-1} (Carmichael et al., 1992). No drinking water guidelines for anatoxin-a(s) have been established to the author's knowledge.

Saxitoxin and its variants are neurotoxic alkaloids produced by a number of strains of cyanobacteria, including *Anabaena circinalis*, *Lyngbya wollei*, and *Aphanizomenon flos-aquae* (Dell'Aversano et al., 2003). There are approximately 20 known saxitoxin variants (Dell'Aversano et al., 2005). These variants are categorized into three groups depending on their side chain: carbamoyl, decarbamoyl or sulfocarbamoyl (Dell'Aversano et al., 2005). Two carbamoyl variants (saxitoxin and neosaxitoxin), and two decarbamoyl variants (decarbamoyl saxitoxin and decarbamoyl neosaxitoxin) were examined in this study. The carbamoyl saxitoxins are the most toxic group (Dell'Aversano et al., 2005), with saxitoxin itself having an LD_{50} of approximately 10 µg kg^{-1} (Carmichael et al., 1992).

Saxitoxin and its variants act by binding to and blocking the extracellular pore of voltage-gated sodium channels on excitable cells (Narahashi et al., 1972 as referenced in Dell'Aversano et al., 2005). This blockage halts the inward flux of sodium and membrane depolarization, which subsequently arrests nerve conduction. Unlike the other cyanobacterial toxins, saxitoxin is frequently associated with human poisonings. Toxicosis due to direct ingestion of water that contains saxitoxin is uncommon, but indirect poisonings of humans due to ingestion of shellfish, termed "paralytic shellfish poisoning" has been described for centuries. Toxicity of this type is

caused by the ingestion of shellfish that have become contaminated by saxitoxin and its variants through filter-feeding on toxic phytoplankton (Turrell et al., 2008). Paralytic shellfish poisoning is an acute syndrome characterized primarily by neurological symptoms, including initial paraesthesia of the mouth and throat that progresses to the neck and extremities, dizziness, weakness, ataxia and muscular and respiratory paralysis (Fitzgerald et al., 1999). Death can occur within minutes (Rapala et al., 2005). No official drinking water guidelines could be found for saxitoxin and its variants, although a health alert at 3 µg STX-eq/L has been suggested (Fitzgerald et al., 1999, Australian Government, 2004).

1.2.2 Cyanobacterial hepatotoxins

Microcystins are a group of cyclic peptides produced by several genera of cyanobacteria, including *Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc* and *Anabaenopsis* (McElhiney et al., 2005). There have been close to 90 variants of microcystin identified (Welker and von Döhren et al., 2006), all with a similar general structure containing three D-amino acids (alanine, β linked erythro-β-methylaspartic acid, and γ-linked glutamic acid), two variable L-amino acids, R_1 and R_2, and two unusual amino acids, *N*-methyldehydroalanine (Mdha) and (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4(E),6(E)-dienoic acid (Adda) (Barco et al., 2005) Variation of the amino acid R groups as well as the methlyation or demethlyation of the stable amino acids is responsible for variants of microcystins. Microcystin-LR, in which R_1 and R_2 are arginine and leucine, respectively, is one of the most common and best described microcystin variants (Lam et al., 1995, Fitzgerald et al., 1999). Consequently, concentrations or toxic activity of other microcystin variants are often reported as microcystin-LR equivalents.

Microcystins are potent toxins, with a rapid and specific mechanism of action. Orally ingested toxins are absorbed into the portal blood and taken up by the liver through the use of an

organic anion transporting polypeptide (Billam et al., 2008). Once in the liver, these toxins act by binding to and inhibiting protein phosphatases 1 and 2A, enzymes the functions of which are to remove phosphate groups from serine and threonine hydroxyl groups on specific proteins (Falconer, 2005). The specific proteins that become inhibited are often enzymes the activities of which are dependent on whether they are phosphorylated or dephosphorylated. These enzymes are also often part of a larger enzyme cascade. Thus; inhibition of these enzymes results in disruption of normal cell regulation. Acute lethality due to exposure of humans and animals to microcystins has been documented. Studies in rodents have shown that an acute lethal dose of microcystins results in breakdown of structural elements of the liver, which results in hemorrhage, and the eventual death due to hepatic insufficiency (Falconer, 2005, Billam et al., 2008). Subchronic and chronic exposures of rodents to microcystins have been known to cause hepatocyte necrosis and fibrosis, loss of weight, and liver failure (Falconer, 2005). Exposure of humans to microcystins can result in nausea, vomiting, diarrhea, irritant/allergenic reactions, convulsions, hepatotoxicosis, and death (Pouria et al., 1998). The WHO provisional guideline for microcystins in drinking water (in the form of microcystin-LR equivalents) is 1 µg/L (Barco et al., 2005), while Canada's guideline concentration is 1.5 µg/L (Davies et al., 2003).

1.3 Ecological advantages of cyanobacteria

Cyanobacteria are able to survive a range of chemical and physical conditions, some of which are extreme. In mid to late summer months, blooms of cyanobacteria can dominate fresh, surface waters in temperate climates (Falconer, 2005), especially when water temperatures are ≥ 20˚C (McQueen and Lean et al., 1987).

Availability of photosynthetically active radiation is also important for cyanobacteria, although optimal light intensity is species-specific. Because cyanobacteria are photosynthesizing

organisms, they need to remain at a depth within the water column where light can be utilized. The depth to which this is possible is called the euphotic zone, and is defined as the depth to which 1% of surface light can be detected.

Some cyanobacteria have the ability to move up or down within the water column by adjusting buoyance by use of gas-filled vacuoles (Falconer, 2005). If decreasing light penetration or other water quality characteristics requires upward movement in the water column, the cell depletes its concentrations of carbohydrates and/or proteins in order to rise, and vice versa (Falconer, 2005). This gives cyanobacteria an advantage over other phytoplankton by allowing them to move to a depth with more favorable temperature, light availability or nutrient concentrations. In addition to the ability to move vertically in the water column, cyanobacteria contain the photosynthetic pigments phycocyanin and allophycocyanin, both of which are blue-green in colour, and phycoerythrin, which is red. These pigments allow cyanobacteria to trap light at lesser intensities than most green algae (Falconer, 2005).

Cyanobacteria are able to store phosphorus (as can all algae), so when phosphorus becomes low, cyanobacteria can continue to grow until phosphorus concentrations are greater and more optimal for growth (Downing et al., 2001, Chorus and Bartram, 1999). However, unlike other algae, cyanobacteria can also store nitrogen, which is advantageous. As well, some cyanobacteria possess heterocysts which are also responsible for nitrogen fixation, which is the process of converting dissolved nitrogen gas into ammonium ions (Falconer, 2005). This ability gives cyanobacteria an advantage over green algae and diatoms in nitrogen-depleted water (Falconer, 2005). Cyanobacteria can dominate eutrophic systems with low concentrations of available nitrogen (Schindler et al., 1977). However, other research has shown that the contribution of nitrogen that has been “fixed” by cyanobacteria is minimal compared to other sources of

nitrogen, possibly due to energy demands or light requirements (Ferber et al., 2004) and is therefore not an advantage over phytoplankton.

1.4 Land use influences on water quality

Nutrient enrichment of aquatic habitats can cause blooms of both toxic and non-toxic cyanobacteria, depletion of oxygen with consequent fish kills, loss of biodiversity, including species important for commerce and recreation, and damage to indigenous aquatic plants (Carpenter et al., 1998). In particular, water bodies containing high concentrations of nutrients and located in areas with warm temperatures and sufficient sunlight can be increasingly susceptible to blooms of algae and/or cyanobacteria (Carmichael et al., 2001, Chorus and Bartram, 1999).

Saskatchewan is a vast province containing 81 631 km^2 of water (Government of Saskatchewan, 2011) and 44% of Canada's cultivated land (Government of Saskatchewan, 2008a). The agricultural zone in the southern half of the province is part of the prairie pothole region, which is characterized by many small lakes, ponds and wetlands adjacent to cultivated cropland or permanent grass pastureland used for livestock grazing. The summer months in Saskatchewan are sunny and warm (average temperatures ranging from approximately 15-35˚C). The combination of abundant, relatively shallow water bodies exposed to warm temperatures and long hours of sunlight creates a situation favourable to formation of cyanobacterial blooms, providing water nutrient concentrations are sufficient.

Soils found in southern Saskatchewan include brown, dark brown, and black Chernozemic soils. Chernozemic soils typically have dark topsoils due to their high organic matter content. This high organic matter results from the decomposition of plant matter associated with a grassland community. The brown Chernozemic soils found in southern-most

Saskatchewan typically have the lowest organic matter content of the three Chernozemic soil types listed above.

Agricultural land use and practices have been shown to affect water quality, especially through enrichment of surface waters (Chow et al., 2011, Touchette et al., 2007, Camargo et al., 2006, Carpenter et al., 1998). Practices such as over-fertilization of crops and topsoil erosion can lead to movement of nutrients due to overland runoff and leaching. Two macronutrients commonly implicated in eutrophication of water bodies are phosphorus and nitrogen (Chow et al., 2011, Carpenter et al., 1998).

Phosphorus is a common component in fertilizers because it is necessary for promoting and maintaining plant growth (USDA, 2003). Additions of phosphorus to agricultural soils are made based on factors including soil properties, past land use and deficiency based on projected crop requirements (McDowell et al., 2001). Although phosphorus is often considered quite immobile in soil (Pote et al., 1999), repeated application of phosphate fertilizers over time may increase the concentrations in soil to such a point where it is no longer taken up by plants, allowing it to be taken up in solution and transported via surface or subsurface runoff to nearby water bodies (McDowell et al., 2001, Sharpley et al., 1992).

Nitrogenous compounds are added to soil as fertilizers in order in increase both yield of biomass and the protein content of crops (Government of Saskatchewan, 2009). The addition of nitrogen to soils is based on factors including existing nitrogen concentrations and moisture content of the soil (Government of Saskatchewan, 2009). Movement of nitrogenous compounds in soil post-fertilization is an extremely complicated process. Nitrate or ammonium fertilizers, routinely used in Saskatchewan (Government of Saskatchewan, 2009), can be used relatively quickly by the growing crop following application (Government of Saskatchewan, 2009).

Alternatively, it is possible that nitrates will become lost by denitrification (Gruber and Galloway et al., 2008, Delwiche et al., 1970), become converted to ammonia through mineralization of organic matter (Delwiche et al., 1970), or become mobile and be transported to local water bodies (Camargo et al., 2006, Delwiche et al., 1970, Sekhon et al., 1995).

Agricultural activity leading to inputs of nitrogen and phosphorus as fertilizers often exceeds output as produce (Carpenter et al., 1998). Touchette et al. (2007) positively correlated agricultural land use with total kjeldahl nitrogen, total nitrogen, and total phosphorus in water. Total phosphorus has been reported to be a predictor of cyanobacterial abundance. When a meta-analysis of 99 temperate lakes was conducted, it was determined that development of blooms of cyanobacteria was correlated with total nitrogen and total phosphorus in water (Downing et al., 2001). Concentrations of individual variants of microcystins was correlated with concentrations of either nitrate or ammonium in water (Wu et al., 2006). As indicated above, phosphorus can be lost due to runoff from agricultural soils (Hart et al. 2004), potentially accelerating eutrophication (Sharpley et al., 1994, Carpenter et al., 1998). Similar scenarios with nitrogenous compounds as contributors to eutrophication have been reported (Camargo et al. 2006; Carpenter et al. 1998).

1.5 Occurrence of cyanobacteria in prairie provinces of Canada

Like some types of algae, when environmental conditions are favourable, cyanobacteria have the potential to form blooms on the surface of lakes, ponds, and rivers. Blooms of cyanobacteria have been reported in the Canadian prairie provinces of Alberta (Kotak et al., 1993, Kotak and Zurawell et al., 2007), Manitoba (Whitaker et al., 1978) and Saskatchewan (Hammer et al., 1968). The prairie provinces are similar in having small to mid-sized water bodies in their southern agricultural regions, as well as warm summer temperatures with many

hours of sunlight. Alberta's Ministry of the Environment oversees a provincial program that routinely samples many larger water bodies for the presence of cyanobacteria (Kotak and Zurawell et al., 2007). Manitoba Environment also has a program that monitors for the presence of toxic cyanobacteria (Kotak and Zurawell et al., 2007). Consequently, both provinces have substantially increased their knowledge of the presence of local cyanobacteria and cyanotoxin production. Saskatchewan does not currently have a comparable monitoring program, and thus knowledge of the occurrence of blooms of toxic cyanobacteria is relatively limited.

1.5.1 History of cyanobacteria in Saskatchewan

One of the earliest cases of cyanobacterial poisoning in Saskatchewan documented the deaths of a number of dogs and geese in 1959 (Dillenberg and Dehnel et al., 1960). Since then, either deaths or sickness due to blooms of toxic cyanobacterial blooms in Saskatchewan have been observed in 1961, 1962, 1964, 1965, 1966, 1975 and 1989 (as summarized in Ressom et al., 1994). The Diagnostic Toxicology Lab at the Western College of Veterinary Medicine (currently Prairie Diagnostics Services) located in Saskatoon, Saskatchewan has records of wildlife, domestic animal and livestock poisonings associated with cyanobacterial exposure for the years 1972-2011. Evidence implicating cyanobacteria as the cause of death in cases like these includes clinical signs of poisoning, histopathology and results of toxicity tests with mice; the strength of evidence varies among cases. Other than these cases of animal poisonings relatively little information is available on cyanotoxins in Saskatchewan.

The most thorough historic study of cyanobacteria in Saskatchewan is presented by Hammer et al. (1968). That paper describes the collection of water from approximately 50 lakes in Saskatchewan where the toxic effects of anatoxin-a or microcystins were observed. It also contains descriptions of suspect poisonings by toxic cyanobacteria from 1961-1967. In addition,

Woo (1995) summarizes cases of suspect cyanotoxin poisonings from all over the world, including occurrences in Saskatchewan from 1954 to 1989. To the author's knowledge, there has been little work done besides that described above to determine the occurrence of toxigenic cyanobacterial blooms and/or the production of cyanotoxins in Saskatchewan. The present field study offered the opportunity to describe, in greater detail, the extent to which Saskatchewan is susceptible to cyanobacterial blooms and toxin production, and investigate any potential links with agricultural practices and land use.

1.6 Determination of field sites

ArcGIS Desktop Version 9.3 in combination with the most recent Saskatchewan water body layer was used to locate water bodies surrounding sites with a history of cyanotoxin livestock poisonings in the first sampling season. While some field work was completed using 16 sites chosen in this way, the design of this study was unsuitable for the purpose of comparing land uses. Consequently, this same design was not used the following year, and the information compiled was organized into a pilot study (Appendix A). The following year, three study areas were selected across south-central Saskatchewan for this investigation: Aberdeen area, Elrose area, and Swift Current area. ArcGIS was used again, but unfortunately the information provided consisted of a large proportion of potential sample sites that were either non-accessible or non-existent (e.g. seasonal or drained wetlands). Consequently, the study design was modified in the second sampling year by selecting three different Prairie Farm Rehabilitation Administration (PFRA) community pastures located in each of the general study areas indicated above. The PFRA community pastures consisted of blocks of natural grassland or permanent pasture used primarily for grazing cattle, and are not cultivated or subjected to fertilization. There are as many as 60 PFRA pastures in 9 different districts located in Saskatchewan (Saskatchewan Ministry of

Agriculture, personal communication). The three selected PFRA pastures were chosen in an attempt to represent as many of the dominant soil zones found in south-central Saskatchewan as possible while still being reasonably close to the laboratory at the University of Saskatchewan to enable prompt sample processing. There are three main soil zones found in southern Saskatchewan: brown, dark brown and black. The three sampling clusters were in the brown and dark brown zones.

Once a permanent PFRA pasture was identified as a study area, 16 specific small water bodies were chosen as sampling sites. Eight water bodies were located within the blocks of permanent pasture, and eight additional water bodies within arable land surrounding the permanent pasture. Water bodies were randomly selected, although accessibility had to be taken into account due to the amount of equipment used. Water bodies had estimated surface areas of 1 to 2 acres, and were approximately 0.5 – 4 m in depth. The overall study design therefore consisted of three geographically distinct clusters of sample sites. Each cluster was a group of eight small water bodies located within permanent pasture and a group of eight small water bodies located within arable/cultivated fields which surrounded the blocks of permanent pasture. Co-locating the groups of sample sites was intended to minimize potential effects of regional weather differences and soil types on cyanobacterial bloom formation and toxin production, in order to enable identification of effects (if any) of agricultural land use and practices.

1.6.1 Water sampling techniques

A potential limitation of collection of representative water samples in the field is thermal stratification, which can result in water bodies with non-homogenous distributions of cyanobacteria or nutrients throughout the water column (Chorus and Bartram, 1999). However, this limitation is more common in deeper water bodies (5-7m in depth), and was therefore less

applicable in this study. In the present study, water was collected at the centre and four corners of each water body using an integrated sampler consisting of a piece of flexible plastic tubing, open at both ends and weighted at one. This design allowed for a columnar sample of water to be taken to a depth of 1m. Pooling of individual water samples produces a composite sample assumed to be representative of the entire water body, to enable determination of the total concentration of each parameter within a water body (Chorus and Bartram, 1999). During sampling and transportation water samples were stored in a cooler containing ice packs to prevent degradation of possible toxins (Chorus and Bartram, 1999).

1.7 Chlorophyll-a, phosphorus and nitrogen as predictors of cyanobacteria

Concentrations of chlorophyll-a are commonly used to represent the total biomass of cyanobacteria in water, or more simply as a predictor of the potential presence of cyanotoxins (Chorus et al., 2000, Wu et al., 2006, Touchette et al., 2007). However, since all photosynthesizing organisms are included in this measurement, unless the water body is dominated by cyanobacteria, it is possible to overestimate concentrations of cyanotoxins by use of concentrations of chlorophyll-a.

The role of phosphorus in predicting cyanobacterial abundance and toxin production has been the topic of research all over the world. Since it has a tendency to bind to soil, phosphorus is often received in surface waters through surface flow rather than groundwater (Correll et al., 1999). The form of phosphorus that is bioavailable to bacteria, algae and plants is dissolved, or soluble, reactive phosphorus (Correll et al,. 1999). Unfortunately, due to high turnover rates (Correll et al, 1999) and resulting high variability (Håkanson et al., 2007), measures of soluble reactive phosphorus are poor predictors of total amounts of available phosphorus in water, especially if phosphorus is limiting.

Total concentrations of phosphorus in water are commonly used as predictors of concentrations of chlorophyll-a, cyanobacterial abundance and even toxin production. Total phosphorus was a better predictor of chlorophyll-a and abundances of cyanobacteria than was total nitrogen approximately in 500 temperate lakes and coastal systems (Håkanson et al. 2007). Laboratory studies performed by Rapala et al. (1997) demonstrated that growth and intracellular concentrations of cyanotoxins were proportional to total concentrations of PO_4 in the *Anabaena* strains examined. Changes in concentrations of microcystins in three hypereutrophic Canadian lakes were positively correlated with abundance of *Microcystis aeruginosa*, concentrations of total and dissolved PO_4, and chlorophyll-a in water (Kotak et al. 1995). Total concentrations of PO_4 and chlorophyll-a were determined to be significant predictors of cyanobacterial abundance (Touchette et al. 2007).

Nitrogen is another macronutrient that has been linked to occurrence of cyanobacterial blooms. Nitrogen was originally considered a limiting nutrient exclusively in marine systems, but has received increasing attention as a limiting nutrient in the growth of phytoplankton in freshwater systems (Elser et al., 1990, Chorus and Bartram, 1999, Lewis et al., 2008). Forms of nitrogen, including nitrate and ammonia, are available for use by cyanobacteria. Phytoplankton tend to primarily use nitrogen as ammonium and nitrate use secondarily (Ferber et al., 2004). Total nitrogen can be estimated through the addition of total kjeldahl nitrogen (ammonium plus organic nitrogen) to nitrate and nitrite concentrations, and is often correlated with other predictors such as chlorophyll-a and total toxin concentration in studies of cyanobacteria.

As with other potential predictors, there is controversy over the role played by nitrogen in cyanobacterial growth and toxin production. There was no significant difference caused by either nitrate starvation or nitrate excess in the expression of the gene responsible for synthesizing the

toxic component of microcystins or in the concentration of microcystins present (Sevilla et al. 2010). However, Correll et al. 1999 states that while the log of chlorophyll-a vs. the log of total concentrations of TP tends towards a sigmoid relationship, reaching a plateau at a threshold concentration of phosphorus, additions of nitrogen will greatly increase eutrophication. This is possibly because nitrogen becomes limiting in conditions where phosphorus concentrations are greatly increased.

Research has shown that N:P ratios can potentially affect the ecology of cyanobacteria (Downing et al., 2001, Wu et al., 2006). It has been proposed that cyanobacteria will dominate in eutrophic waters at lesser ratios of N:P (Schindler et al. 1977). In a study of temperate lakes it was concluded that dominance by cyanobacteria will occur at total N:P ratios of $\leq 29{:}1$ by mass, and that cyanobacteria will be rare at ratios greater than that (Smith et al. 1983). This paradigm is still generally accepted, although additions to it have been made. Earlier work was amended by proposing that heterocystous cyanobacteria will likely dominate at low N:P ratios due to their nitrogen fixing abilities (Levine and Schindler et al. 1999). A study of the relationship between cyanobacterial dominance and nitrogen fixation in a hypereutrophic lake found that fixation of nitrogen contributed relatively little to the phytoplankton blooms, and it was concluded that lesser N:P ratios in water were only one of the factors involved in cyanobacterial dominance (Ferber et al. 2004). Research on this topic is ongoing.

1.8 Measurement of cyanobacterial toxins

1.8.1 High performance liquid chromatography tandem mass spectrometry analysis

HPLC (high performance liquid chromatography) is one of the most widely used analytical techniques in the measurement of cyanotoxins (McElhiney et al., 2005, Hoeger et al., 2004, Botana, 2008). HPLC combined with UV detection, using a photodiode array UV detector or

PDA, or alternatively being combined with a mass spectrometer (MS) are two commonly used methods (McElhiney et al., 2005, Hoeger et al., 2004). Both methods allow for sensitive determination of specific cyanotoxin variants through the use of reference materials. Although the use of HPLC is common, it can be quite time consuming, as an initial clean-up step such as solid-phase extraction is often necessary. This can result in added expense for the overall analyses. Also, due to the complex nature of the equipment, skilled personnel are required. If they are not available, the analyses would have to be contracted out. In addition, only specific cyanotoxin variants for which purified reference standards are available can be detected.

1.8.2 Protein phosphatase inhibition assay analysis

Assays of inhibition of protein phosphatase are a simple bio-analytical method producing rapid, semi-quantitative screening for the presence of cyanotoxins. The combination of an instrumental and bio-analytical method where detection is based on functional activity with one based on detection of specific structural components and therefore specific variants is often desired in the study of cyanotoxins. The colorimetric protein phosphatase inhibition assay as described by An and Carmichael et al. (1994) is commonly used in the analysis of microcystins (McElhiney et al., 2005, Hoeger et al., 2004, Botana, 2008). This assay is especially useful when a rapid screening method is needed, since it requires no preparatory clean-up steps other than lysing the cells. This method is also very sensitive, with detection limits in the ppb (μg/L) range.

The combination of HPLC and protein phosphatase inhibition assays (or some other pairing of physico-chemical and biological assays) is common in the study of cyanotoxins. These methods allow for determination of both total toxic activity and the presence (or absence) of specific common variants. These two methods of quantification of cyanotoxins are often relatively well correlated. However, due to the nature of both assays, it is possible to detect

inhibition of protein phosphatase but not a quantifiable peak in HPLC-MS/MS analysis. This is most likely due to the fact that HPLC will only detect the reference materials that have been optimized.

1.9 Research objectives and hypotheses

The goal of this study was to survey small water bodies across three distinct regions in south-central Saskatchewan for the presence of specific neurotoxins and hepatotoxins produced by certain cyanobacteria, as well as to determine any associations between cyanotoxins and specific agricultural land use, geographical region, water quality measures, and soil and water nutrients.

The objectives of the study have been divided into the following testable null hypotheses:

1. Determine if there is an association between cyanobacterial blooms and cyanotoxin production and agricultural land use and geographic region in small Saskatchewan water bodies (e.g. dugouts, ponds and wetlands).

Ho: There is no association between cyanotoxin production and land use and region in small south-central Saskatchewan water bodies.

2. Determine if there is a difference between water and soil nutrient characteristics associated with small water bodies located within permanent pasture compared with water bodies located within arable land.

Ho_1: There is no difference between nutrient levels in soil from drainage basins surrounded by arable land vs. nutrient levels in soil from drainage basins surrounded by permanent pasture.

Ho_2: There is no difference between nutrient levels in surface water in water bodies surrounded by arable land and surface water found in water bodies surrounded by permanent pasture.

3. Determine the principal environmental factors associated with cyanotoxin production (if any), including water quality and water and soil nutrient characteristics.

Ho_1: There is no association between concentration of cyanotoxins present in water (if any) and water quality characteristics in water bodies.

Ho_2: There is no association between concentration of cyanotoxins present in water (if any) and water nutrient characteristics in water bodies.

Ho_3: There is no association between concentrations of cyanotoxins present in water (if any) and soil nutrient characteristics of the drainage basins surrounding water bodies.

CHAPTER 2

2 CYANOBACTERIAL NEUROTOXINS IN SMALL SOUTH-CENTRAL SASKATCHEWAN WATER BODIES

2.1 Introduction

Certain genera of cyanobacteria have the ability to produce potent neurotoxins. Three neurotoxins known to be produced by freshwater cyanobacteria include anatoxin-a, anatoxin-a(s) and saxitoxin (Aráoz et al., 2010). Anatoxin-a acts as an acetylcholine analogue, binding to post-synaptic receptors at neuromuscular junctions and causing muscle cell stimulation. Since acetylcholinesterase is unable to break down anatoxin-a prolonged overstimulation of the muscle occurs. Clinical effects of anatoxin-a poisoning may include muscle spasms or twitching (Carmichael et al., 1992), leaping movements (Carmichael et al., 2001), convulsions, paralysis and death due to respiratory arrest (Carmichael et al., 1992). Bioassays using intraperitoneal injection of mice determined an LD_{50} of 200 μg kg^{-1}, death occurring within minutes (Carmichael et al., 1992).

Anatoxin-a(s) also affects neuromuscular junctions. While anatoxin-a acts by binding to acetylcholine receptors, anatoxin-a(s) instead binds to acetylcholinesterase, therefore blocking its ability to break down acetylcholine. This also results in continuous stimulation of muscle. Signs of anatoxin-a(s) intoxication include hypersalivation ("s" standing for salivation factor), diarrhea, ataxia, muscle tremors and death by respiratory paralysis (Carmichael et al., 1992). LD_{50} determined at 20 μg kg^{-1} for i.p. injection in mice (Carmichael et al., 1992).

Saxitoxin, the cyanobacterial neurotoxin of interest in this study, is a potent alkaloid neurotoxin with an i.p. LD_{50} of approximately 10 μg kg^{-1} in mice (Carmichael et al., 1992). These alkaloid neurotoxins are classified into three related groups: saxitoxins being the non-

sulphated type (Chorus and Bartram, 1999). The other two groups include singly-sulphated toxins (the gonyautoxins) and those that are doubly-sulphated (the C-toxins) (Chorus and Bartram, 1999). The present study focused on four variants of non-sulphated saxitoxins: saxitoxin (STX), neosaxitoxin (NEO-b), decarbamoyl saxitoxin (dcSTX) and decarbamoyl neosaxitoxin (dcNEO-b). Saxitoxin acts by binding to and blocking voltage-gated sodium channels on axon terminals in the brain (Carmichael et al., 1994). This causes a halt in the inward flux of sodium, preventing the release of acetylcholine at synapses. The result of this blockage is that muscle cells receive no stimulation (Kotak et al., 2007). Physically, an intoxication of saxitoxin would initially cause paresthesia of the mouth and lips, following with headaches and dizziness, eventually leading to paresthesia of the arms and legs, and ending in respiratory paralysis and death (Kao, 1993). Time from intoxication to death is dependent on the amount consumed and the specific toxin ingested (Kao, 1993), with death occurring in as little as 30 minutes or as long as a few hours (Trevino et al., 1998).

The discovery that saxitoxin was produced by some cyanobacteria was relatively recent. The toxin was first observed to be produced by *Aphanizomenon flos-aquae* and appeared to be similar, if not the same saxitoxin which was known to be produced by various genera of marine algae (Jackim and Gentile, 1968). Before this discovery, saxitoxin was commonly known as a toxin implicated in "paralytic shellfish poisoning" (PSP). Paralytic shellfish poisoning results from accumulation of toxins produced by various marine phytoplankton including a number of species in the genus *Alexandrium* by shellfish that filter the cells from the water (Parkhill et al., 1999, Aráoz et al., 2010). The most common shellfish ingested leading to this type of poisoning are bivalve shellfish including mussels, scallops and clams (Kao, 1993). Poisonings through the ingestion of shellfish are not a new occurrence, with records dating back as far as the late 1700s

(Acres et al., 1978, Kao, 1993). Cases of PSP occur all over the world, including the coasts of Canada (Fisheries and Oceans Canada, 2011).

Anatoxin-a and anatoxin-a(s) are only produced by cyanobacteria found in freshwater (Kotak and Zurawell et al., 2007). In addition to the marine dinoflagellates associated with PSP, saxitoxin is now known to be produced by certain genera of cyanobacteria including *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya and Planktothrix* (Aráoz et al., 2010). Of these, species in the genera *Aphanizomenon* and/or *Anabaena* have been observed in the prairie provinces of Alberta (Kotak et al., 1993), Saskatchewan (Hammer et al., 1968) and Manitoba (Whitaker et al., 1978). Although the presence of these species has been reported, little is known about the potential occurrence of saxitoxin in these areas.

Neurotoxins produced by cyanobacteria have been identified through laboratory analysis or diagnosis using animal models in multiple countries (Negri et al., 1995, Pereira et al., 2000). Although cyanobacterial neurotoxins have been studied in increasing detail in the past few decades, the effort devoted to understanding the neurotoxins is still not comparable to the research being done on cyanobacterial hepatotoxins. Although there have been suspect cases of cyanobacterial neurotoxin poisoning in Saskatchewan (Dr. Barry Blakley, Western College of Veterinary Medicine, personal communication), none have been proven and almost no effort has been made to measure neurotoxin concentrations in water bodies in Saskatchewan. The objective of this study was to determine whether saxitoxin or its variants were being produced in small water bodies in Saskatchewan, and, if saxitoxins were present, to determine whether agricultural land use, geographic region, water and soil nutrient concentrations or other environmental variables were influencing their production.

2.2 Materials and Methods

2.2.1 Experimental design

A factorial design was used to examine whether geographic region and/or agricultural land use influence the production of cyanobacterial toxins in small water bodies. Three regions were selected within the province ranging from south to central, and represented two of three major soil zones (Figure 3.2, Ch.3) Sixteen sampling sites containing a small water body and its drainage basin were selected for study within each region, with half the sites located within permanent pasture managed by the Prairie Farm Rehabilitation Administrations (PFRA) for cattle grazing, and half located in cultivated crop land within a few miles of the pastures. Water bodies sampled included natural wetlands and potholes, as well as man-made dugouts, with size generally ranging from approximately 1 to 2 acres.

2.2.2 Sampling and storage

Water was collected at all 48 sites, each visited twice over the summer. A total of 95 water samples were collected, since one site was unavailable for sampling a second time. The first sampling, which occurred in July/early August 2009 was referred to as "early", and the second, referred to as "late", occurred in late August/September. Changes that occurred over the season (late value – early value) were referred to as "delta". Five 2 L containers of water collected at different points in each water body were pooled to produce a 10 L composite sample per site. A YSI 6600 V2-4 Sonde with YSI 650 handheld display was used to measure water quality parameters, including water temperature, conductivity, dissolved oxygen, pH, chlorophyll-a and phycocyanin in the center of each water body.

For quantification of chlorophyll-a, a 300 mL subsample of water was filtered through Whatman GF/C 47mm glass microfiber filters, stored at 4˚C and submitted to the ALS Laboratory Group in Saskatoon for analysis the following day. Three 500 mL bottles of water

were stored at 4˚C overnight for quantification of total phosphorus, soluble reactive phosphorus, nitrate, nitrite, total kjeldahl nitrogen and ammonia by ALS Laboratory Group. Five 20 mL scintillation vials of water were stored at -20˚C until analysis of toxin concentration using HPLC-MS/MS. Two additional 50 mL subsamples of water were fixed in two bottles containing 50 mL of 10% formalin each for algal identification.

Ten 10 cm soil cores were collected per site from the apparent drainage basin surrounding each water body and stored at -20˚C until analysis for nitrate, ammonium, potassium and phosphorus at a later date.

2.2.3 High performance liquid chromatography tandem mass spectrometry analysis

Four variants of saxitoxin [saxitoxin dihydrochloride (STX-e, 0.5mL at 65µM), neosaxitoxin (NEO-b, 0.5mL at 65µM), decarbamoyl saxitoxin (dcSTX, 0.5mL at 62µM) and decarbamoyl neosaxitoxin (dcNEO-b, 0.5mL at 28.9µM)] were obtained from the National Research Council of Canada (Halifax, Canada). All variants purchased were stored in 0.003M hydrochloric acid. Diluted standards were used to create standard curves to quantify saxitoxin variant concentrations in the water samples.

Samples of water were subjected to a cycle of freeze-thaws using a VWR Incubating Orbital Shaker and the -80˚C freezer in order to lyse the cyanobacterial cells (Eaglesham et al., 1999). This cycle was repeated three times for each vial in order to ensure the complete breakdown of all possible cyanobacterial cells present in the water. Once thawed the water was filtered through 0.2µm Millex nylon syringe driven filter units using 3mL BD luer-lock tip syringes and stored at -20˚C in Agilent Technologies crimp-top auto sampler vials pending analysis.

Separation of the neurotoxic compounds was accomplished using an Agilent 1200 HPLC (Santa Clara, CA) fitted with a TOSOH Bioscience TSK gel Amide 80 (150x4.6mm, 3µm particle size) analytical column. Eluent A consisted of Millipore water and eluent B was a mixture of acetonitrile/water (95:5), both containing 2.0 mM ammonium formate and 3.6 mM formic acid. (Dell'Aversano et al., 2004, Dell'Aversano et al., 2005). The pH of both was adjusted to 3.5 (Dell'Aversano et al., 2005). The finalized method used gradient conditions at 300 µL min-1 flow rate, starting with 85% eluent A and 15% eluent B. Initial conditions were held for 4 minutes and then changed to 100% eluent A at 8 minutes, and held until 10 minutes where it was returned to initial conditions until 15 minutes. Injection volume was optimized to 20µL.

Mass spectra were collected using an Applied Bioscience SCIEX 3000 (Foster City, CA) tandem mass spectrometer, fitted with an electrospray ionization source, operated in the positive ionization mode. Chromatograms were recorded using MRM mode, where at least two transitions per-analyte were monitored where possible. (Hiller et al., 2007). The following instrument parameters were used: desolvation temperature (250˚C); desolvation (curtain) gas 7.0 arbitrary units (AU); nebulizer gas 12 AU; ionspray voltage 3800.0 EV (electron volts); focusing potential 300 AU; and an entrance potential of 7.0 AU. Quantification using these transitions was accomplished by use of Analyst 1.4.1 software provided by Sciex (Applied Bioscience, Foster City, CA).

2.3 Results

2.3.1 Presence of saxitoxins in small Saskatchewan water bodies

A total of 95 water samples, 48 in the early season and 47 in late season were analyzed for the presence of STX-e, NEO-b, dcSTX and dcNEO-b. Limits of detection, based on

calibration curves created for each variant, were approximately 16 ppb for STX-e, 16 ppb for NEO-b, 12.8 ppb for dcSTX, and 100 ppb for dcNEO-b. Where possible, multiple transition ions were monitored. After extensive investigation and standard addition, it was concluded that there were no measureable amounts of saxitoxin or its variants present in any of the water tested (Table 2.1). No other methods of analysis were used as detectable amounts of saxitoxin or variants were not found.

Table 2.1 - Detection of saxitoxin and variants (STX-e, NEO-b, dcSTX and dcNEO-b) using high performance liquid chromatography in small water bodies surrounded by arable land or permanent pasture in three geographic regions of Saskatchewan in the summer of 2009 (n = 8, with the exception of Swift Current pasture late summer where n = 7).

Site	Early Summer (June/July) [Mean ± SD (µg/L)]	Late Summer (August/September) [Mean ± SD (µg/L)]
n	8	7-8
Aberdeen Cultivated	No Detects	No Detects
Aberdeen Pasture	No Detects	No Detects
Elrose Cultivated	No Detects	No Detects
Elrose Pasture	No Detects	No Detects
Swift Current Cultivated	No Detects	No Detects
Swift Current Pasture	No Detects	No Detects

2.3.2 Past and present occurrence of cyanotoxins in Saskatchewan

Annual diagnostic reports from the Veterinary Toxicology Laboratory at the Western College of Veterinary Medicine were examined in order to determine the history of suspected cyanotoxin poisonings in Saskatchewan. Forty years of annual diagnostic reports are summarized in Table 2. In the case of an "abnormal" diagnosis (consistent with cyanotoxin poisoning), reports did not elaborate on whether hepatotoxins or neurotoxins were suspected.

Table 2.2 - History of diagnoses of cyanotoxin poisoning of livestock in Saskatchewan from 1972-2011. Values indicate whether analysis (using standard mouse bioassay) was determined to be normal (not consistent with cyanotoxins) or abnormal (consistent with cyanotoxins). Annual reports did not indicate whether positive mouse bioassay responses appeared to be due to hepatotoxic or neurotoxic cyanotoxins.

Year	Number of Cases Diagnosed as:		Year	Number of Cases Diagnosed as:	
	Normal	Abnormal		Normal	Abnormal
2011	2	0	1991	1	0
2010	4	0	1990	2	0
2009	1	0	1989	0	0
2008	5	1	1988	0	0
2007	2	0	1987	1	0
2006	10	0	1986	0	0
2005	1	0	1985	1	0
2004	1	0	1984	1	0
2003	0	0	1983	4	1
2002	0	0	1982	0	0
2001	4	0	1981	2	0
2000	4	0	1980	1	0
1999	2	1	1979	0	0
1998	1	0	1978	0	0
1997	1	0	1977	0	0
1996	0	0	1976	4	0
1995	2	0	1975	0	0
1994	1	0	1974	0	0
1993	3	0	1973	0	0
1992	0	0	1972	0	0

2.4 Discussion and conclusions

Poisoning of humans and animals by saxitoxin has been diagnosed in many parts of the world. Some geographic areas commonly report cases of PSP (Garcia et al., 2004, Rodrigue et al., 1990, Trevino et al., 1998). In recent years, saxitoxins produced by various genera of cyanobacteria causing large-scale losses of livestock have received increasing attention (Steffensen et al., 2008).

No measureable concentrations of saxitoxin, neo-saxitoxin, decarbamoyl saxitoxin or decarbamoyl neosaxitoxin were found in any of the water bodies tested in this study. Unlike the hepatotoxins, cyanobacterial neurotoxins are known for their ephemeral nature and their rapid degradation, making them more challenging to sample and analyze. Saxitoxin is also known to be quite unstable in basic conditions (Wood et al., 2011, Jones et al., 1997). Most of the water bodies tested in the present study were weakly basic, pH ranging from approximately 7 - 9. If saxitoxins were ever present, this slight basicity could have caused degradation. Some researchers have suggested that solar irradiance may play a part in the degradation of saxitoxin (Wood et al., 2011), but others (Jones et al., 1997) contend that since their UV absorbance is quite low, it is unlikely that they are heavily degraded in this way.

Due to time and money constraints, no attempt was made to measure anatoxin-a or anatoxin-a(s) in these water samples. There is very little data on the occurrence of these neurotoxins in Saskatchewan water bodies. Just like saxitoxin, anatoxin-a and anatoxin-a(s) are ephemeral, which makes them more difficult to sample and analyze.

Alberta Environment has recently done some work investigating the presence of anatoxin-a in water bodies in both Alberta and Saskatchewan. Their most recent report (Government of Alberta, 2010) described finding anatoxin-a at concentrations close to the limit of detection (the highest being 0.5 µg/L) using GC/MS. The species that are known to produce

anatoxin-a are not seen very often in Alberta water bodies, and this study reported detecting trace amounts of toxin in only 11% of samples. The study concluded that anatoxin-a is present in Alberta and Saskatchewan, but in limited amounts and therefore the risk posed by these toxins is generally low. The present study failed to find any of the saxitoxin variants in water samples from 48 sources over three distinct areas of the province. This represents the most comprehensive effort to identify saxitoxin in Saskatchewan surface water to date. The study is limited in that it comprised only one field season, and cyanotoxin occurrence frequently varies from year to year. Nonetheless, the results indicate that the likelihood of saxitoxin posing a risk to livestock in Saskatchewan is also generally low.

CHAPTER 3

3 OCCURRENCE OF CYANOBACTERIAL MICROCYSTINS IN SMALL SOUTH-CENTRAL SASKATCHEWAN WATER BODIES AND ASSOCIATED ENVIRONMENTAL AND AGRICULTURAL INFLUENCES

3.1 Introduction

The province of Saskatchewan contains 44% of all the cultivated crop land in Canada (Government of Saskatchewan, 2008a) as well as large blocks of uncultivated grassland used for livestock grazing. Saskatchewan is located at the northern extremity of the prairie pothole region, so these lands include large numbers of small water bodies which are used for livestock watering and occasionally for household water use by rural farm families. These fresh water bodies are vulnerable to fertilizer or animal waste contamination by overland runoff from cultivated fields or pastures, or by contaminated groundwater inflow due to being surrounded by agricultural land. A common detrimental impact on water quality is increased nutrient loading through leaching of macronutrients such as nitrogen and phosphorus into water bodies within the surrounding drainage basin (Arbuckle et al., 2001). Nutrient enrichment, or eutrophication, often leads to the growth of potentially toxic cyanobacteria.

Cyanobacteria are robust organisms commonly found in freshwater, as well as marine and brackish systems, and terrestrial environments (Barco et al., 2005, Florencio et al., 2006). The ability of these bacteria to produce lethal toxins, termed cyanotoxins, as well as their ability to survive in such a diverse range of habitats makes them an animal and human health concern. Cyanotoxin occurrences have been documented all over the world (McElhiney and Lawton, 2005), including Australia (Kankaanpää et al., 2005), South America (Irisarri et al., 2001), North America (Carmichael and Li, 2006), Asia (Mohamed and Al Shehri, 2007), Europe (Bláhová et al., 2009), Africa (Nyenje et al., 2010) and even Antarctica (Hitzfeld et al., 2000).

Cyanobacterial blooms have been reported in Alberta (Kotak et al., 1993, Kotak and Zurawell et al., 2007) and Manitoba (Whitaker et al., 1978), as well as in Saskatchewan (Hammer et al., 1968). One of the earliest recorded cases from the prairie provinces involved the deaths of a number of dogs, geese and fish in Echo Lake, Saskatchewan in 1959 (Dillenberg and Dehnel, 1960).

The class of cyanotoxins commonly reported in most freshwater systems is microcystins. Microcystins are potent cyclic peptide hepatotoxins produced by several genera of cyanobacteria including *Microcystis, Anabaena, Oscillatoria, Nostoc* and *Anabaenopsis* (McElhiney et al., 2005). Once ingested, these toxins are transported to the liver through the use of an organic anion transporting polypeptide (Billam et al., 2008) where they act by binding to, and inhibiting, protein phosphatases 1 and 2A. Lethal exposures result in liver necrosis and organ failure (Chorus et al., 2000). Chronic, low dose exposure is associated with promotion of tumor growth (Chorus et al., 2000). The World Health Organization (WHO) provisional guideline for microcystins in water for human consumption is 1 µg microcystin-LR equivalents/L (Barco et al., 2005), while Canada's guideline concentration is 1.5 µg microcystin-LR equivalents/L for human consumption (Davies et al., 2003). Agriculture and Agri-Food Canada (2011) suggested a value of approximately 4.2 µg microcystin-LR equivalents/L as a no observed adverse effect level (NOAEL) in drinking water for cattle.

The cyanobacteria that potentially produce microcystins are widely distributed in many habitats, although little information is available for water bodies in Saskatchewan. Factors that promote cyanobacterial dominance in water are the subject of study worldwide, and include resource ratio (N:P) competition, light requirements, CO_2 competition, buoyancy, high temperature tolerance, avoidance by herbivores, superior cellular storage of nutrients, and

ammonium-N exploitation (Hyenstrand et al., 1998 as cited by Downing et al., 2001). Additionally and specifically, phosphorus and nitrogen have been identified as key macronutrients that promote eutrophication of surface waters (Carpenter et al., 1998) and encourage bloom formation.

Land use and certain agricultural practices may influence several of the environmental factors associated with cyanobacterial bloom formation, particularly the potential for enrichment of water bodies with critical nutrients that could otherwise limit growth. Water bodies surrounded by pasture used for livestock grazing might be contaminated with manure, which is a rich source of many macronutrients. Water bodies in cultivated land are subject to potential nutrient loading from application of inorganic fertilizers to cropland.

Phosphorus is a component of fertilizers because it is necessary for promoting and maintaining plant growth (USDA, 2003), especially during early growth stages and for seed formation (Government of Saskatchewan, 2006). Amounts of phosphorus applied to agricultural land as fertilizer is a function of factors including soil properties, past land use, and specific deficiency relative to crop needs (McDowell et al., 2001). Although phosphorus is generally considered immobile in soil (Pote et al., 1999), long-term repeated application of phosphate fertilizers might raise the concentration in soil to such a point where it is no longer taken up by plants, allowing it to enter solution and be transported via surface or subsurface runoff to nearby water bodies (McDowell et al., 2001, Sharpley et al., 1992).

Nitrogenous compounds are added to soil as fertilizers in order in increase both the biomass (yield) and the protein content of the crop (Government of Saskatchewan, 2009). The addition of these compounds to soil is based on factors including existing nitrogen concentrations and moisture content of the soil (Government of Saskatchewan, 2009). Nitrate or ammonium

fertilizers routinely used in Saskatchewan may be used relatively quickly by the growing crop following application (Government of Saskatchewan, 2009). Alternatively, nitrates may be lost by denitrification (Gruber and Galloway et al., 2008, Delwiche et al., 1970), and organic nitrogen forms become converted to ammonia through mineralization of organic matter (Delwiche et al., 1970), or become mobile and be transported to local water bodies (Camargo et al., 2006, Delwiche et al., 1970, Sekhon et al., 1995).

Saskatchewan is a moderately nutrient-rich province. Saskatchewan soils can contain relatively low plant available forms of nitrogen and phosphorus (Government of Saskatchewan, 2006, 2008b), creating the need for fertilization in regions that are cropped. Fertilization practices leading to increased concentrations of nutrients in soil that may become mobile and transported to small water bodies can potentially support baseline concentrations of cyanobacteria. Differences in surrounding land use and subsequent differences in external nutrient inputs may result in differences in concentrations of cyanotoxins in water.

The objective of this study was to determine whether agricultural practices influence the occurrence of cyanotoxins or water quality in Saskatchewan water bodies. Cyanotoxin concentrations were compared among small water bodies surrounded by arable land and water bodies located within permanent pasture used for livestock grazing over three geographic regions in southern and central Saskatchewan. This study also evaluated whether nutrient concentrations in soil and water, and specific water quality parameters were predictors of cyanotoxin abundance in water.

3.2 Materials and Methods

3.2.1 Experimental Design

A factorial design was used to examine how geographic region and agricultural land use influence the abundance of cyanotoxins in small water bodies. Three regions were selected within the province (Figure 3.1) ranging from south to central, and represented two out of three dominant soil zones found in Saskatchewan (Fig. 3.2). Sixteen sampling sites containing a small water body and its drainage basin were selected for study within each region, with half of the sites located within permanent pasture managed by the Prairie Farm Rehabilitation Administration (PFRA) for cattle grazing, and half located in cultivated crop land within a few miles of the pastures. Water bodies sampled included natural wetlands and potholes, as well as man-made dugouts. The surface area of the water bodies selected ranged from an estimated 1 to 2 acres.

Figure 3.1 - Map of southern Saskatchewan, Canada. Stars indicate locations of three sampling regions (Saskatoon/Aberdeen, Elrose/Rosetown, Swift Current), each consisting of 16 sampling sites (eight within arable land, eight within permanent pasture).

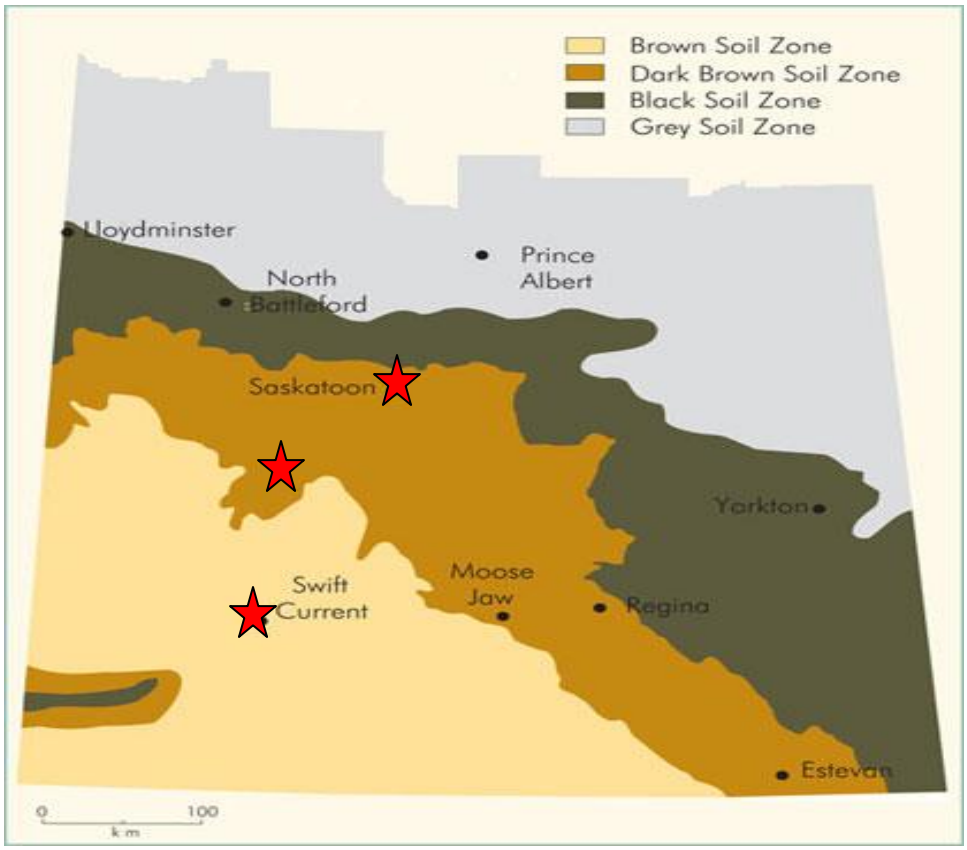


Figure 3.2 - Map of southern Saskatchewan soil zones. Stars indicate approximate locations of three sampling regions (Saskatoon/Aberdeen, Elrose/Rosetown, Swift Current).

3.2.2 Collection of water and soil samples

Water samples were collected at all 48 sites, with each site visited twice over the summer. A total of 95 samples of water were collected, since one site was unavailable for sampling a second time. The first sampling event (Early) occurred in July/early August, 2009 and the second samples (Late) were collected in late August/September, 2009. Change over the season (Late value – Early value) is referred to as Delta.

The center of each water body was estimated visually, and a suite of water quality parameters including water temperature, conductivity, dissolved oxygen, pH, and chlorophyll-a and phycocyanin concentrations were measured using a YSI 6600 V2-4 Sonde with YSI 650 handheld display at that location. A 2 L integrated sample of surface water was collected to a

depth of 1 m at this same location, and at four other points in each water body. All five 2 L collections were then pooled to produce a representative 10 L sample from each water body.

Five 20 mL aliquots were removed from the pooled sample and stored at -20˚C in plastic scintillation vials pending analysis of individual hepatotoxin variant concentrations and total toxin activity using HPLC-MS/MS and a protein phosphatase inhibition assay, respectively. Two additional 50 mL subsamples of water were fixed in two Boston bottles with the addition of 50 mL of 10% formalin, and stored at room temperature for potential future algal identification.

A 300 mL subsample of the remaining water was filtered through Whatman a 47 mm GF/C glass microfiber filter to collect phytoplankton for chlorophyll-a determination. Filters were stored at 4˚C and submitted to ALS Laboratory Group (Saskatoon, SK) the following day. Three additional 500 mL subsamples of water were stored at 4˚C overnight and delivered to the same analytical laboratory for determination of total phosphorus, soluble reactive phosphorus, nitrate, nitrite, nitrate + nitrite, total kjeldahl nitrogen and ammonia.

Ten soil cores were collected to a depth of 10 cm from the apparent drainage basin surrounding each water body for analysis of topsoil nutrient composition. Soil cores were combined and stored at -20˚C pending analysis for concentrations of nitrate and ammonium (Keeney and Nelson et al., 1982) and potassium and phosphorus (Qian et al., 1994) concentrations.

3.2.3 High performance liquid chromatography tandem mass spectrometry analysis

Water samples for microcystin analysis were sonicated for 20 s using an ultrasonic probe (An and Carmichael, 1994). Extraction of microcystins from sonicated samples was accomplished using the method of Triantis et al. (2010). Extraction was performed using Oasis

HLB 6cc (200mg) extraction cartridges (Waters Corp., USA). Samples were re-dissolved following extraction in two washes of 125 µL Millipore water and stored at -20˚C.

Standards for microcystins LR (MC-LR, 0.5 mL at 10 µM), -RR (MC-RR, 0.5 mL at 9.8 µM), -dmLR (7-dmMC-LR, 0.5 mL at 9.6 µM) and nodularin (NOD, 0.5 mL at 12.4 µM) (a related cyclic peptide hepatotoxin) were purchased from the National Research Council of Canada (Halifax, Canada). All standards were diluted in 50% methanol and stored at -20˚C. Diluted standards were used to create standard curves for HPLC analysis.

Separation of compounds was accomplished using an Agilent 1200 HPLC (Santa Clara, CA) fitted with a Betasil C18 100 mm, 2.1 ID column. High-purity water (A) and acetonitrile (B), both containing 0.5% formic acid (Triantis et al., 2010) were the mobile phase solutions. Gradient conditions were applied at 300 µL/min and the injection volume was 20 µL. Mass spectra were collected using an Applied Bioscience SCIEX 3000 (Foster City, CA) tandem mass spectrometer fitted with an electrospray ionization source and operated in the positive ionization mode. Chromatograms were recorded using MRM mode (Hiller et al, 2007), where at least two transitions per-analyte were monitored where possible. The following instrument parameters were used: desolvation temperature (550˚C); desolvation (curtain) gas 10 arbitrary units (AU); nebulizer gas 10 AU; collision gas 12 AU; ionspray voltage 3800.0 EV (electron volts); focusing potential 300 AU; declustering potential 30 AU; and a collision cell exit potential of 10 AU. Quantification was accomplished using Analyst 1.4.1. software provided by SCIEX (Applied, Bioscience, Foster City, CA).

Two transitions were monitored for each analyte; one for identification, one for confirmation. The parent compounds for MC-LR, MC-RR, dmMC-LR and NOD are 995.7,

519.7, 981.5 and 825.5 m/z, respectively. The transitions for the above are 135 and 213 m/z for MC-LR, MC-RR and dmMC-LR, and 135 and 227 m/z for NOD.

3.2.4 Protein phosphatase inhibition assay

Microcystin-LR standard was obtained from the National Research Council of Canada (Halifax, Canada). Purified rabbit protein phosphatase-1 enzyme and para-nitrophenyl phosphate substrate were purchased from Sigma Aldrich.

Toxin activity was determined using a well-established protein phosphatase inhibition assay (An and Carmichael, 1994) as modified by Dr. Ron Zurawell (Limnologist/Water Quality Specialist, Alberta Environment). Water samples were sonicated for 20 s using an ultrasonic probe. Water samples or microcystin standards were added to each well of a 96 well plate (NUNC®), followed by 20 µL of protein phosphatase-1 enzyme and 40 µ/L para-nitrophenyl phosphate for a total of 350 µ/L per well. Water samples and standards were run in triplicate while colour correction wells were run in duplicate. Colour intensity was read with a Molecular Devices SpectraMax 190 colorimetric plate reader at 405 nm after incubation at 37˚C for approximately 3 hours. Data were analyzed using SoftMax Pro 4.0 microplate data software, and total toxin activity (as MC-LR equivalents) was expressed in µg/L. Toxin concentration in samples was determined from a standard curve created using microcystin-LR at concentrations of 2 – 44 ppb concurrently with samples. Assay detection ranged from 10 - 80% inhibition.

3.2.5 Soil nutrient analysis

Pooled soil cores were air-dried and ground to pass through a 2 mm sieve prior to analysis for nutrient content. Nitrate and ammonium concentrations were determined by preparing a 2 M KCl solution (Keeney and Nelson et al., 1982) by dissolving 3000 g of KCl in 20 L of distilled water. About 5.00 to 5.09 g of soil was added to an extraction bottle followed by

50 mL of 2 M KCl solution. The bottles were placed on a rotary shaker at 142 rpm for one hour then filtered through VWR 454 filter paper into vials. The vials were capped and stored at 4˚C pending analysis with an AutoAnalyzer (Technicon AutoAnalyzer II, Tarrytown, NY).

Available phosphorus and potassium were determined using the Modified Kelowna method (Qian et al., 1994). A Kelowna solution was prepared by combining 28 mL of acetic acid, 38.5 mL of ammonium acetate, and 1.11 g of ammonium fluoride in a 2 L bottle. 3 g of soil was then added to 30 mL of Kelowna solution in plastic screw top bottles and shaken horizontally at 160 rpm for 5 min. Soil extracts were filtered through VWR 454 filter paper into vials, and stored at 4˚C pending colourimetric analysis on the AutoAnalyzer.

Automated colourimetry methods were used to determine the inorganic nitrogen and available phosphorus in the soil extraction solutions. Technicon AutoAnalyzer II automated colourimetry involves the ions in the solution reacting with chemical reagents to form a coloured complex which absorbs light at specific wavelengths. The absorbance of the light by the coloured complex is measured using a spectrophotometer. Atomic absorption spectroscopy (Varian 220 AA/FE) was used to determine potassium concentration in the extracts.

3.2.6 Statistical analysis

Analysis of variance (ANOVA) was used to investigate whether statistical differences existed between region and land use, and cyanotoxin abundance in water. Additionally, ANOVA was used to determine whether statistical differences existed between land uses for water chemistry parameters, water quality parameters, and soil nutrient concentrations. Land use was treated as a fixed effect, region as a random effect. Endpoints were analyzed for Early season samples, Late season samples, and the change over the season (Delta). Where analysis of variance revealed a failure of Levene's Test, a Mann-Whitney U test for independent samples

was used to compare means. Multiple regression analyses were used to identify potential drivers of cyanotoxin abundance from all environmental variables measured. Natural log transformations were used to reduce positive skewness and improve visual examination of relationships. Significance was associated with ($p \leq 0.05$).

3.3 Results

3.3.1 No effect of land use

Two-way analysis of variance of region by land use revealed no significant effect of region, nor any significant region by land use interaction in any of the endpoints measured ($p > 0.05$). Therefore, all data from all regions were pooled for final analyses related to land use.

3.3.2 Chlorophyll-a and phycocyanin concentrations in water bodies

Mean ± standard deviation (SD) concentrations of chlorophyll-a in all water bodies in permanent pastures declined over the summer from 119.3 ± 209.9 mg/L to 66.2 ± 97.5 mg/L, while concentrations in water bodies in arable land increased from 42.2 ± 68.7 mg/L to 84.2 ± 216.4 mg/L during the same time period. While chlorophyll-a concentrations were not significantly different between land uses in Early or Late seasons individually, they were significantly different over the change in season (Delta) ($p = 0.031$). Phycocyanin concentrations were not significantly different between land uses during either Early or Late sampling seasons.

Linear regression indicated a significant positive correlation of chlorophyll-a concentrations with total phosphorus ($p < 0.001$, $r^2 = 0.30$) and kjeldahl nitrogen concentrations ($p < 0.001$, $r^2 = 0.291$) in Late season sampling. Also, a significant correlation was seen for delta chlorophyll-a and kjeldahl nitrogen ($p = 0.003$, $r^2 = 0.18$), but not for total phosphorus. Chlorophyll-a had a significant positive correlation with total microcystins concentrations in Late season ($p = 0.021$, $r^2 = 0.11$) and Delta ($p < 0.001$, $r^2 = 0.28$), but not Early season.

Both lesser and greater microcystins concentrations were observed in association with lesser concentrations of chlorophyll-a in Late season sampling. Similarly, lesser phycocyanin concentrations in Late season sampling were also seen to be associated with both lesser and greater concentrations of microcystins. Greater concentrations of microcystins were observed in water bodies found in arable land.

3.3.3 Water quality parameters

Experimental design allowed for the minimization of potential effects of variation in air temperature and precipitation on observed differences between land uses and within region. No significant differences in depth of water body, water temperature, dissolved oxygen, pH or specific conductivity were observed among land use or region (Table 3.1). Water temperature was positively correlated with total microcystins concentrations in Late season water samples ($p = 0.016$, $r^2 = 0.12$).

Table 3.1 - Water quality parameters and physical factors measured in water bodies found on both arable land (A.L.) and permanent pasture (P.P.) during 2009 field season. Measurements include Early, Late and Delta values for each land use. Values are expressed as mean ± standard deviation of the mean. Sample size (n) indicated within table.

Analyte	Early Summer (July/August)		Late Summer (August/September)		Delta (Late - Early)	
	P.P.	A.L.	P.P.	A.L.	P.P.	A.L.
N	24	24	23	24	23	24
Depth (m)	1.46 ± 0.71	1.35 ± 1.01	1.37 ± 0.69	1.13 ± 0.83	-0.13 ± 0.23	-0.22 ± -0.29
Water Temperature (˚C)	18.5 ± 1.27	18.6 ± 1.92	16.7 ± 1.48	18.1 ± 1.43	-1.78 ± 1.27	-0.48 ± 1.81
Specific Conductance (mS/cm)	4.07 ± 8.15	6.17 ± 11.5	4.40 ± 8.43	7.61 ± 15.0	0.15 ± 1.09	1.44 ± 3.50
Dissolved Oxygen (mg/L)	6.86 ± 4.07	9.04 ± 3.13	6.88 ± 3.25	8.38 ± 3.38	-0.27 ± 4.96	-0.30 ± 3.53
pH	8.62 ± 0.82	8.72 ± 0.53	8.71 ± 0.55	8.87 ± 0.43	0.08 ± 0.71	0.15 ± 0.47
Air Temperature (˚C)	21 ± 3.9	21 ± 4.1	24 ± 5.9	23 ± 4.2	3.30 ± 6.74	1.63 ± 4.91

3.3.4 Total phosphorus and soluble reactive phosphorus

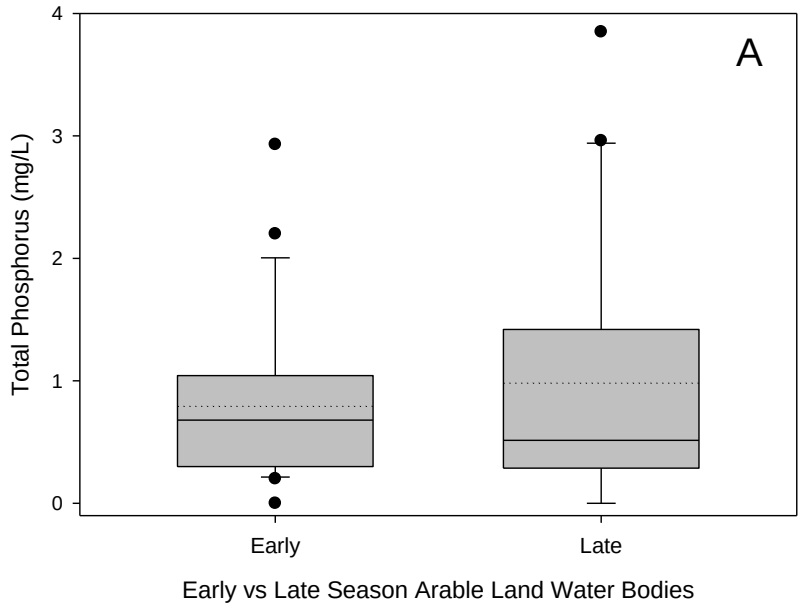
Mean concentrations of total phosphorus in water were significantly greater in water bodies surrounded by arable land in Late season (Table 3.2, p = 0.008) and Delta season (Table 3.2, p= 0.028) compared to water bodies located in permanent pastures. The mean concentration (± standard deviation) of total phosphorus in water bodies surrounded by arable land increased from 0.93 ± 0.93 to 0.99 ± 1.1 mg/L , while concentrations decreased from 0.55 ± 0.72 to 0.33 ± 0.28 mg/L in water bodies located within permanent pasture over the same time period (Figure 3.3). A similar trend of nutrient concentrations increasing for water within arable land while nutrient concentrations decreased for water within permanent pasture over the same time period

was also seen for total kjeldahl nitrogen (although the means were not significantly different between land uses, unlike total phosphorus).

Total phosphorus concentrations had greater variation in water bodies located in arable land in comparison with permanent pasture, although distributions for both land uses display a number of major outliers (Figure 3.4). Early season concentrations of total phosphorus in water were fairly comparable in mean, median and general variance between land uses (Figure 3.4). Mean concentrations of total phosphorus in water were not significantly different ($p > 0.05$ as determined by one-way ANOVA) between Early and Late season for water bodies found in arable land or permanent pasture. Distribution of total phosphorus concentrations in Late season in permanent pasture water samples were small and displayed few major outliers, while distributions of late season concentrations of total phosphorus in arable land water bodies were wider and contained two major outliers. Mean concentrations of total phosphorus in water in Late season was significantly different between land uses ($p = 0.008$).

Linear regression demonstrated a positive correlation between natural log-transformed total phosphorus and natural log-transformed total microcystins concentrations in Late season water samples (Figure 3.5, $p = 0.001$, $r^2 = 0.21$, $F = 12.00$).

Concentrations of soluble reactive phosphorus were significantly greater in water bodies located in arable land in Early season samples (Table 3.2, $p = 0.022$) as well as in Late season (Table 3.2, $p = 0.010$), compared with permanent pasture sites.



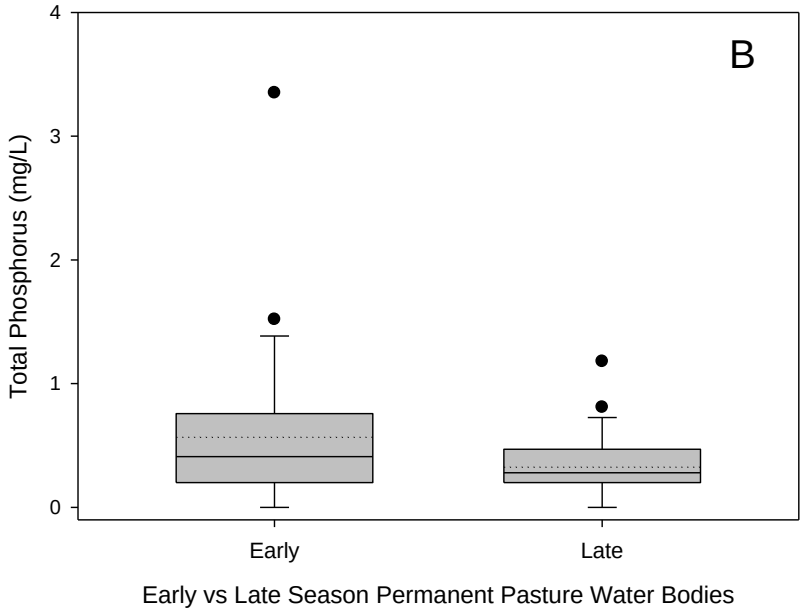


Figure 3.3 - Early and Late season distributions of total phosphorus concentrations (mg/L) from water bodies located within arable land (A, n= 48) and permanent pasture (B, n = 47). Box plots indicate median (solid line) and mean (dotted line) with error bars denoting confidence intervals of 10 and 90%. Filled circles represent outliers. No significant difference was found between mean concentrations of total phosphorus in arable land or permanent pasture water bodies over the same time period.

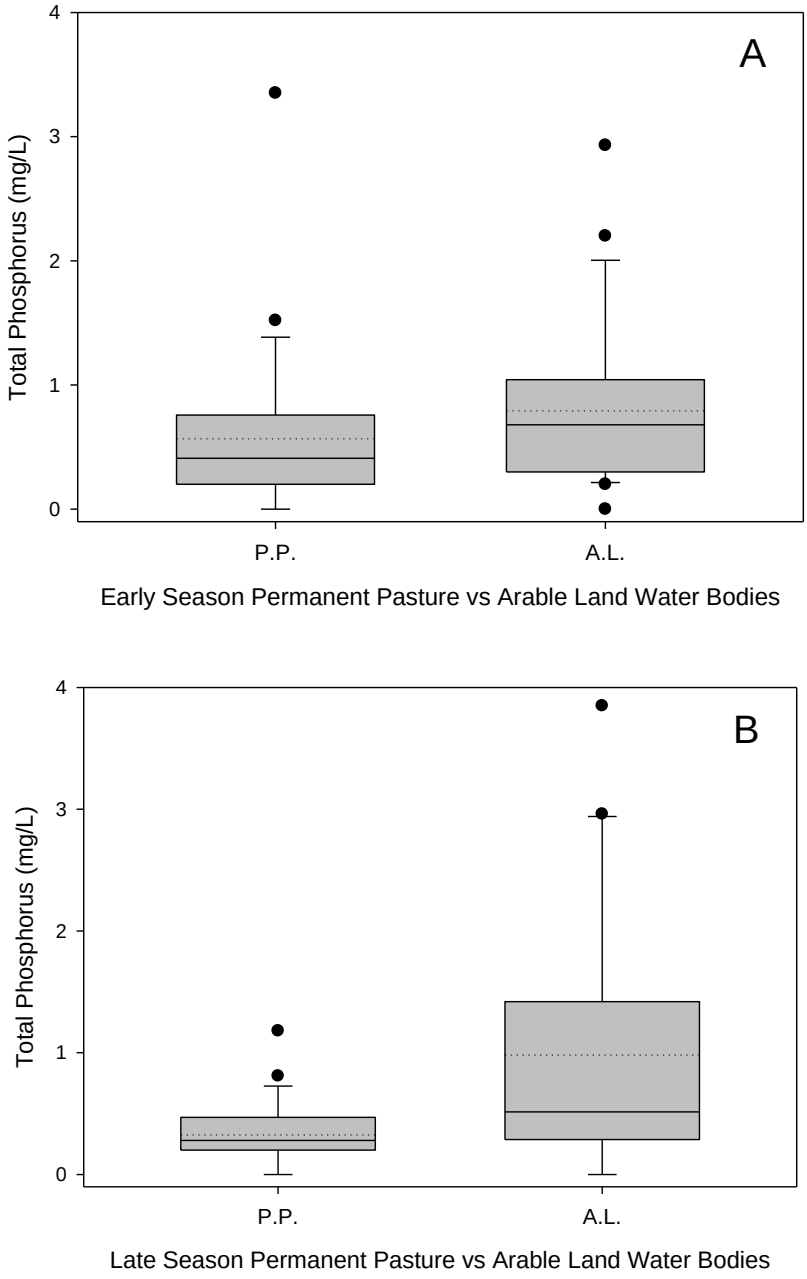


Figure 3.4 - Distributions of total phosphorus concentrations (mg/L) in permanent pasture (P.P.) and arable land (A.L.) for water bodies from Early (A, n = 48) and Late sampling events (B, n = 47). Box plots indicate median (solid line) and mean (dotted line) with error bars denoting confidence intervals of 10 and 90%. Filled circles represent outliers. Mean concentration of total phosphorus in water in Late season (B) was significantly different between land uses ($p = 0.008$).

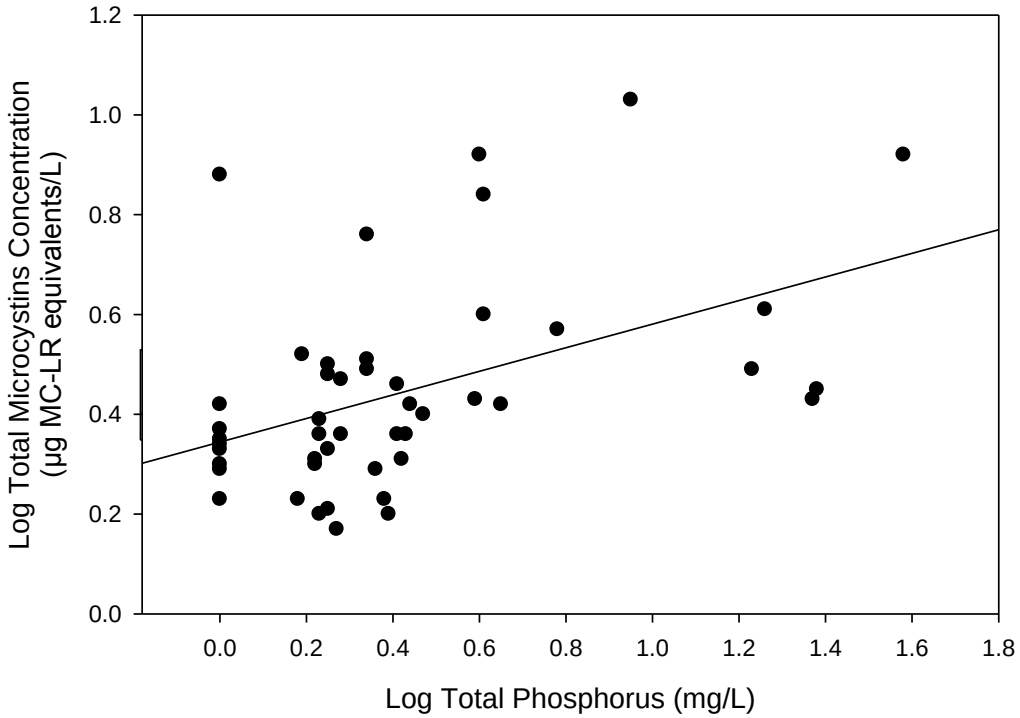


Figure 3.5 - Positive correlation between natural log-transformed total phosphorus and natural log-transformed total microcystins concentrations in water samples from water bodies found within arable land and permanent pasture (n = 47, p = 0.001, r^2 = 0.21) in Late season sampling event.

3.3.5 Total Kjeldahl Nitrogen

Concentrations of nitrate, nitrite, and nitrate + nitrite in water samples were almost entirely below detection limits. Total nitrogen is composed of nitrate + nitrite, ammonia and organic nitrogen, and is often assumed to be an important variable in the study of environmental factors associated with cyanotoxins. Total kjeldahl nitrogen is composed of ammonia and organic nitrogen. Since nitrate and nitrite were below detection in most of the water samples, total kjeldahl nitrogen was used as a proxy for total nitrogen for the purpose of this study.

While the mean concentration of total kjeldahl nitrogen in water bodies within arable land increased slightly from early to late season, the mean concentration decreased slightly in permanent pasture sites during the same time period (Figure 3.6). This trend was also observed in mean concentrations of total phosphorus. As with total phosphorus concentrations, the distribution of total kjeldahl nitrogen concentrations tended to be wide, and contained outliers (Figure 3.6). The distributions of the concentrations of total kjeldahl nitrogen for both land uses were similar in Early season samples. Late season total kjeldahl nitrogen concentrations in water bodies located in arable land demonstrated a more pronounced deviation from the mean, as well as more pronounced outliers compared to total kjeldahl nitrogen concentrations from permanent pasture sites (Figure 3.7). The mean concentrations of total kjeldahl nitrogen from both Early and Late sampling events were not significantly different, regardless of land use. Mean concentration of total kjeldahl nitrogen in water bodies in arable land was significantly different from those located within permanent pasture during Delta season (Table 3.2, $p = 0.039$).

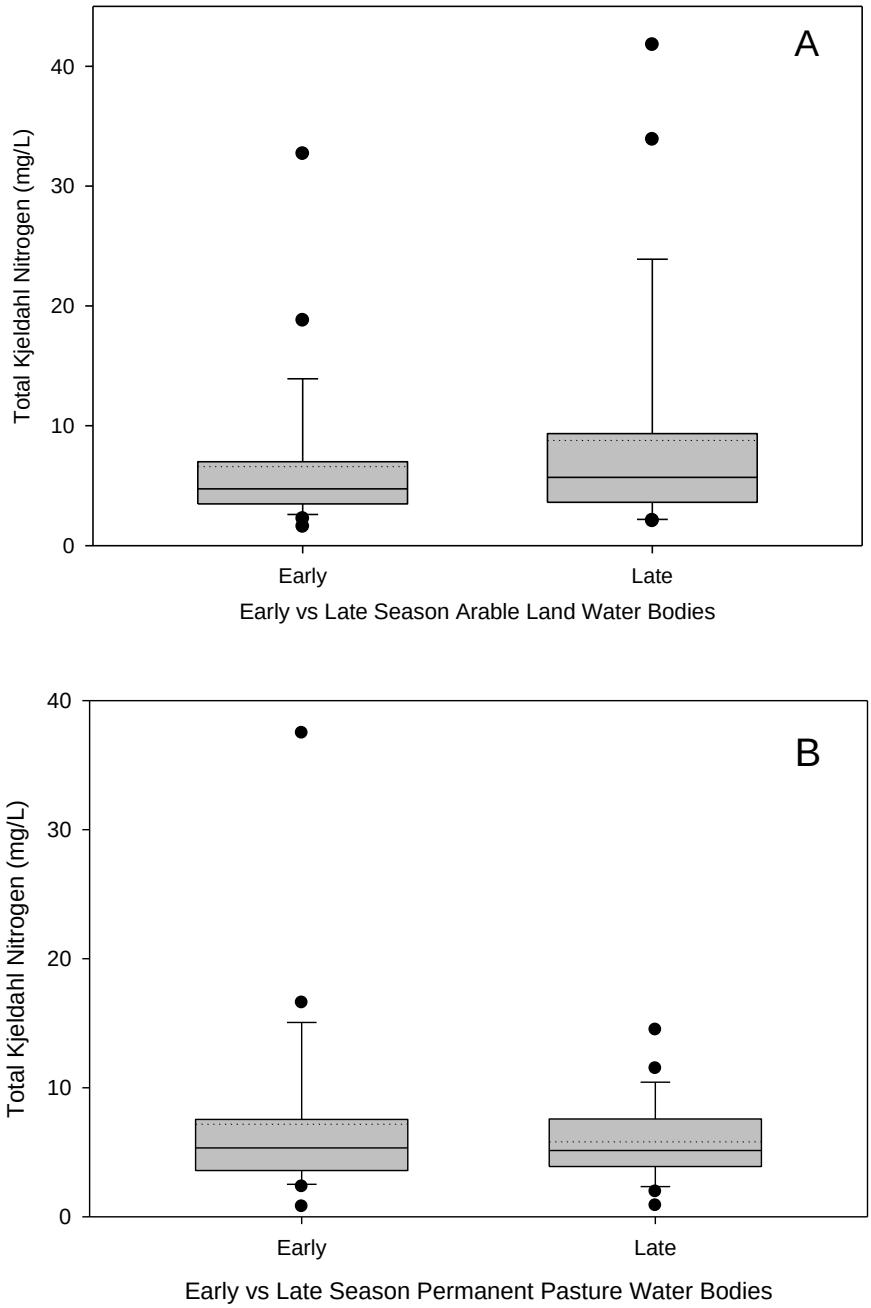


Figure 3.6 - Early and late season distributions of total kjeldahl nitrogen (mg/L) from water bodies located within arable land (A, n= 48) and permanent pasture (B, n = 47). Box plots indicate median (solid line) and mean (dotted line) with error bars denoting confidence intervals of 10 and 90%. Filled circles represent outliers. No significant difference was found between mean concentrations of total kjeldahl nitrogen in arable land or permanent pasture water bodies over the same time period.

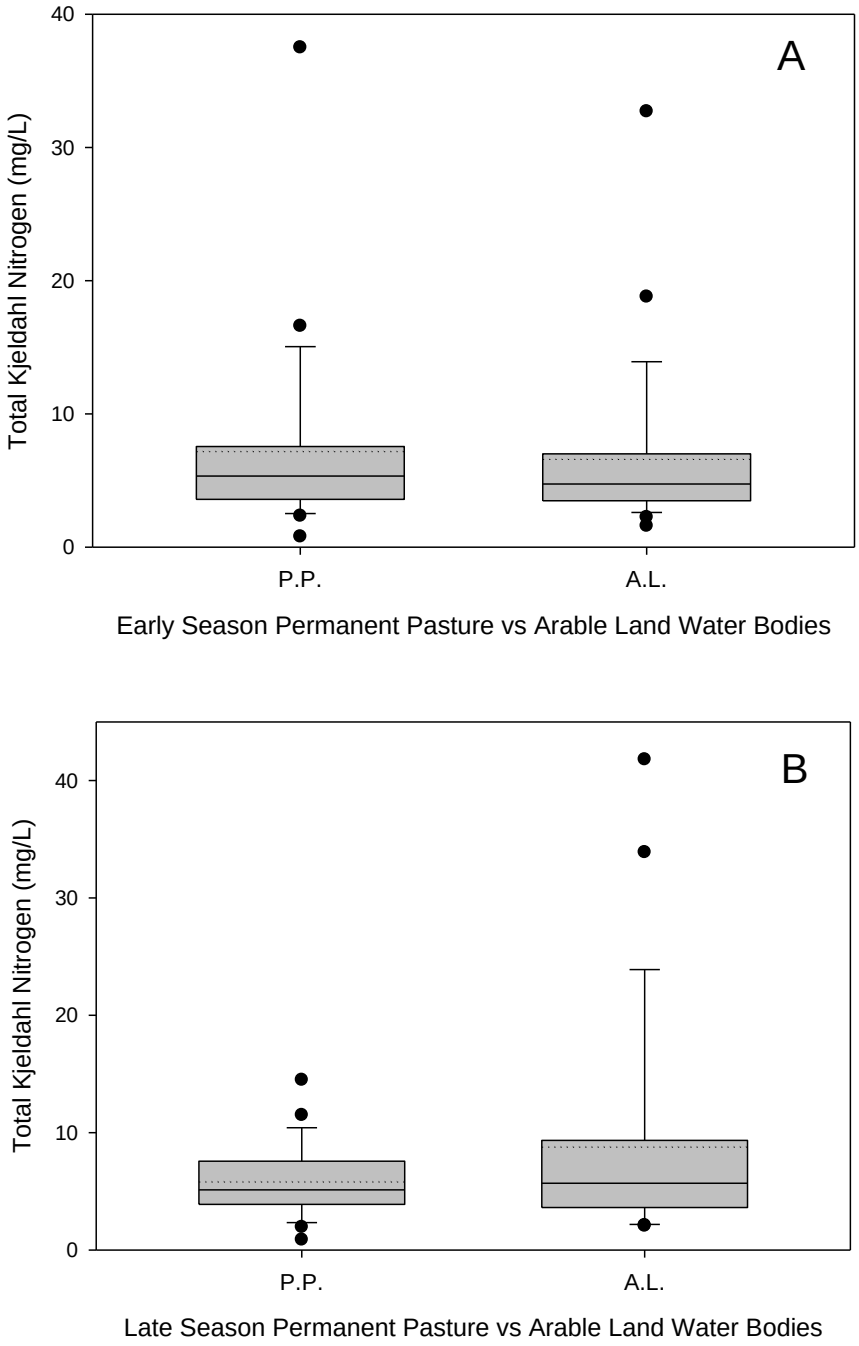


Figure 3.7 - Distributions of total kjeldahl nitrogen concentrations (mg/L) in permanent pasture (P.P.) and arable land (A.L.) for water bodies from Early (A, n = 48) and Late sampling events (B, n = 47), respectively. Box plots indicate median (solid line) and mean (dotted line) with error bars denoting confidence intervals of 10 and 90%. Filled circles represent outliers. No significant difference in mean concentrations of total kjeldahl nitrogen between land uses for Early or Late season samples.

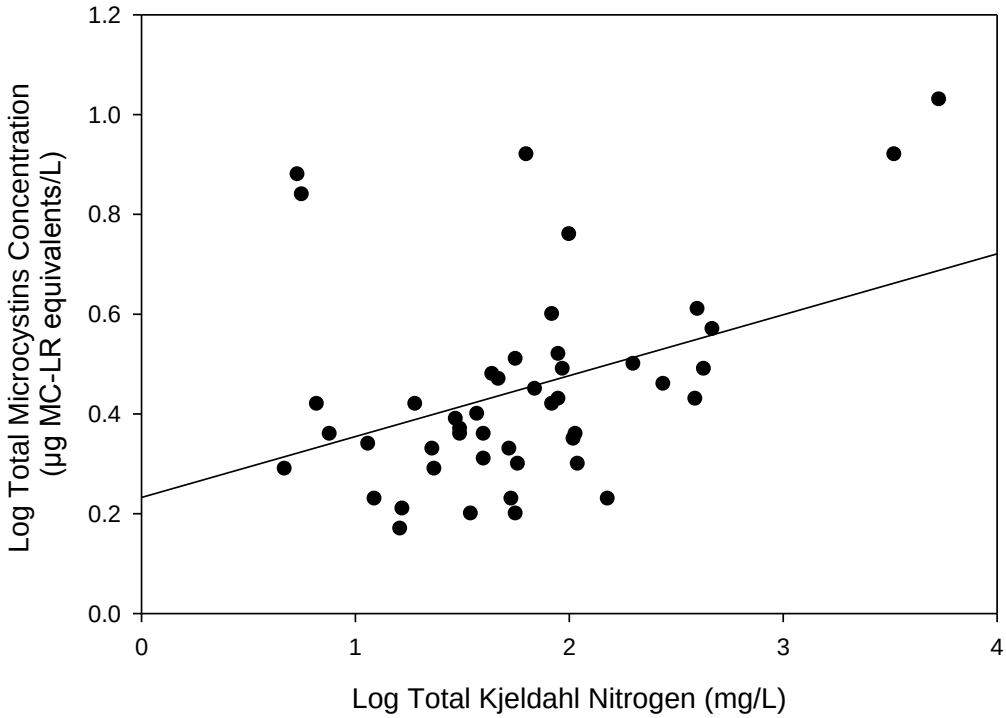


Figure 3.8 - Positive correlation between natural log-transformed total kjeldahl nitrogen and natural log-transformed total microcystins concentrations in water samples from water bodies found within arable land and permanent pasture (n = 47, p = 0.004, r^2 = 0.17) in Late season sampling event.

Table 3.2 - Summary of water nutrient concentrations measured in water bodies surrounded by arable land (A.L.) or permanent pasture (P.P.) during 2009 field season. Measurements include Early, Late, and Delta values for each land use. Values are expressed as mean ± standard deviation of the mean. Sample size (n) indicated within table. Significant differences were detected using one-way ANOVA with land use as a fixed effect. Asterisk denotes significant differences in concentrations found between water bodies located within arable land and permanent pasture (*$p \leq 0.05$, **$p \leq 0.01$).

Analyte	Early Summer (July/August)		Late Summer (August/September)		Delta (Late - Early)	
	P.P.	A.L.	P.P.	A.L.	P.P.	A.L.
N	24	24	23	24	23	24
Total Phosphorus (mg/L)	0.55 ± 0.72	0.93 ± 0.93	0.33 ± 0.28	0.99 ± 1.1**	-0.27 ± 0.69	0.19 ± 0.69*
Soluble reactive phosphorus (mg/L)	0.193 ± 0.361	0.574 ± 0.691*	0.206 ± 0.306	0.710 ± 0.839**	0.007 ± 0.25	0.14 ± 0.47
Nitrate (mg/L)	< 0.50	< 0.50	< 0.50	< 0.50	0	0
Nitrite (mg/L)	< 0.050	< 0.050	< 0.050	< 0.050	-0.002 ± 0.007	0.003 ± 0.01
Total Kjeldahl Nitrogen (mg/L)	7.21 ± 7.36	6.62 ± 6.53	5.85 ± 3.05	8.81 ± 9.64	-1.64 ± 7.99	2.19 ± 3.70*
Ammonia (mg/L)	0.856 ± 2.23	0.388 ± 0.721	0.275 ± 0.368	0.490 ± 0.597	-0.62 ± 2.22	0.10 ± 0.35

3.3.6 Soil ammonium and soil nitrate concentrations

Multiple regression was run in forward, backward, enter, remove, and stepwise modes, and included the variables soil phosphorus, soil ammonium and Late season conductivity, soluble reactive phosphorus, total kjeldahl nitrogen and total phosphorus. Three out of five modes (forward, backward and stepwise) indicated total kjeldahl nitrogen and soil ammonium as predictors of total microcystins concentrations in water (from forward mode: $p < 0.001$, $r^2 = 0.497$, $F = 21.72$), the remaining two indicated soil ammonium alone (data not shown). However, no significant differences in mean concentrations of soil ammonium or nitrate were observed between soil cores collected within the drainage basins of water bodies located in arable land and permanent pasture (Table 3.3).

3.3.7 Soil phosphorus and soil potassium concentrations

Analysis of variance detected a significant difference between concentrations of soil phosphorus in drainage basins found in arable land and permanent pasture ($p = 0.01$). However, the ANOVA failed to pass Levene's test of homogeneity of variance. The analysis was repeated using the Mann-Whitney U test. Results of the nonparametric test were in agreement with the initial ANOVA results (Table 3.3, $p = 0.006$). Analysis of variance also indicated a significant difference between concentrations of soil potassium in drainage basins found in arable land and permanent pasture ($p = 0.02$). Mann-Whitney U test results supported the ANOVA in this case as well (Table 3.3, $p = 0.005$).

Table 3.3 - Summary of soil nutrient concentrations detected in soil cores collected from drainage basins surrounding water bodies located within arable land (A.L.) and permanent pasture (P.P.) during 2009 field season. Kel- indicates analysis by modified Kelowna method. Values expressed as mean ± standard deviation of the mean. Sample size (n) indicated within table. Significant differences were detected using one-way ANOVA with land use as a fixed effect. Asterisk denotes significant differences in concentrations found between soil collected from drainage basins surrounding arable land and permanent pasture (*$p \leq 0.05$, **$p \leq 0.01$).

Analyte	P.P.	A.L.
n	24	24
Nitrate (µg/g)	10.0 ± 6.10	13.8 ± 17.8
Ammonium (µg/g)	12.8 ± 6.36	17.6 ± 19.1
Kel-Phosphorus (µg/g)	2.0 ± 2.56	5.50 ± 3.87**
Kel-Potassium (µg/g)	353 ± 178	489 ± 211**

3.3.8 Cyanotoxin concentrations

A total of 95 water samples (48 from Early season and 47 from Late season) were analyzed for the presence of microcystins-LR, -RR, -dmLR and nodularin using HPLC-MS/MS. Calibration curves were created for nodularin and each microcystin variant. The instrument detection limit was determined to be 1.25 ppb for MC-RR and 2.5 ppb for MC-LR, dmMC-LR and NOD. The method detection limit (with solid phase extraction) was determined to be 32 ppt for MC-RR and 63 ppt for MC-LR, dmMC-LR and NOD. Of the 95 water samples tested, 87% contained detectable concentrations of MC-LR, 97% contained dmMC-LR, 41% contained MC-RR, and 2% contained NOD. Differences in total concentrations of specific variants were determined by adding concentrations of individual variants found within each land use and

region (Figure 3.9). ANOVA indicated that the differences in concentrations of MC-RR in Late season water samples were significant between land uses ($p = 0.04$), with greater mean concentrations observed in water bodies within arable land.

Protein phosphatase inhibition assays detected low level concentrations of microcystins in all water bodies sampled. Concentrations of total microcystins in water samples ranged from 0.17 µg/L to 1.8 µg/L. No significant difference in total microcystins concentrations were seen between land uses in Early season samples. However, microcystins concentrations were significantly greater in water bodies in arable land than those in permanent pasture in Late season samples (Figure 3.10 and Figure 3.11, $p = 0.004$).

Linear regression indicated a positive correlation between HPLC-MS/MS results (when concentrations of all four microcystin variants were summed in each individual water body to produce a total microcystins concentration) and protein phosphatase inhibition assay results for total microcystins concentrations ($p < 0.001$, $r^2 = 0.34$). Total microcystins concentrations as determined by protein phosphatase assays and the summation of all four individual variants as determined by HPLC-MS/MS are shown graphically (Figure 3.12).

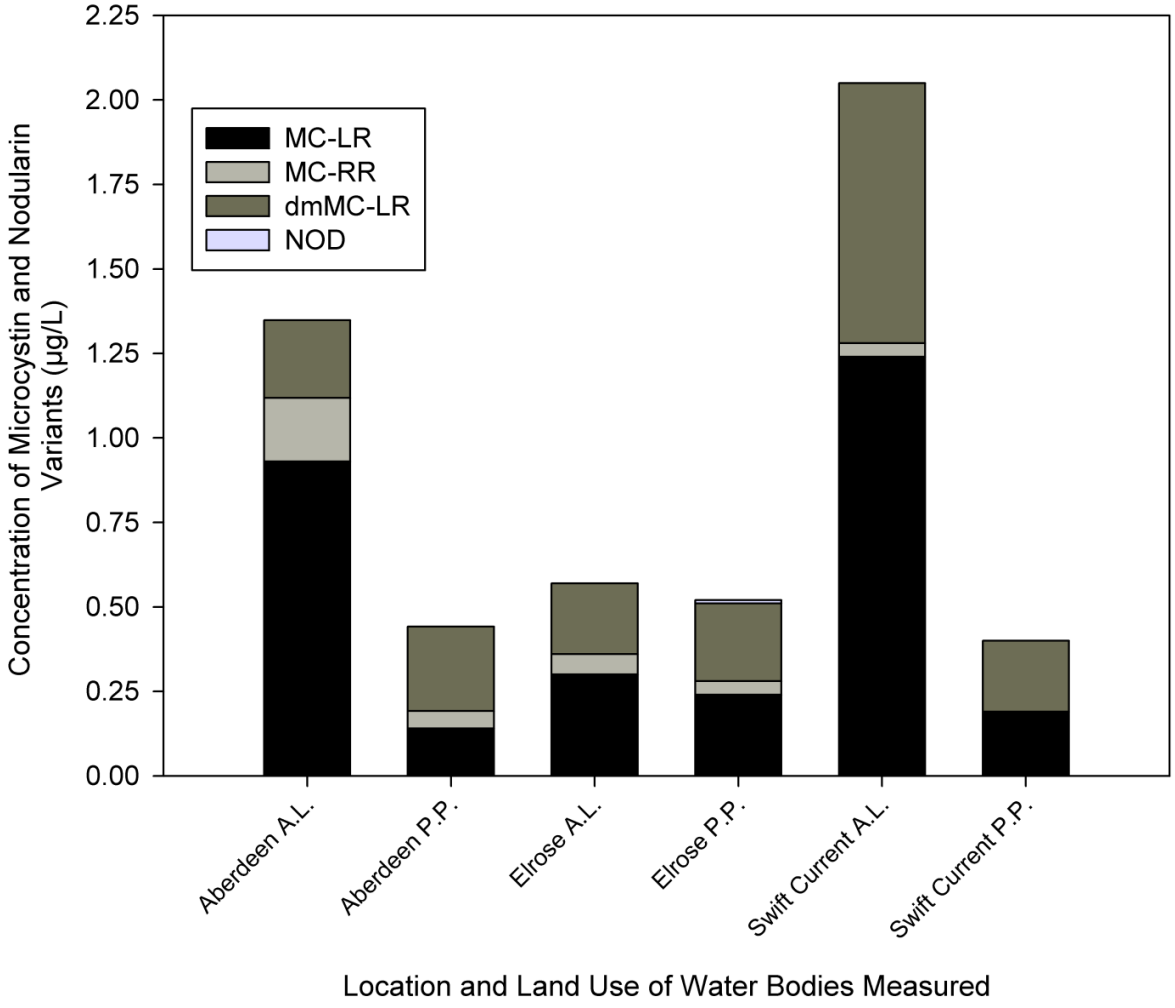


Figure 3.9 - Total concentration of four individual variants, MC-LR, MC-RR, dmMC-LR and NOD (µg/L) found within each region and land use (n = 8, except Swift Current P.P., where n = 7) in Late season water bodies, as determined by HPLC-MS/MS analysis.

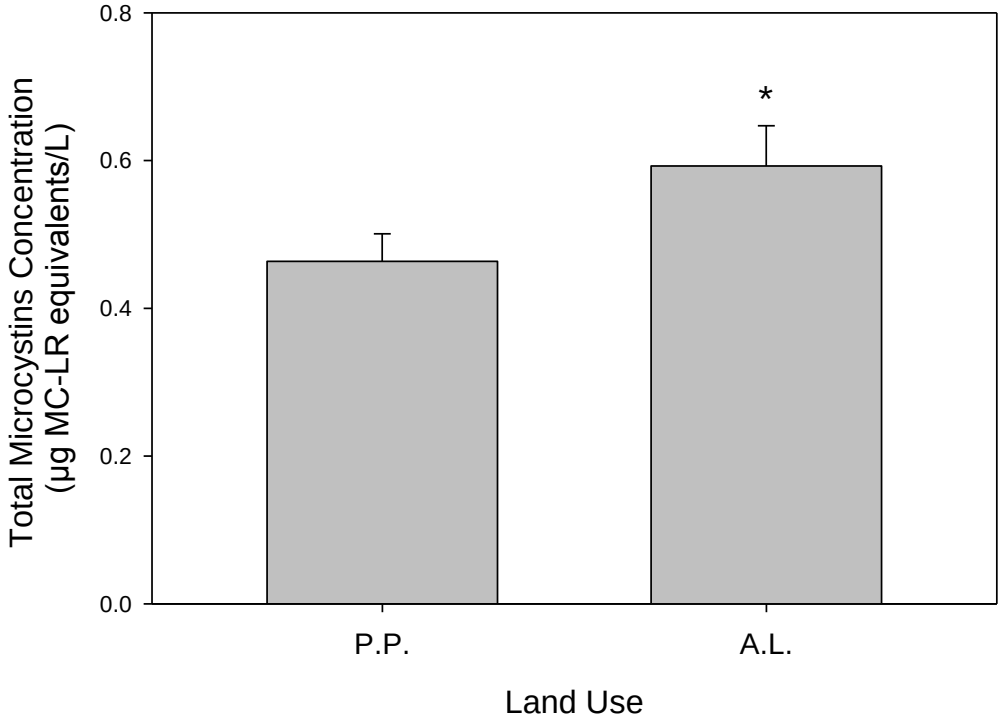


Figure 3.10 - Mean concentrations of total microcystins in water samples collected in 2009 field season from water bodies located within permanent pasture (P.P., n = 23) and arable land (A.L., n = 24) in Late season sampling. Bar graph denotes mean concentration of total microcystins ± standard error of the mean. Significant differences were detected using one-way ANOVA with land use as a fixed effect. Asterisk denotes significant difference in total microcystins concentrations between arable land and permanent pasture (*p = 0.004).

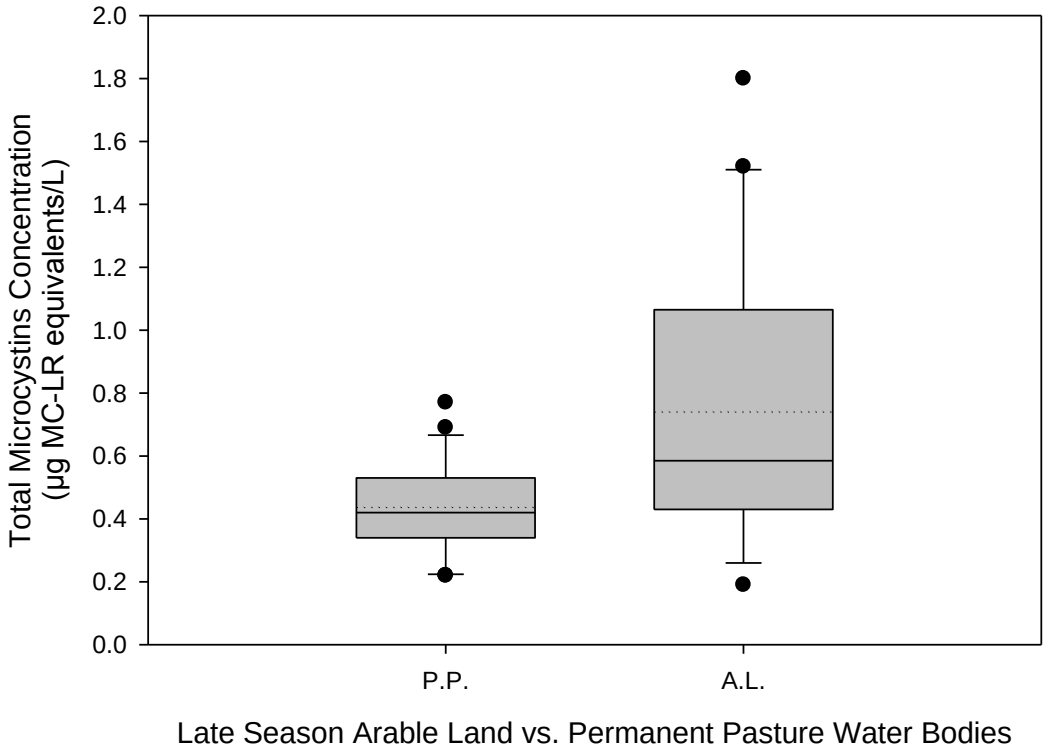


Figure 3.11 - Distributions of total microcystins concentrations (µg MC-LR equivalents/L) in permanent pasture (P.P., n = 23) and arable land (A.L., n = 24) in Late season sampling. Box plots indicate median (solid line) and mean (dotted line) with error bars denoting confidence intervals of 10 and 90%. Filled circles represent outliers. A significant difference between land uses was detected using one-way ANOVA with land use as a fixed effect ($p = 0.004$).

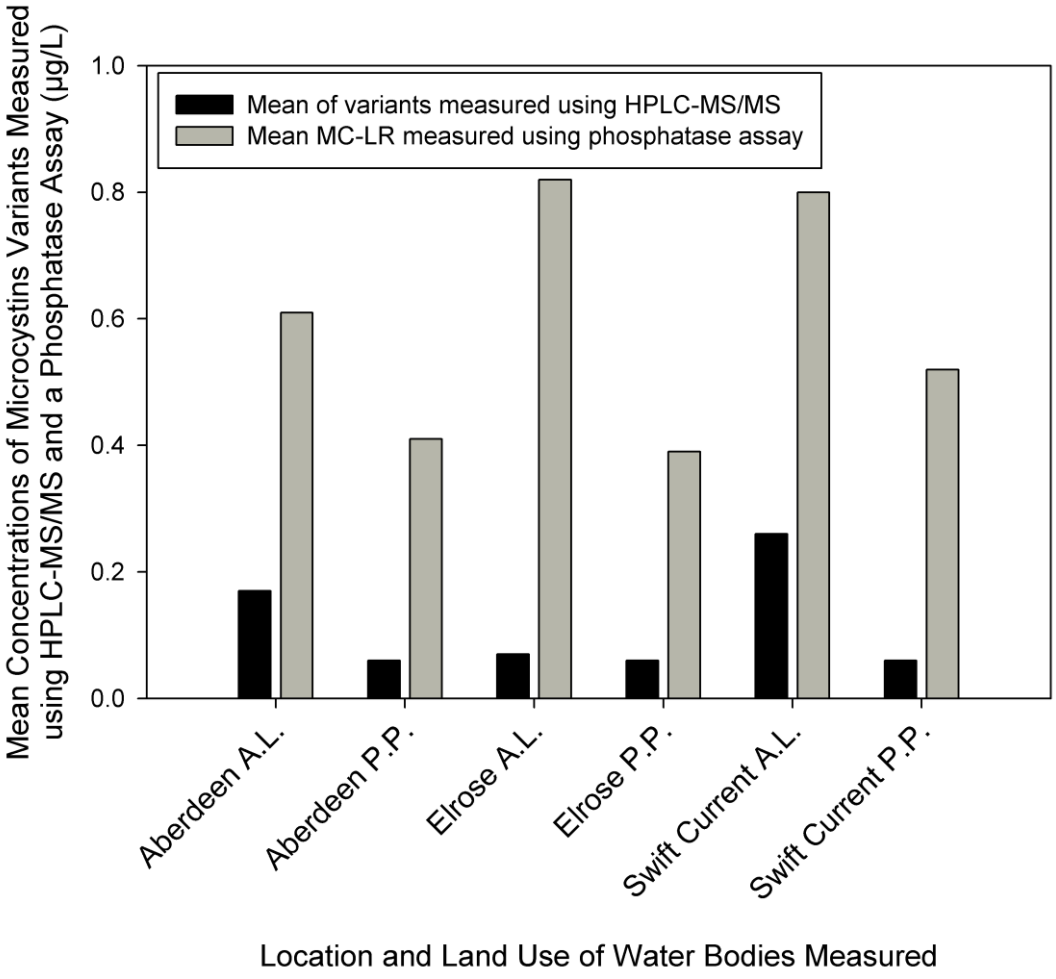


Figure 3.12 - Total concentration of microcystins measured in Late season water bodies in all regions and land uses (n = 8, except Swift Current P.P., where n = 7). Different colour bars represent either concentration of total microcystins measured by protein phosphatase assays or the concentration of microcystins measured through summation of microcystin and nodularin variants as measured by HPLC-MS/MS.

3.4 Discussion

Microcystins concentrations were significantly greater in water bodies surrounded by arable land as compared to permanent pasture water bodies. Total phosphorus concentrations were high (eutrophic-hypereutrophic, USEPA, 1996) in most of the water bodies examined. Water concentrations of nitrogenous compounds (specifically total kjeldahl nitrogen) and ammonium concentrations in soil from drainage basins surrounding water bodies were found to be significant factors in predicting microcystin concentrations in water already containing high concentrations of phosphorus. However, it was observed that the relationships described were often influenced highly by a small number of observations. Although the results suggest nitrogenous compounds as important predictors of cyanotoxin concentrations in water, it is probable that there are a number of other unmeasured or unrelated factors also involved. Regardless, this study does indicate that cyanobacterial microcystins are more abundant in small water bodies located within crop lands relative to pastures in Saskatchewan. Furthermore, concentrations of total kjeldahl nitrogen in water and ammonium in soil can both be considered potential predictors of total microcystins concentrations in these same water bodies.

Almost all water bodies in both arable land and permanent pasture contained some detectable concentration of microcystins, indicating that lesser concentrations of microcystins might be common in small Saskatchewan water bodies during summer months. Interestingly, microcystins concentrations in water samples from the Early sampling season were comparable between arable land and permanent pasture. However as summer progressed, microcystins concentrations in permanent pasture remained relatively unchanged, while concentrations in arable land increased to a point to which there was a significant difference between land uses.

Microcystins were observed in permanent pastures where the only major outside input of nutrients is most likely contamination with livestock manure. However, cycling of natural

background levels (e.g., nutrients in snowmelt run-off from leaching of soluble nitrogen and phosphorus in surface thatch and dead grass) could also be sufficiently high to support cyanobacterial growth. Hall et al. (1999) used fossil analyses to show that Pasqua Lake (a larger water body in the Qu'Appelle Valley drainage system in Saskatchewan) was a eutrophic water body that supported cyanobacteria prior to agricultural practices (ca. 1776-1890).

Microcystins were observed in lesser concentrations in the water bodies located within permanent pasture sites, demonstrating that activities occurring in permanent pasture, including contamination of water bodies through manure, are high enough to support the growth of toxic cyanobacteria. While microcystins were found in these water bodies, the mean concentrations were significantly greater in arable land water bodies, implicating the main difference between the water bodies studied: land use and external inputs of nutrients.

The findings of this study are consistent with the hypothesis that increased nutrient loading produced by agricultural activity has the potential to increase microcystins concentrations in water bodies receiving nutrient input. Fertilizers common to the types of crops grown in Saskatchewan contain nitrogen and phosphorus (Government of Saskatchewan, 2009, Government of Saskatchewan, 2006), two macronutrients commonly implicated in eutrophication (Chow et al., 2011, Carpenter et al., 1998), which can lead to cyanobacterial growth and possibly cyanotoxin production.

Phosphate fertilizers are commonly used on cropland in Saskatchewan (Government of Saskatchewan, 2006). Although phosphorus tends to bind to soil more strongly than nitrates, many authors agree that it is still possible to enrich topsoil to a point where movement of phosphorus overland to water bodies or leaching to ground water is possible (Sharpley et al., 1994). A significant difference in mean concentrations of soil phosphorus was seen between

permanent pasture and arable land. This result indicates that practices occurring on arable land (e.g. fertilization), as compared to the external inputs in permanent pasture, are likely influencing the greater concentrations of phosphorus found in the soil within the drainage basins of these Saskatchewan water bodies. These increased concentrations of soil phosphorus may, through leaching or overland runoff, contribute to the eutrophication of local water.

Phosphorus is often implicated as one of the most important potentially limiting nutrients in freshwater systems (Schindler et al., 1977). Increased concentrations of phosphorus in water is considered a major contributor in the decrease of water quality through eutrophication (USDA, 2003). In the present study, soluble reactive phosphorus concentrations in both Early and Late season samples, and total phosphorus concentrations in Late season samples were present in significantly greater concentrations in water bodies surrounded by arable land. Additionally, phosphorus concentrations in soil were also greater in the drainage basins of water bodies found within arable land. While neither soluble reactive phosphorus or soil phosphorus were positively correlated with total microcystin concentrations in water, total phosphorus was positively correlated in Late season samples. Research has shown that total phosphorus in water is often a predictor of cyanobacterial abundance, and even possibly cyanotoxin concentrations. However in this study, phosphorus concentrations were rarely limiting in either land use, and it is likely because of this that it was not indicated as a predictor of cyanotoxin abundance.

Although phosphorus concentrations were significantly greater in water bodies located within arable land, phosphorus concentrations in most water bodies, (within both land uses), was sufficient to cause a water body to be considered eutrophic to hypereutrophic (USEPA, 1996). The range of phosphorus concentrations in water that could potentially support a substantial population of cyanobacteria has been estimated to be 0.03-0.1 mg/L (Ansari, 2010). Most water

bodies in both arable land and permanent pasture had total phosphorus concentrations well above this range during both Early and Late season sampling. This indicates that phosphorus was present in more than sufficient concentrations to sustain baseline cyanobacterial populations. Since it appears that concentrations of phosphorus were not limiting in water bodies within either land use, but arable land water bodies experienced significantly higher concentrations of microcystins, another variable(s) is most likely influencing microcystins abundance in the studied water bodies. Other research suggested that once phosphorus in a water body reached a certain concentration, its influence on eutrophication plateaus, but subsequent additions of nitrogen will increase eutrophication (Correll et al., 1999). This is possibly because nitrogen becomes limiting in conditions where phosphorus concentrations are greatly increased.

Nitrogenous compounds have also been considered significant factors in the growth of cyanobacteria and/or cyanotoxin production. Analysis of variance did not detect a difference in mean concentrations of total kjeldahl nitrogen from water bodies surrounded by arable land compared to permanent pasture sites in Late season samples, even though it was indicated as a predictor of microcystins abundance. This lack of significant difference could be due to the high variability that is intrinsic in an observational study. This work is a first step in determining the role nitrogenous compounds may play in determining microcystins abundance in small Saskatchewan water bodies.

Fertilization practices over several years may lead to a build-up of nitrogenous compounds in the soil. Jalil et al. (1996) demonstrated that soil ammonium levels can be used as an indicator of soil nitrogen release potential by mineralization, as affected by management of the land. In addition to fertilization practices, naturally occurring soil organic matter can be converted to ammonia, which can subsequently be converted to nitrite and finally to nitrate; a

mobile nutrient (Fetter, 1993). Mobile nitrogenous compounds can be transported via surface or subsurface runoff and leaching to water bodies within the same drainage basin. In the present study, multivariate regression analysis consistently implicated soil ammonium concentrations as being positively correlated with microcystins concentrations ($p < 0.001$, $r^2 = 0.497$). Rainfall events might also lead to spikes of nitrogen in local water, regardless of land use, and aid in the growth of toxic cyanobacteria. However, precipitation was not measured in this study. Future work in Saskatchewan may include precipitation as a possible predictor in cyanotoxin abundance.

The role of nitrogenous compounds in the growth of cyanobacteria and the production of cyanotoxins is controversial. Where phosphorus was previously considered the most important factor in primary production, research has shown that nitrogen also plays an important role. However, the extent to which either macronutrient acts individually or in combination is still under question. A study on 241 lakes was conducted that included a range of trophic statuses to examine the relationship between microcystin-LR concentrations and total nitrogen in the water (Graham et al. 2004). Their results suggested that the relationship between the two variables was not linear, but strongly correlated. The work demonstrated a relationship described by a unimodal curve, with maximal concentrations of microcystin-LR occurring between 1500 – 4000 µg/L total nitrogen, concentrations slightly lower than the mean concentrations observed in the present study. In this previous study, microcystin-LR concentrations decreased at total nitrogen values exceeding 4000 µg/L (Graham et al., 2004). A similar study to Graham et al. (2004) was conducted using 30 shallow, subtropical eutrophic - hypereutrophic lakes along the Yangtze River (China) (Wu et al. 2006). Their results indicated that the relationship between different microcystin variants and total nitrogen could be described by a unimodal curve. In this same

work, the highest concentrations of the microcystins variants examined occurred between total nitrogen concentrations of 250 – 1000 µg/L (Wu et al. 2006).

Another study used laboratory based approach to determine the effects of nitrogen and phosphorus and the interactive effects of these nutrients on microcystin concentrations, as well as on the growth of toxic vs. non-toxic strains of *Microcystis* (Vézie et al. 2002). Their study revealed an interactive effect of nitrogen and phosphorus, with greatest microcystins concentrations being detected in the treatments with the greatest nitrogen and phosphorus concentrations. However, high microcystins concentrations were also detected in the lesser treatments of nitrogen and phosphorus, which they were unable to explain. This study concluded that high concentrations of nutrients favoured the growth of toxic strains of *Microcystis* as opposed to non-toxic strains, explaining that toxic strains may need higher nutrient concentrations due to their toxin-producing ability, which is energy-consuming (Vézie et al. 2002).

It has been suggested that maintaining concentrations of total nitrogen in water < 0.5 – 1.0 mg/L could prevent eutrophication in aquatic ecosystems (Camargo et al. 2006). Total kjeldahl nitrogen (a proxy for total nitrogen in this study) concentrations in the water bodies from both arable land and permanent pastures exceeded this range.

It is important to note that the regressions that implicated both soil ammonium and total kjeldahl nitrogen in water appear to be driven by a few influential cases. Although this is true, it is also the defining difference between the land uses. Permanent pasture water bodies rarely, if ever, experienced extreme values for any variable measured, while these extreme values occurred frequently within arable land water bodies. This is likely due to the variability in external loading of nutrients that is common in arable land. Increased nutrient loading can lead to

increases in nutrient concentrations in soil and ultimately in water, which are then more likely to support the growth of cyanobacteria.

The hydrology of permanently grassed pastureland and arable land might also contribute to differences in nutrient concentrations, and therefore differences in microcystins abundance between land uses. Research on the prairie region in southern Saskatchewan demonstrated the differences in water retention between cultivated land and adjacent undisturbed grassland (Van der Kamp et al., 1999, 2003). They observed that the soil found in permanent grassland has a much higher infiltrability than soil, when both frozen and unfrozen, than soil found on cultivated land. These results imply that the majority of snowmelt runoff and summer precipitation, along with the nutrients they carry, will likely infiltrate into the soil in permanent grasslands, but will become overland runoff in arable land.

The WHO describes microcystin concentrations in terms of microcystin-LR equivalents; -LR being one of the most toxic and also one of the most commonly found variants (Falconer, 2005). The results of this study are generally consistent with this observation. Some detectable concentration of MC-LR and dmMC-LR was found in almost every water body, regardless of land use. MC-LR and dmMC-LR were also found in the highest concentrations of any of the four variants measured for most land uses and regions (Figure 3.9). However, the only variant that was found to be linked to land use was microcystin-RR. It has been proposed that the presence of individual variants might be due to the availability of specific nutrients in the water (Van de Waal et al. 2010). Using this reasoning, Van de Waal et al. (2010) demonstrated that microcystin-RR might be the dominant variant when nitrogen availability was increased; a situation observed in the present study, especially in water bodies located within arable land.

Microcystin-LR concentrations were not significantly different between water bodies found in arable land and permanent pasture.

Linear regression revealed a positive correlation between total microcystin-LR equivalents and the sum of variants data derived from protein phosphatase assays and HPLC-MS/MS, respectively. However, a graphical representation of the concentrations of microcystins revealed that for all regions and land uses, the sum of variants as measured by HPLC is a relatively small portion of the total MC-LR equivalents concentration as measured by enzyme inhibition (Figure 3.12). Since HPLC-MS/MS will only detect the reference materials that have been quantified, the difference in concentrations seen between these two methods is likely due to only quantifying four reference materials of the almost 90 microcystins variants that have been identified.

Chlorophyll-a is a commonly used surrogate for cyanobacterial abundance (Chorus et al., 2000, Wu et al., 2006, Touchette et al., 2007). In the present study, chlorophyll-a was positively correlated with total kjeldahl nitrogen and total phosphorus concentrations in water in Late season samples. This finding indicates that total phosphorus and total kjeldahl nitrogen might be contributing to an increase in the total burden of phytoplankton containing chlorophyll-a, which may or may not indicate cyanobacteria specifically. Chlorophyll-a concentrations were also positively correlated with microcystins concentrations in water. However, this correlation appeared to be driven by a single influential case (Figure 3.3). Both lesser and greater concentrations of microcystins were observed in association with lesser concentrations of chlorophyll-a. Our findings suggest that chlorophyll-a might not always be an ideal predictor of cyanotoxin abundance (Figure 3.3). Phycocyanin was also a weak predictor of microcystins concentrations in water in this study (Figure 3.4). However, as with chlorophyll-a, the

relationship between phycocyanin and microcystins abundance may be due to the relatively small number of observations. More work is needed to investigate these relationships. Associations of phycocyanin or chlorophyll-a with cyanobacterial abundance were not investigated in this study.

Water temperature was positively correlated with total microcystins concentrations in water in Late season samples. Other researchers have observed that increased water temperature may increase cyanobacterial growth and/or toxin production (Jiang et al., 2008, Yang et al., 2012). However, the range of optimal temperature for most toxigenic cyanobacterial species is quite large, and water temperatures observed in the present study fell within this range. Consequently, water temperature is most likely a relatively minor factor working in combination with other, more important factors during typical prairie summers.

No significant differences between land uses were observed in any of the other water quality parameters measured. Previous researchers (Jacoby et al., 2000), have speculated that pH may influence cyanobacterial dominance indirectly, because cyanobacteria are more efficient than other phytoplankton at utilizing CO_2. However, water bodies in the present study did not show significantly different levels of pH between land uses.

3.5 Conclusions

Significant differences in microcystins concentrations in surface water were observed between water bodies located in arable land as compared to those in permanent pasture. This suggests that local agricultural practices may be impacting local small water bodies and supporting the growth of toxin-producing cyanobacteria. Secondly, this study identified nitrogenous compounds in soil and water as being correlated with microcystins concentrations in small Saskatchewan water bodies which were also found to have phosphorus concentrations

present in excess. This finding supports the hypothesis that fertilization practices leading to increases in water nitrogen concentrations might be a trigger for cyanotoxin production in some situations. Additionally, this study illustrated the high natural variability present in measures of nutrients in soil and water in southern and central Saskatchewan. Consequently, a single field season was not sufficient to establish these findings as concrete; more work is necessary. Cyanobacterial blooms and toxin production are notoriously unpredictable year to year, so studies over multiple seasons are essential to identify key local drivers.

CHAPTER 4

4 GENERAL DISCUSSION

4.1 Introduction

The goal of my thesis research was to investigate the occurrence of cyanobacterial neurotoxins and hepatotoxins in small water bodies in southern and central Saskatchewan in relation to land use practices and environmental factors. The study was conducted using a factorial design, comparing water and soil nutrients from different water bodies and their respective drainage basins located within either cultivated farm land or permanent grass pastures from three different geographic regions.

4.2 2009 field work

The original study design was based on a monitoring-type approach (Appendix A), in which sample site selection was based on knowledge of historic/previous cyanotoxin poisonings of livestock. This approach to site selection was discarded in the second year because it was concluded that potential effects of temperature, sunlight and precipitation (which can vary significantly across the province) might mask or confound differences in cyanotoxin production related to land use. Therefore, a factorial design was adopted for the second year of field work. This two-by-three factorial design was better suited to test the main hypothesis of whether a significant difference was present in the mean concentrations of cyanotoxins between land uses.

The 2009 field work (on which the majority of this thesis is based) was accomplished with the use of an integrated sampler for water collection, a YSI 6600 V2-4 Sonde with a YSI 650 handheld display for water quality measurements, and a backsaver probe for soil collection. Parameters measured at each water body included total depth, water temperature, dissolved oxygen, pH, conductivity, chlorophyll-a, phycocyanin, total phosphorus, soluble reactive phosphorus, nitrate, nitrite, total kjeldahl nitrogen, and ammonia concentrations in water, and

nitrate, ammonium, potassium and phosphorus concentrations in soil. Sampling techniques and parameters measured in this study are similar to a number of other studies investigating either cyanobacterial populations or cyanotoxins in water, including Kotak et al. (2000), Wu et al. (2006), Jacoby et al. (2000), and Gillett et al. (2011). The majority of the above studies were also based on water bodies classified as eutrophic to hypereutrophic.

The present study fills a gap in the literature on cyanotoxin occurrence in small water bodies. The majority of research on cyanotoxins (or cyanobacterial populations) in water is focused on larger, deeper water bodies. The emphasis on small water bodies in this study was necessary because in the prairie pothole regions of the Great Plains, small water bodies are not only abundant, but frequently used as watering holes for livestock or wildlife, and occasionally for household use by rural farm families.

4.3 Statistical analysis approach

Analysis of variance was used to determine significant differences between means. Where Levene's test of homogeneity of variance failed, the nonparametric Mann-Whitney U test was used. Multiple regression analysis was used to explore the relationship between environmental variables and cyanotoxin abundance in water. 30 variables were initially screened, producing a model with approximately 3 observations per predictor. Over-fitting is a risk when exploring limited data (Babyak et al., 2004). Potential predictors were further narrowed down to 6 based on significant ANOVAs as well as nutrients implicated as potential predictors in the scientific literature. The resultant model was comprised of two variables: soil ammonium and total kjeldahl nitrogen. These two variables were consistently indicated as predictors that explained the most variability in microcystins abundance when regression analyses were run in enter, remove, forward, backward, and stepwise modes.

4.4 The examination of small Saskatchewan water bodies for the presence of saxitoxins

Chapter 2 described a field study conducted during the summer months of 2009, which resulted in the collection of water and soil from 48 different water bodies located within arable land or permanent pasture across three regions of southern and central Saskatchewan. A portion of the water collected was used for investigating whether saxitoxins were present in these small water bodies, and, if so, whether there was a significant difference in concentration between water bodies located within arable land, and those located within permanent pasture. To determine this, water collected during this field season was analyzed using an HPLC-MS/MS method developed from Dell'Aversano et al. (2004) and Dell'Aversano et al. (2005) and compared to certified reference materials. No detectable concentrations of saxitoxins were found in any water bodies.

Less is known about the occurrence of cyanobacterial neurotoxins than the hepatotoxins. This is at least partly due to the ephemeral nature of the neurotoxins, which makes their detection difficult. If water or bloom samples are not collected relatively quickly and stored and shipped properly following a poisoning event, the neurotoxins will likely have degraded by the time they are tested. The present study was the most extensive and systematic effort to measure saxitoxins in Saskatchewan. While the saxitoxins have not been detected in Saskatchewan, there are reasons to believe that they occur. Firstly, at least two genera of cyanobacteria known to produce these toxins have been observed in the prairie provinces (Kotak et al., 1993, Hammer et al., 1968, Whitaker et al., 1978). Secondly, clinical cases of suspect cyanotoxin livestock poisoning submitted to the toxicology laboratory at the WCVM (Saskatoon, SK) have been consistent with saxitoxin exposure (Dr. Barry Blakley, Western College of Veterinary Medicine, personal communication), but the analytical method to confirm this was not available in Saskatchewan until it was developed in conjunction with this study.

More work is necessary to determine whether saxitoxins are present in Saskatchewan, and if they are, in what concentrations. The field work and HPLC-MS/MS method developed could be a model for future research on saxitoxins in Saskatchewan. Analysis for anatoxin-a or anatoxin-a(s), the other cyanobacterial neurotoxins, was outside the scope of this study. Future work may focus on developing analytical methods of detection for anatoxin-a and anatoxin-a(s) in Saskatchewan.

4.5 Nutrient concentrations in Saskatchewan water and soil

This study provided insight into nutrient concentrations present in small Saskatchewan water bodies, for which routine monitoring is not a common practice. Phosphorus, a nutrient known to be required for the growth of cyanobacteria, was present in almost all sites, regardless of land use, at concentrations surpassing the 0.03-0.1 mg/L range thought to support a substantial cyanobacterial population (Ansari, 2010). However, concentrations of total phosphorus in water and soil in arable land sites were significantly higher than water and soil from permanent pasture sites. While concentrations of plant available phosphorus in water and soil in Saskatchewan are naturally present in moderate to low concentrations, these concentrations can be increased depending on the land use in the surrounding area. Phosphate fertilization is a common practice in Saskatchewan, and is likely influencing the increased concentrations of phosphorus found in water and soil surrounded by arable land, an effect not seen in pasture surrounded water bodies.

It has been suggested that maintaining concentrations of total nitrogen in water at < 0.5 – 1.0 mg/L could prevent eutrophication in most aquatic ecosystems (Camargo et al. 2006). Total kjeldahl nitrogen (a proxy for total nitrogen in this study) concentrations in water bodies from both arable land and permanent pastures exceeded this range. Fertilizers of nitrogenous compounds are commonly used in Saskatchewan. Concentrations of total kjeldahl nitrogen were

high in water bodies associated with both land uses, although not significantly different between land uses. Failure to observe a statistically significant difference related to land use, in spite of frequent additions of nitrogenous fertilizer to arable land, is possibly due to the high variability of measurements like nutrient concentrations in natural systems. A larger sample size might be required to demonstrate a difference.

Soil ammonium and total kjeldahl nitrogen in water were identified as potential predictors of microcystin abundance in small Saskatchewan water bodies. Although phosphorus has long been considered the most critical limiting nutrient, research has shown nitrogen to be influential as well, although to what extent is uncertain. Although total phosphorus concentrations in most water bodies were fairly high, phosphorus was not identified as a predictor of microcystins abundance in water. Other research has suggested that once phosphorus in a water body reaches a certain concentration, its influence on eutrophication plateaus, but subsequent additions of nitrogen will increase eutrophication (Correll et al., 1999). The extent to which nitrogenous compounds influence cyanotoxin abundance is still unknown. This research suggests that in eutrophic waters, nitrogenous compounds might be the most important predictors of microcystins abundance and that a pulse in nitrogen concentrations (e.g., following fertilization of arable land) might trigger or increase the occurrence of these toxins.

This research revealed that many small Saskatchewan water bodies might be classified as eutrophic – hypereutrophic (USEPA, 1996). Water that is eutrophic, stagnant and located in an area with warm temperatures and long hours of sunlight provides conditions in which cyanobacteria can dominate and potentially produce cyanotoxins. These conditions are seen in small Saskatchewan water bodies. Future work may focus on taking measurements from water bodies and soils that could truly be considered natural (i.e., unaffected by agricultural practices),

such as those found within waterfowl refuges or Grassland National Park, in order to determine what nutrient loads might be considered "natural background" in Saskatchewan soil and water. Regular monitoring of small (and large) water bodies might be important to gain a better understanding of natural background concentrations of these nutrients in Saskatchewan in order to determine what impact agricultural practices are having on concentrations of nutrients in soil and water.

4.6 Influential observations in regression analyses

As noted in Chapter 3, the multiple regression analyses which indicated soil ammonium and total kjeldahl nitrogen as predictors of microcystins abundance were highly influenced by a relatively small number of observations. However, these influential observations are likely a feature of the system under study. It is important to note that the data sets that contained these highly influential points were found only within samples from water or soil found within arable land. I believe that this susceptibility to extreme values (likely due to external nutrient loading) is truly the defining difference between the two land uses, and as such, it is important to not exclude such values from statistical analyses.

4.7 The examination of small Saskatchewan water bodies for the presence of microcystins

Chapter 3 described a field study conducted during the summer months of 2009, which resulted in the collection of water and soil from 48 different water bodies located in either arable land or permanent pasture across three regions of southern and central Saskatchewan. A portion of the water collected was used for investigating whether microcystins and nodularin were present in these small water bodies, and, if so, whether there was a significant difference in concentration between water bodies located within arable land and those located within permanent pasture. To determine this, water collected from the field was tested using an HPLC-

MS/MS method adapted from Triantis et al. (2010) and a protein phosphatase inhibition assay (An and Carmichael et al., 1994) and compared to certified reference materials. Both methods of analysis detected microcystins in all water bodies studied regardless of surrounding land use. This indicates the possibility that low level concentrations of these toxins occur naturally in Saskatchewan. However, concentrations were significantly greater in water surrounded by arable land. Agricultural activity leading to increased nutrient concentrations is likely the cause of increased concentrations of microcystins in arable land water bodies.

4.8 Conclusions

Agricultural activity is very common in southern and central Saskatchewan, a province that also boasts 81 631 km^2 of water. Agricultural activity has frequently been implicated in the eutrophication of surface waters (Chow et al., 2011, Touchette et al., 2007, Camargo et al., 2006, Carpenter et al., 1998). Most water bodies in the present study could be classified as eutrophic – hypereutrophic (USEPA, 1996), regardless of surrounding land use. Water with such nutrient-rich conditions can promote cyanobacterial growth and toxin production, which is a hazard to human health, wildlife, domestic animals, and livestock. Microcystins were found in all water bodies in lesser concentrations, regardless of the surrounding land use. However, greater concentrations of microcystins in water surrounded by arable land may be due to nutrient enrichment resulting from fertilization practices. Some water bodies exhibited microcystin concentrations exceeding the WHO drinking water standard of 1 μg/L. This represents a potential hazard to human health, as well as to wildlife, domestic animals, and livestock. Regression analyses specifically implicated nitrogenous compounds in water and soil as predictors of microcystins abundance. While this is likely true, these systems are complex, and therefore microcystins abundance is not likely the result of concentrations of a single nutrient.

No detectable concentrations of saxitoxins were measured in small Saskatchewan water bodies in this study (Chapter 2), although results of a single year's sampling of 48 water bodies are not conclusive.

This research has provided insight into cyanotoxin abundance in Saskatchewan in relation to land use and nutrient enrichment. Although the results are based on a relatively small sample size, the conclusions may provide useful information for future risk management. For example, the findings that soil ammonium and water kjeldahl nitrogen concentrations are predictors of microcystins concentrations reinforces the adoption of agricultural practices such as low or no-till farming to minimize surface runoff of fertilizer to water bodies and avoidance of fertilizer application in the drainage basin of permanent water bodies, as well as fencing to minimize cattle entry into water bodies and subsequent contamination with manure and nitrogenous waste.

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APPENDIX A

2008 PILOT STUDY

Introduction

This research, supervised by Dr. Mark Wickstrom was originally the master's project of Amanda Hitchings, a graduate from the Toxicology undergraduate program. Amanda ran the first field season, which, due to lateness of funding was based on a design focused on historic livestock poisonings. Unfortunately, Amanda Hitchings decided to leave the program in the fall following her field season. At this time the author was already engaged as an undergraduate student on the project through the Toxicology 480 honors thesis research course. This project was rolled from the Toxicology 480 project into the author's master's research. Results of the first field season are included in this appendix because of differences in design, not differences in methods used in sampling and analysis. The problems inherit in a design where sampling is based on historical hotspots include difficulties with statistical analyses, inadequate sample size to compare land use effects, and potential confounding effects of variability in temperature, sunlight and water levels. It is also included in the appendix because the author did not take part in this work.

Materials and Methods

The original design of this project was to sample from sites that were deemed "historical hotspots". These hotspots were identified from information provided verbally by rural veterinarians located throughout southern Saskatchewan. A total of 65 rural clinics were contacted with less than 35% able to provide useful information on historical diagnoses of cyanobacterial poisonings. Sixteen sampling locations were chosen using the information from these rural veterinarians as well as information provided by individuals from various PFRA

pastures and from the Prairie Diagnostics Centre (Saskatoon, SK). These sites included a combination of lakes and small water bodies. 8 lakes containing 9 sampling sites were chosen (Table A.1). The remaining 7 sites (Table A.1) were dugouts or sloughs, and were named based on the landowner's name or the location

Table A.1 - Summary of historical hotspot sites and water body type sampled during summer 2008. Site specific descriptions given where available.

Site Name	Water Body Type	Site Notes
Moose Mountain Lake	Small bay off lake	Evidence of cyanobacteria along shore.
Pasqua Lake	Lake	Sampled off dock. Lots of green algae, possibly some cyanobacteria present.
Martin Pond 1	Slough	
Mission Lake	Small bay off lake	Site sheltered from wind. Possible cyanobacteria present.
Last Mountain Lake (Arlington Beach)	Lake	Sheltered marina off main lake area.
Last Mountain Lake (Regina Beach)	Lake	
Monet Site 1	Pasture Dugout	Filled with *Aphanizomenon.*
Focca 1	Dugout	Many aquatic plants present.
Martin Pond 2	Slough	
Staker Dugout	Dugout	Half covered in duckweed.
Burton Pond	Slough	Green algal bloom present at time of sampling.
Cowan Lake	Lake	*Aphanizomenon* present. Algae also present.
Ivan 1	Dugout/Slough	Arable land surrounded. Many aquatic plants present.
Katepwa Lake	Lake	No evidence of cyanobacteria.
Echo Lake	Lake	Lots of algae present, possibly some cyanobacteria.
Buffalo Pound Lake	Small bay off lake	No evidence of cyanobacteria. Site near campgrounds.

Water and soil sampling and analysis, HPLC-MS/MS and protein phosphatase inhibition assay analysis were performed as described in Chapter 3.

Results

Presence of hepatotoxins in historical cyanotoxin poisoning hotspots

Analysis for the determination of total toxin concentrations present using protein phosphatase inhibition assay concluded that all water bodies contained detectable concentrations of microcystin-LR equivalents (Table A.2). Concentrations ranged from 0.28 – 1.20 μg/L. Three water bodies out of the sixteen total sites (19%) exhibited concentrations of MC-LR above the WHO limit of 1μg/L MC-LR in water, while two additional water bodies contained just below that level.

Table A.2 Total microcystins concentrations for water bodies sampled in 2008. Values reported as MC-LR equivalents based on the protein phosphatase inhibition assay.

Site	Total MC-LR concentration (µg/L)
Moose Mountain Lake	1.03
Pasqua Lake	1.14
Martin Pond 1	0.39
Mission Lake	0.55
Last Mountain Lake (Arlington Beach)	0.32
Last Mountain Lake (Regina Beach)	0.43
Monet Site 1	0.50
Focca 1	0.31
Martin Pond 2	0.41
Staker Dugout	0.42
Burton Pond	1.20
Cowan Lake	0.99
Ivan 1	0.41
Katepwa Lake	0.28
Echo Lake	0.99
Buffalo Pound Lake	0.34

Specific hepatotoxin variants present in historical cyanotoxin poisoning hotspots

All 16 water bodies were analyzed for microcystin variants MC-LR, MC-RR, dmMC-LR and nodularin using high performance liquid chromatography tandem mass spectrometry. Calibration curves were created for all variants to which field water samples were compared. Instrument detection was determined to be 2.5 ppb for all variants. The detection limit for MC-RR is possibly lower than 2.5 ppb, but due to a lack of available standard, this could not be confirmed. Method detection limit (with solid phase extraction) was determined to be 63 ppt for

all variants. Of all samples analyzed only one sample (Pasqua Lake) contained a detectable concentration of any variant (74 ppt MC-LR).

Water chemistry parameters

Nutrient concentrations were quite variable among water bodies (Table A.3). Staker dugout and Monet 1 contained among the highest concentrations of chlorophyll-a, total kjeldahl nitrogen, soluble reactive phosphorus, total phosphorus and ammonia. Nitrate and nitrite were not observed due to a high limit of detection.

Table A.3 Summary of nutrient concentrations including NO_{3-} (nitrate), NO_{2-} (nitrite) NO_{3-} + NO_{2-} (nitrate plus nitrite), TKN (total kjeldahl nitrogen), SRP (soluble reactive phosphorus), TP (total phosphorus), NH_{4+} (ammonia) and Chl a (chlorophyll-a) as measured for all water bodies during summer 2008.

Site Name	Chl a (µg/L)	NO_{3-} (mg/L)	NO_{2-} (mg/L)	NO_{3-} + NO_{2-} (mg/L)	TKN (mg/L)	SRP (mg/L)	TP (mg/L)	NH_{4+} (mg/L)
Martin 1	2	<0.5	<0.05	<0.5	4.5	0.64	0.7	2.58
Martin 2	2	<0.5	<0.05	<0.5	3.8	0.99	1	0.44
Staker Dugout	390	<0.5	<0.05	<0.5	14	3.22	3.8	3.96
Monet 1	440	<0.5	<0.05	<0.5	6.2	0.04	0.6	0.11
Burton Pond	17	<0.5	<0.05	<0.5	1.9	0.74	0.9	0.12
Ivan 1	3	<0.5	<0.05	<0.5	4.9	1.08	1.2	0.24
Focca 1	5	<0.5	<0.05	<0.5	1.4	0.17	0.4	0.08
Cowan Lake	11	<0.5	<0.05	<0.5	3.1	0.05	<0.2	0.5
Moose Mountain Lake	110	<0.5	<0.05	<0.5	3	0.03	0.2	<0.05
Regina Beach	51	<0.5	<0.05	<0.5	1.6	0.1	0.2	<0.05
Buffalo Pound Lake	25	<0.5	<0.05	<0.5	0.8	0.03	<0.2	<0.05
Arlington Beach	14	<0.5	<0.05	<0.5	1.7	<0.02	<0.2	0.18
Pasqua Lake	72	<0.5	<0.05	<0.5	1.6	0.16	0.3	0.48
Echo Lake	71	<0.5	<0.05	<0.5	1.3	0.13	0.3	0.05
Mission Lake	54	<0.5	<0.05	<0.5	1.4	0.18	0.3	0.22
Katepwa Lake	64	<0.5	<0.05	<0.5	1.6	0.11	0.2	0.3

Water quality parameters

Water quality parameters were minimally variable between as well as within water bodies (Table A.4). Depth ranged from 1 – 4 m, but was not correlated significantly with any water quality parameters.

Table A.4 Summary of water quality parameters as measured for all water bodies during summer 2008. Values expressed as mean ± standard deviation of the mean where applicable.

Site Name	Depth (m)	Water Temperature (˚C)	Conductivity (mS/cm)	Dissolved Oxygen (mg/L)	pH	Turbidity (NTU)
Martin 1	1	14.82 (0.01)	2.67 (0.001)	6.89 (0.06)	8.25 (0.02)	2.13 (2.59)
Martin 2	1	15.10 (0.06)	1.18 (0.001)	9.65 (0.12)	8.92 (0.01)	0.97 (9.87)
Staker Dugout	2.75	11.42 (0.20)	1.35 (0.001)	0.81 (0.55)	7.69 (0.01)	22.4 (0.58)
Monet 1	2.75	12.44 (0.03)	0.39 (0)	11.4 (0.08)	9.52 (0)	39.5 (7.28)
Burton Pond	2.5	15.67 (0.40)	0.69 (0.003)	9.64 (0.52)	9.06 (0.01)	11.7 (0.38)
Ivan 1	4	12.94 (0.20)	1.96 (0.001)	8.40 (0.02)	8.67 (0.02)	1.88 (0.59)
Focca 1	1	11.26 (1.74)	0.24 (0.01)	3.90 (1.76)	9.15 (0.73)	9.23 (6.34)
Cowan Lake	2.12	14.90 (0.10)	0.90 (0)	6.68 (0.18)	8.10 (0.09)	7.18 (2.01)
Moose Mountain Lake	1	13.02 (0.04)	1.00 (0.001)	8.04 (0.18)	9.23 (0.06)	32.5 (1.69)
Regina Beach	2.4	15.33 (0.46)	2.07 (0.004)	12.2 (0.31)	9.07 (0.03)	5.80 (0.87)
Buffalo Pound Lake	2.25	14.60 (0.09)	0.47 (0.002)	9.26 (0.04)	8.32 (0.14)	3.88 (0.10)
Arlington Beach	1	14.36 (0.17)	2.42 (0.002)	6.76 (0.07)	8.28 (0.03)	72.1 (138)
Pasqua Lake	1.3	16.99 (0.02)	1.46 (0.001)	9.44 (0.07)	8.45 (0.06)	15.7 (2.99)
Echo Lake	1.35	14.76 (2.30)	1.76 (0.001)	14.2 (0.06)	8.87 (0.02)	14.4 (0.66)
Mission Lake	1.6	16.72 (0.43)	1.75 (0.001)	10.4 (0.19)	8.72 (0.02)	7.15 (1.12)
Katepwa Lake	2.5	15.92 (0.01)	1.61 (0.001)	7.84 (0.04)	8.61 (0.02)	6.10 (0.47)

Discussion

This 2008 study supports the work done by the author in Ch. 3 in concluding that low level concentrations of cyanobacterial hepatotoxins may be common in small (and possibly large) water bodies in the late summer months in southern Saskatchewan. Although this conclusion was quite clear in the earlier chapter, it is less so here because of the nature of the experimental design. It can only be speculated that microcystins are common in larger water bodies, as the sampling design in this study is not representative of these water bodies as a whole. More research will need to be done (ideally on an annual basis) on larger Saskatchewan lakes in order to confirm this conclusion.

Interestingly, although the sampling design was not ideal, it was the larger, lake-type water bodies that collectively had higher concentrations of MC-LR in comparison to the smaller dugout-type water bodies, although nutrient levels were almost exclusively higher in the smaller dugout water bodies as compared to the lakes. Again, it must be noted that these observations are slightly superficial, as the water bodies deemed "lakes" were not sampled in a manner that allows them to be representative of these lakes.

Unfortunately, the HPLC-MS/MS work in this study did not correlate well with the protein phosphatase inhibition assay. While the assay detected microcystins in all water bodies, the HPLC-MS/MS work only detected a single variant (MC-LR) in one water body (Pasqua Lake). While these two methods are commonly used together in the study of cyanobacterial toxins, they do not always correlate well. As indicated in an earlier chapter, this could be simply due to the fact that while the protein phosphatase inhibition assay detects all possible microcystin variants, HPLC-MS/MS will only detect those reference materials it has been optimized to detect.

Conclusions

This study was based on a monitoring-type approach in which historical hotspots were investigated for the presence of cyanobacterial hepatotoxins. While these toxins were readily identified, the experimental design did not lend itself well to statistical comparisons. While it is interesting that cyanotoxins were once again identified in these "hotspots," more work is needed to determine whether this is due to specific characteristics of these sites, or whether it is simply because low level concentrations of cyanobacterial hepatotoxins are common in water bodies throughout southern Saskatchewan.

APPENDIX B

CYANOBACTERIAL IDENTIFICATION

Blooms of cyanobacteria can occur, but not produce measureable concentrations of toxins. Composite integrated water samples preserved in 10% formalin were examined microscopically for the presence of five cyanobacterial genera (*Microcystis, Anabaena, Oscillatoria, Aphanizomenon* and *Lyngbya*) commonly associated with clinical poisonings worldwide and widely distributed in freshwater environments (Lee et al., 2008).The aim of this pilot study was to improve our understanding of the abundance of these organisms in small Saskatchewan water bodies.

Quantitative determination of the abundance of several toxic cyanobacterial genera in water samples using the standard Utermöhl method is a labour-intensive procedure requiring significant training to master. The amount of time and effort required was considered to be beyond the scope of this project, and contracting the analysis out was cost prohibitive. Therefore, a simple, qualitative method was developed for identification. As the identification of cyanobacteria/algae is often considered more of an art form than a science, a number of different references were combined and used as a key (Graham et al., 2009, Huynh and Serediak et al., 2006, Lee et al., 2008, Prescott, 1970, Sheath and Wehr et al., 2003).

Four slides per water body (40 µL of formalin fixed water per slide) were examined for the presence or absence of the genera of cyanobacteria being investigated. Two slides were prepared from each of the two Boston bottles collected per water body. The 40 µL subsample examined was collected from the bottom of the Boston bottle in order to examine as much organic material as possible. A wet mount was prepared for each subsample, with slides systematically scanned using a compound microscope at 400X magnification. Slides were

scanned from the top left corner to the bottom right corner with a maximum time of 10 min per slide.

Although the method adopted was only qualitative, examination of water samples revealed all five genera of potentially toxigenic cyanobacteria in many water bodies, in both land uses and all regions. Unfortunately, due to limited resources and the relative inexperience of the technician providing the analysis, no firm conclusions could be made from this work, but the tentative conclusions support the observation that cyanobacterial hepatotoxins were detected in virtually all water samples using both HPLC –MS/MS and the protein phosphatase inhibition assay.

APPENDIX C

ADDITIONAL DATA TABLES

Table B.1 Summary of water nutrient concentrations measured in water bodies surrounded by arable land (A.L.) and permanent pasture (P.P.) in the Aberdeen study region during early and late summer 2009 (n = 8). Values expressed as mean ± standard deviation of the mean.

Analyte	Early Summer (July/August)		Late Summer (August/September)	
	P.P.	A.L.	P.P.	A.L.
n	8	8	8	8
Total Phosphorus (mg/L)	0.45 ± 0.38	0.60 ± 0.36	0.17 ± 0.22	0.49 ± 0.28
Soluble Reactive Phosphorus (mg/L)	0.162 ± 0.244	0.449 ± 0.333	0.137 ± 0.165	0.418 ± 0.348
Nitrate (mg/L)	< 0.50	< 0.50	< 0.50	< 0.50
Nitrite (mg/L)	< 0.050	< 0.050	< 0.050	< 0.050
Total Kjeldahl Nitrogen (mg/L)	4.42 ± 1.17	4.27 ± 1.20	5.06 ± 1.92	4.63 ± 1.63
Ammonia (mg/L)	0.413 ± 0.719	0.125 ± 0.073	0.224 ± 0.247	0.285 ± 0.418

Table B.2 Summary of water nutrient concentrations measured in water bodies surrounded by arable land (A.L.) and permanent pasture (P.P.) in the Elrose study region during early and late summer 2009 (n = 8). Values expressed as mean ± standard deviation of the mean.

Analyte	Early Summer (July/August)		Late Summer (August/September)	
	P.P.	A.L.	P.P.	A.L.
n	8	8	8	8
Total Phosphorus (mg/L)	0.30 ± 0.27	1.1 ± 0.94	0.35 ± 0.18	1.1 ± 0.94
Soluble Reactive Phosphorus (mg/L)	0.082 ± 0.147	0.559 ± 0.485	0.221 ± 0.327	0.559 ± 0.485
Nitrate (mg/L)	< 0.50	< 0.50	< 0.50	< 0.50
Nitrite (mg/L)	< 0.050	< 0.050	< 0.050	< 0.050
Total Kjeldahl Nitrogen (mg/L)	5.82 ± 3.65	10.6 ± 10.3	5.11 ± 2.75	10.6 ± 10.3
Ammonia (mg/L)	0.116 ± 0.096	0.684 ± 1.18	0.137 ± 0.061	0.684 ± 1.18

Table B.3 Summary of water nutrient concentrations measured in water bodies surrounded by arable land (A.L.) and permanent pasture (P.P.) in the Swift Current study region during early and late summer 2009 (n = 7-8). Values expressed as mean ± standard deviation of the mean.

Analyte	Early Summer (July/August)		Late Summer (August/September)	
	P.P.	A.L.	P.P.	A.L.
n	8	8	7	8
Total Phosphorus (mg/L)	0.91 ± 1.1	0.71 ± 0.69	0.48 ± 0.37	1.1 ± 1.3
Soluble Reactive Phosphorus (mg/L)	0.334 ± 0.555	0.716 ± 1.09	0.269 ± 0.419	1.00 ± 1.23
Nitrate (mg/L)	< 0.50	< 0.50	< 0.50	< 0.50
Nitrite (mg/L)	< 0.050	< 0.050	< 0.050	< 0.050
Total Kjeldahl Nitrogen (mg/L)	11.4 ± 11.5	5.01 ± 2.44	7.59 ± 3.98	7.12 ± 4.81
Ammonia (mg/L)	2.04 ± 3.65	0.357 ± 0.345	0.492 ± 0.580	0.428 ± 0.503

Table B.4 Water quality parameters and physical factors measured for water bodies surrounded by arable land (A.L.) and permanent pasture (P.P.) in the Aberdeen study region during early and late summer 2009. Values expressed as mean ± standard deviation of the mean.

Analyte	Early Summer (July/August)		Late Summer (August/September)	
	P.P.	A.L.	P.P.	A.L.
n	8	8	8	8
Depth (m)	1.27 ± 0.67	1.2 ± 0.65	1.19 ± 0.58	1.07 ± 0.56
Water Temperature (˚C)	18.0 ± 1.79	19.1 ± 1.76	15.7 ± 1.79	19.0 ± 1.08
Conductivity (mS/cm)	1.64 ± 2.08	1.58 ± 0.53	1.84 ± 2.52	1.68 ± 0.69
Dissolved Oxygen (mg/L)	7.33 ± 2.41	9.68 ± 2.05	8.52 ± 1.78	9.88 ± 1.63
pH	8.61 ± 1.07	8.82 ± 0.31	8.73 ± 0.45	9.06 ± 0.41
Air Temperature (˚C)	18.3 ± 3.88	21.4 ± 3.78	27.5 ± 4.14	23.1 ± 4.26

Table B.5 Water quality parameters and physical factors measured for water bodies surrounded by arable land (A.L.) and permanent pasture (P.P.) in the Elrose study region during early and late summer 2009. Values expressed as mean ± standard deviation of the mean.

Analyte	Early Summer (July/August)		Late Summer (August/September)	
	P.P.	A.L.	P.P.	A.L.
n	8	8	8	8
Depth (m)	1.67 ± 0.80	0.98 ± 0.88	1.57 ± 0.86	0.82 ± 0.81
Water Temperature (˚C)	18.7 ± 0.55	17.8 ± 2.41	16.6 ± 0.60	18.0 ± 1.70
Conductivity (mS/cm)	0.55 ± 0.38	9.31 ± 17.89	0.58 ± 0.44	11.0 ± 21.92
Dissolved Oxygen (mg/L)	6.92 ± 3.03	9.64 ± 4.61	5.96 ± 1.29	9.44 ± 4.64
pH	8.75 ± 0.82	8.72 ± 0.66	8.97 ± 0.58	8.99 ± 0.34
Air Temperature (˚C)	21.3 ± 3.65	19.4 ± 2.97	18.9 ± 4.97	24.5 ± 3.82

Table B.6 Water quality parameters and physical factors measured for water bodies surrounded by arable land (A.L.) and permanent pasture (P.P.) in the Swift Current study region during early and late summer 2009. Values expressed as mean ± standard deviation of the mean.

Analyte	Early Summer (July/August)		Late Summer (August/September)	
	P.P.	A.L.	P.P.	A.L.
n	8	8	7	8
Depth (m)	1.45 ± 0.67	1.89 ± 1.28	1.34 ± 0.63	1.51 ± 1.00
Water Temperature (˚C)	18.7 ± 1.17	18.9 ± 1.42	18.0 ± 0.69	17.34 ± 1.03
Conductivity (mS/cm)	8.62 ± 11.11	6.09 ± 6.85	9.83 ± 11.60	7.37 ± 8.55
Dissolved Oxygen (mg/L)	6.33 ± 6.24	7.81 ± 2.04	6.06 ± 5.25	5.82 ± 1.41
pH	8.49 ± 0.57	8.63 ± 0.60	8.38 ± 0.53	8.56 ± 0.38
Air Temperature (˚C)	23.13 ± 2.17	22.6 ± 5.15	26.3 ± 4.75	20.6 ± 4.10

Table B.7 Measurements of photosynthetic pigments found within cyanobacteria, cyanotoxin abundance, and specific microcystins and nodularin variants detected in water bodies located within arable land (A.L.) and permanent pasture (P.P.) in the Aberdeen study region during early and late summer 2009. Values expressed as mean ± standard deviation of the mean.

Analyte	Early Summer (July/August)		Late Summer (August/September)	
	P.P.	A.L.	P.P.	A.L.
n	8	8	8	8
Chlorophyll-a (mg/L)	17.3 ± 20.1	11.9 ± 13.9	33.3 ± 43.2	13.7 ± 19.9
Phycocyanin (cells/mL)	2240 ± 844.4	2657 ± 1745	2793 ± 1852	1652 ± 468.9
Total Microcystins (µg/L)	0.43 ± 0.069	0.46 ± 0.22	0.41 ± 0.059	0.61 ± 0.33
MC-LR (ng/mL)	0.03 ± 0.02	0.03 ± 0.02	0.02 ± 0.02	0.01 ± 0.02
MC-RR (ng/mL)	0	0.002 ± 0.004	0.007 ± 0.004	0.02 ± 0.03
dmMC-LR (ng/mL)	0.03 ± 0.01	0.03 ± 0.005	0.03 ± 0.01	0.03 ± 0.01
NOD (ng/mL)	$3 \times 10^{-6} \pm 7 \times 10^{-6}$	0	0	0
Total Variants (ng/mL)	0.06 ± 0.03	0.06 ± 0.02	0.06 ± 0.03	0.17 ± 0.28

Table B.8 Measurements of photosynthetic pigments found within cyanobacteria, cyanotoxin abundance, and specific microcystins and nodularin variants detected in water bodies located within arable land (A.L.) and permanent pasture (P.P.) in the Elrose study region during early and late summer 2009. Values expressed as mean ± standard deviation of the mean.

Analyte	Early Summer (July/August)		Late Summer (August/September)	
	P.P.	A.L.	P.P.	A.L.
n	8	8	8	8
Chlorophyll-a (mg/L)	173 ± 262	80.7 ± 104	92.2 ± 144	199 ± 360
Phycocyanin (cells/mL)	26832 ± 55023	10666 ± 11844	20523 ± 42738	21883 ± 34925
Total Microcystins (µg/L)	0.47 ± 0.50	0.43 ± 0.13	0.39 ± 0.17	0.82 ± 0.60
MC-LR (ng/mL)	0.03 ± 0.004	0.03 ± 0.007	0.03 ± 0.01	0.04 ± 0.04
MC-RR (ng/mL)	0.001 ± 0.003	0.003 ± 0.004	0.005 ± 0.005	0.007 ± 0.007
dmMC-LR (ng/mL)	0.03± 0.008	0.03 ±0.008	0.03 ±0.004	0.03 ± 0.01
NOD (ng/mL)	0	0	0.001 ± 0.004	0
Total Variants (ng/mL)	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.05

Table B.9 Measurements of photosynthetic pigments found within cyanobacteria, cyanotoxin abundance, and specific microcystins and nodularin variants detected in water bodies located within arable land (A.L.) and permanent pasture (P.P.) in the Swift Current study region during early and late summer 2009. Values expressed as mean ± standard deviation of the mean.

Analyte	Early Summer (July/August)		Late Summer (August/September)	
	P.P.	A.L.	P.P.	A.L.
n	8	8	7	8
Chlorophyll-a (mg/L)	146 ± 201	33.9 ± 41.3	74.0 ± 78.2	39.7 ± 27.6
Phycocyanin (cells/mL)	25021 ± 33464	4324 ± 3819	15877 ± 17657	6173 ± 2300
Total Microcystins (µg/L)	0.46 ± 0.21	0.53 ± 0.29	0.52 ± 0.18	0.80 ± 0.44
MC-LR (ng/mL)	0.03 ± 0.01	0.05 ± 0.04	0.03 ± 0.01	0.2 ± 0.3
MC-RR (ng/mL)	0.006 ± 0.005	0.003 ± 0.004	0	0.005 ± 0.005
dmMC-LR (ng/mL)	0.03 ± 0.004	0.04 ± 0.02	0.03 ± 0	0.1 ± 0.2
NOD (ng/mL)	0	0	0	0
Total Variants (ng/mL)	0.06 ± 0.01	0.09 ± 0.05	0.06 ± 0.01	0.26 ± 0.37