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TEMPORAL AND SPATIAL TRENDS IN TOXIC CYANOBACTERIA AS IDENTIFIED THROUGH LAKE SEDIMENT DNA

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ABSTRACT

Cyanobacterial and algal blooms can negatively affect water quality particularly when producing toxins that affect human health and wildlife. While reports of blooms are on the rise globally, their underlying causes remain unclear. The goal of this thesis was to determine temporal changes in cyanobacterial abundance and composition through sediment cores in relation to (1) altered land-use leading to cultural eutrophication and (2) warmer air temperatures that have been recorded in the past few decades. This involved evaluating the use of DNA extracted from lake sediments to quantify cyanobacterial abundance and composition.

Lake sediments preserved under appropriate storage conditions showed the potential to yield high quality DNA for downstream molecular applications. Cyanobacteria, quantified using the 16S rRNA gene, were found to have increased over the last three decades in comparison to historical averages (since the 1850s) both inside and outside a protected area in western Quebec, Canada, in concert with recent regional warming. Copy numbers of 16S rRNA genes specific to cyanobacteria largely correlated to temporal trends in cyanobacterial pigments. Larger percent increases were seen in cyanobacterial genes in recent times compared to changes in the eubacterial glutamine synthetase (*glnA*) gene. The *mcyD* gene was quantified as a proxy for microcystin production through sediment cores from two lakes of western Canada. Copy numbers of both *mcyD* and *Microcystis* 16S rRNA correlated with chemical analyses of microcystin through time in cores. Cyanobacteria in the more eutrophic of these lakes shifted toward less diverse assemblages and more toxigenic taxa in recent years. Lastly, temporal and spatial changes in cyanobacterial diversity were analyzed through pyrosequencing of cyanobacterial 16S rRNA along a latitudinal transect representative of northern Canada. Significant shifts towards less diverse assemblages were found, composed of potentially toxigenic strains, suggestive of climate warming in northern latitudes. These results support recent reports of increased abundance and geographic expansion of cyanobacteria and point to increases in cyanotoxins in some cases. Using DNA archived in sediments to determine the historical state of cyanobacterial abundance and diversity could help inform lake management policies.

RÉSUMÉ

Les cyanobactéries et les proliférations d'algues peuvent nuire à la qualité de l'eau en particulier quand elles produisent des toxines qui affectent la santé humaine et la faune. Alors que les incidences de proliférations semblent à la hausse à l'échelle mondiale, les causes restent indéterminées. L'objectif de cette thèse était de déterminer les changements temporels dans l'abondance et la composition des cyanobactéries par des carottes de sédiments en fonction de (1) les activités anthropiques conduisant à l'eutrophisation et (2) la tendance au réchauffement de l'air qui a été enregistrée au cours des dernières décennies. Cette thèse évalue le potentiel de l'ADN conservé dans les sédiments de lac à quantifier l'abondance et la diversité des cyanobactéries.

Les sédiments conservés dans des conditions de stockage appropriées ont montré un potentiel pour l'obtention d'ADN de haute qualité à des fins d'applications moléculaires. Les cyanobactéries, quantifiées en utilisant le gène d'ARNr 16S, ont augmenté au cours des trois dernières décennies par rapport aux moyennes historiques (depuis environ 1850) tant à l'intérieur qu'à l'extérieur d'un espace protégé dans l'ouest du Québec (Canada) en parallèle avec le réchauffement régional. Le nombre de copies de gènes d'ARNr 16S spécifiques aux cyanobactéries était corrélé avec les concentrations en pigments de cyanobactéries. Les gènes de cyanobactéries ont augmenté relativement plus rapidement dans les années récentes en comparaison avec les variations plus stables du gène universel eubactérien de la glutamine synthétase (*glnA*). Le gène *mcyD* a été quantifié comme un indice de la production des microcystines à travers des carottes de sédiments de deux lacs de l'ouest du Canada. Les nombres de copies de *mcyD* et d'ARNr 16S de *Microcystis* ont été corrélés avec des analyses chimiques de microcystines à travers les carottes. Les cyanobactéries dans le lac le plus eutrophique étaient moins diversifiées et comprenaient plus de taxons toxigène dans les sédiments récents. Enfin, les changements temporels et spatiaux dans la diversité des cyanobactéries ont été analysés par pyroséquençage de l'ARNr 16S des cyanobactéries au long d'un transect représentant un gradient latitudinal vers le pôle Nord. Des changements significatifs vers des communautés moins diversifiées et des souches potentiellement toxigènes ont été trouvés; en apparence due au réchauffement climatique. Ces résultats confirment les rapports récents de l'augmentation de l'abondance et de l'expansion géographique des cyanobactéries et démontrent une hausse de cyanotoxines dans certains cas. L'analyse de l'ADN conservé dans les sédiments pour déterminer l'état historique des cyanobactéries pourrait aider à concevoir des politiques de gestion pour la protection du milieu lacustre dans l'avenir.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ASE	accelerated solvent extraction
CFCS	Constant Flux Constant Sedimentation
CIC	Constant Initial Concentration
C_q	quantification cycle
CRS	Constant Rate of Supply
CYAN	16S rRNA gene primers specific to cyanobacteria
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent analysis
glnA	glutamine synthetase gene
GSL	Great Slave Lake
HPLC	High Performance Liquid Chromatography
IPCC	Intergovernmental Panel on Climate Change
LOI	Loss on Ignition
MC	Microcystin
mcy	microcystin-synthesizing gene cluster
MICR	16S rRNA gene primers specific to *Microcystis*
MS	mass spectrometer
NGS	Next Generation Sequencing
NPRS	non-ribosomal peptide synthetase
ORF	open reading frame
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PKS	polyketide synthase
QIIME	Quantitative Insights Into Microbial Ecology (software)
Qpcr	quantitative Polymerase Chain Reaction
RDP	Ribosomal Database Project
SDS	sodium dodecyl sulfate
TKN	total Kjeldahl nitrogen
TP	total phosphorous
T-RFLP	Terminal Restricting Fragment Length Polymorphism

CHAPTER 1: GENERAL INTRODUCTION

1.1 Cyanobacteria in the face of environmental change

Cyanobacteria are the oldest phototrophic organisms on the planet and are important primary producers in aquatic ecosystems (Whitton and Potts 2012). They are found in a broad range of habitats including fresh and marine water bodies, microbial polar mats, and deserts. When they grow profusely and accumulate in surface waters, they may lead to visible blooms. Cyanobacterial and algal blooms are natural occurrences, but in many lakes their intensity and frequency have become a major problem because of the negative effects on water quality (e.g. Reynolds and Walsby 1975; Smith 2003). The increase in blooms is in part due to enhanced nutrient loading (especially phosphorus) into lakes from anthropogenic activities, otherwise known as cultural eutrophication (Schindler 2006). The link between phosphorus and cyanobacterial growth has been well established for some time (e.g. Schindler 1974; Pick and Lean 1987). Seasonally, in temperate regions, cyanobacteria tend to dominate over other phytoplankton during warmer temperatures and greater water column stability (Sommer *et al.* 1986; Jöhnk *et al.* 2008; Paerl and Huisman 2008). Effects of cyanobacterial blooms include oxygen depletion, toxic metabolites, taste and odour problems for drinking water, and decreased light penetration for other phototrophs (Skulberg *et al.* 1984).

Reports of cyanobacterial blooms have increased, even in temperate lakes historically not subject to blooms (LeBlanc *et al.* 2008; Winter *et al.* 2011). This has brought the role of climate warming into question, especially in combination with nutrient loading from human activities (Beaulieu *et al.* 2013; Rigosi *et al.* 2014). Warmer temperatures, predicted by the Intergovernmental Panel on Climate Change

(IPCC 2013), and longer growing seasons in temperate regions are likely to favour increases in cyanobacteria (Taranu *et al.* 2012; Beaulieu *et al.* 2013; Deng *et al.* 2014).

Characteristics of Planktonic Cyanobacteria

A number of physiological and morphological traits may enable cyanobacteria to dominate over other phytoplankton in response to increased nutrient loading and a changing climate (Markensten *et al.* 2010; O'Neil *et al.* 2012). For example, their size variation alone confers a range in nutrient uptake and use. The picocyanobacteria (0.2-2 µm) are ubiquitous but tend to dominate phytoplankton in oligotrophic systems because of their small size and high surface: volume ratio, (Pick 2000; Callieri 2002). Large and sometimes macroscopic colonial taxa, which have nutrient storage capacities, tend to dominate phytoplankton when lake nutrient levels increase (Watson 1992).

Heterocystous cyanobacteria of the order Nostocales can thrive when the N: P (nitrogen: phosphorus) ratio is low, due to their ability to fix atmospheric nitrogen (Schindler 1977). Anthropogenic sources of nutrients are numerous. In the case of sewage, the ratio of N:P in effluents is quite low, which could benefit N-fixing taxa. Generally, high nutrient concentrations are associated with low N:P ratios (Downing *et al.* 2001) thus potentially favouring N-fixing cyanobacteria.

Cyanobacteria of the genus *Microcystis* and other buoyant planktonic bloom-formers possess gas vesicles that allow them to rise to the surface of water columns, particularly when conditions are stable (Jöhnk *et al.* 2008). A longer growing season, which is one consequence of a warming climate in temperate regions, could further promote the growth of buoyant cyanobacteria (Paerl and Huisman 2008). Climate change also includes effects on precipitation patterns.

Cyanobacteria in the order Nostocales including the genera *Anabaena*, *Cylindrospermopsis*, and *Nodularia* form thick-walled resting cysts (akinetes) during unfavourable conditions (Adams and Duggan 1999), which can persist in sediments. This trait would give such cyanobacteria a competitive advantage during times of drought, which are projected in some regions (Carey *et al.* 2012).

Cyanotoxins

Cyanobacteria produce a number of unique metabolites, which may provide a competitive advantage over other phytoplankton, in addition to the traits mentioned earlier. One group of such metabolites are known as cyanotoxins. The purpose of cyanotoxin synthesis and the environmental factors involved remain somewhat unclear. Several reasons for the production and release of cyanotoxins are plausible including defense against grazers, allelopathic interactions, and intercellular signalling (Holland and Kinnear 2013). Both acute and chronic toxicity have been associated with many cyanotoxins (Funari and Testai 2008). When ingested by vertebrates, toxins can be hepatotoxic (such as microcystins or nodularin) or neurotoxic (such as anatoxins) or can also act as dermal irritants (Chorus and Bertram 1999).

Microcystins compose the most ubiquitous group of cyanotoxins found in freshwater systems. There are 80 different isoforms reported that may differ in their toxicity (Meriluoto 2008). Microcystins can be chemically quite stable and persist in water columns and sediments for months (Lahti 1997; Zastepa *et al.* 2014). As they specifically target the liver, they can lead to liver diseases through acute exposure. Examples of biota that have been exposed to microcystin-contaminated water include fish species (Malbrouck and Kestemont 2006; Puerto *et al.* 2011), amphibians (Dvořáková *et al.* 2002), rhinoceros (Oberholster *et al.* 2009), and sea otters (Miller *et al.* 2010). In 1996, dialysis patients in Brazil were exposed to microcystin through water given intravenously which eventually led to 52 deaths (Jochimsen *et al.* 1998). Chronic exposure suggests that there are potential carcinogenic properties of microcystins (Hernández *et al.* 2009; Svirčev *et al.* 2010). In 2003, concern for exposure lead the World Health Organization to set a drinking water guideline at 1.0 µg/L for microcystin-LR, which is one of the most prevalent and well-studied isoforms (World Health Organization 2003).

Microcystins are produced by non-ribosomal peptide synthetases (NRPS) coupled with polyketide synthases (PKS) known as the microcystin synthetase (Dittmann *et al.* 2001). The microcystin synthetase gene (*mcy*) is present in all strains of cyanobacteria that are known producers of microcystin (Neilan *et al.* 1999) and is fully sequenced (Tillett *et al.* 2000). The *mcy* gene is a 55-kb gene cluster with 10 open reading frames (ORFs): six large ORFs (*mcyA-E* and *G*) and four smaller ORFs (*mcyF* and *H-J*). The six large ORFs are responsible for eventual NRPS/PKS translation while the four smaller ORFs have tailoring functions (Tillett *et al.* 2000). The *mcy* gene is considered constitutive, meaning that if the gene is present in a strain, it is expressed (Kaebernick *et al.* 2000; Ngwa *et al.* 2014).

The larger gene fragments of the *mcy* gene cluster (A through E) have been detected and quantified in water samples in the course of studying potentially toxigenic cyanobacteria and microcystin dynamics (Ouahid *et al.* 2005; Mankiewicz Boczek *et al.* 2006). Significant correlations between gene copy numbers of *mcyD* in the water column with concentrations of microcystin have been reported (Fortin *et al.* 2010; Druga *et al.* 2013; Pimental and Giani 2013). Transcription of the gene has been shown to be related to growth rate in culture-based studies (Renaud *et al.* 2011; Ngwa *et al.* 2014).

1.2 Sediments as historical archives

Modern monitoring records typically do not extend far enough back in time to pre-date effects of anthropogenic stressors that have intensified in recent decades (Smol 2010). The onset of significant cultural eutrophication varies from one region to another depending on human settlement and changes in land-use such as increased agriculture. The condition of lakes prior to increased nutrient loading usually cannot be determined using modern monitoring records. The same problem arises when assessing the impact of warmer air temperatures, which have been higher in the last three decades (since 1980) than the global climate normals set prior to this time (IPCC 2013). Sediment cores taken from lakes can be used to hindcast temperature, nutrient concentrations, and abundance of certain preserved organisms to reveal the pristine or pre-anthropogenic state of a lake system (Smol 2008). The length of time represented by a sediment core depends on the coring method and the sedimentation rate of a particular site. Data gleaned from sediment cores can be used to determine associations between cyanobacterial blooms and physiochemical variables and help predict changes in cyanobacterial abundance in relation to future environmental conditions. Paleolimnological data can be used to inform lake management practices when striving to return a lake to pristine or pre-disturbance conditions (e.g. Adams *et al.* 2014; Moos *et al.* 2014).

Organisms that preserve well in sediments are most often used as proxies of environmental variables in sediment analyses. Diatoms, in particular, are well-preserved due to their silica frustules and are frequently used as indicators of phosphorus concentrations and pH levels (e.g. Dixit 1999). Similarly, chironomid head capsules can be used to infer past oxygen conditions (e.g. Frossard *et al.* 2013) and pollen can be used to infer climatic conditions (e.g. Gajewski and Atkinson 2003). Akinete-forming cyanobacteria are useful in paleolimnology and can be detected and quantified using microscopy or through germination tests (van Geel *et al.* 1994; Eilers *et al.* 2004; Wood *et al.* 2009). Preserved akinetes can be used to approximate the onset of cultural eutrophication of lakes, even as far back as ~6000 years before present (Bradbury *et al.* 2004; Hillbrand *et al.* 2014). Cyanobacteria have also been tracked using carotenoid pigments, some of which are indicative of total cyanobacteria and other pigments which are markers of particular orders (Leavitt and Findlay 1994; Leavitt and Hodgson 2001). Using fossil pigments, temporal changes in phytoplankton community composition have been documented in numerous lakes (e.g. Watanabe *et al.* 2012) along with the dominance of cyanobacteria over other phytoplankton (Overmann *et al.* 1993). Cyanobacterial pigments can also aid in characterizing the effect of anthropogenic stressors on lake systems, especially if the core pre-dates the onset of significant human impact (Deshpande *et al.* 2014).

DNA in sediments

Many organisms do not leave fossil remains behind in sediments and therefore cannot be detected through down core analyses using morphological criteria. Pigments of algae and cyanobacteria are useful proxies in paleolimnology but often co-elute, making it difficult to distinguish certain algal groups apart (Leavitt and Hodgson 2001). Assessing temporal trends in species composition using DNA extracted from sediment cores is a potential way to circumvent these issues. Many of the studies that have conducted DNA analyses have been based on cores from Antarctic lakes which are composed of anoxic and cold sediments - ideal storage conditions for DNA (Anderson-Carpenter *et al.* 2011). In these lakes, sedimentation rates are low, meaning that retrieved cores may date back thousands of years in time. Due to faster sedimentation rates, cores of similar length from temperate and tropical lakes do not date back as far as cores from polar regions. Few studies of temperate/tropical lakes have employed sediment DNA, but due to advances in molecular biology methods, applications are increasing. Examples of DNA extracted from sediment cores of various lengths and of both prokaryotes and eukaryotes are presented in Table 1.1. Cyanobacterial DNA has only been extracted from a few dated sediment cores thus far.

Once extracted, DNA can be used to determine the presence or absence of a particular gene (through PCR) or the absolute quantity based on gene copy numbers, through (quantitative PCR). Quantitative real-time PCR (qPCR) has been widely used in the field of environmental microbiology as a rapid, effective method for measuring the abundance of particular microbes (Heid *et al.* 1996; Smith and Osborn 2009). Most of the molecular work involving cyanobacterial genes has been conducted on water samples (reviewed by Ouellette and Wilhelm 2003; Pearson and Neilan 2008). Developing molecular methods for aquatic sciences may enable detection of potentially toxigenic cyanobacteria more efficiently than conventional microscopic methods (e.g. Fortin *et al.* 2010; Bukowska *et al.* 2014) and also eliminate potential user bias. A small number of recent studies have estimated cyanobacterial abundance in sediments along down core profiles using qPCR (Savichtcheva *et al.* 2011; Ye *et al.* 2011; Domaizon *et al.* 2013).

Cyanobacterial genes

A number of molecular probes for specific genes have been developed to study the phylogeny, biogeography, and taxonomy of cyanobacteria (Moreira *et al.* 2013). The 16S rRNA gene has been the most frequently used in these studies and the primers specific to the cyanobacterial 16S rRNA gene were originally designed almost 20 years ago (Nübel *et al.* 1997). Researchers have modified these primers since then for use in qPCR of DNA from sediments (Rinta-Kanto *et al.* 2005; Ye *et al.* 2009; Ye *et al.* 2011). The 16S rRNA gene has been considered the "gold standard" for phylogenetic analyses within the bacterial kingdom due to its conservation in all bacteria (Woese 1987). Because of this, a large number of sequences for the gene are publicly available (e.g. GenBank, European Nucleotide Archive) furthering the popularity of this gene in studies of diversity. The 16S rRNA gene is present as a multi-copy gene in all prokaryotes as it codes for a vital component of the bacterial ribosome (Sun *et al.* 2013). Intragenomic heterogeneity of the multiple copies of this gene does not seem significant at taxonomic levels above species and subspecies, when compared to the single-copy gene *rpoB*, which has also been evaluated for use in cyanobacterial taxonomy (Gaget *et al.* 2011). The *rpoB* gene codes for the RNA polymerase β subunit and is used as a core housekeeping gene (Case *et al.* 2007). The *cpc* gene, which codes for phycocyanin, a major accessory photosynthetic pigment of cyanobacteria, has also been used to estimate total cyanobacterial abundance (Li *et al.* 2011). Cyanobacterial RNA has also been recently quantified through a down-core approach (Savichtcheva *et al.* 2015) but showed low copy numbers through the core, illustrating the lack of activity of cyanobacteria preserved in sediments.

Genes specific to particular cyanobacteria can also be used to study certain functional traits. For example, the *nif* gene, involved in nitrogen fixation, has been used to study the evolution of nitrogen fixation in comparing cyanobacteria to other bacteria capable of fixing nitrogen (e.g. Latysheva *et al.* 2012). Studying nitrogen fixation requires measuring the expression of the *nif* gene, as its expression is under environmental control (Golden 1985). Genes used to estimate toxigenic potential include *mcy* (as discussed earlier), *nda* (for the toxin nodularin; Gehringer and Wannicke 2014), the *ana* gene cluster (for anatoxin-*a*; Rantala-Ylinen *et al.* 2011), and the *cyr* and *sxt* gene clusters which synthesize cylindrospermopsin and saxitoxin respectively (Hoff Risseti *et al.* 2013).

Molecular methods in microbial diversity

Microbial diversity in ecosystems has been assessed semi-quantitatively based on extracted environmental DNA using methods such as denaturing gradient gel electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP). In sediments, cyanobacterial diversity has been estimated through band richness from DGGE analysis (Ye *et al.* 2011) and at the genus level using T-RFLP (Innok *et al.* 2005). This work has mostly involved cyanobacteria in surface sediments (Innok *et al.* 2005; Dadheech *et al.* 2009; Rinta-Kanto *et al.* 2009) where some species overwinter and subsequently repopulate the water body the following growing season (Tan *et al.* 2008).

Recent concurrent advances in extraction efficiency of environmental DNA and next generation sequencing (NGS) methods of DNA have made analyses of microbial communities more quantitative (Shokralla *et al.* 2012). One of the main types of NGS is known as "sequencing by synthesis," which involves PCR amplification and thus sequential addition of bases to the template DNA. Pyrosequencing (also known as 454 sequencing) is one such method where a light signal is emitted with the addition of each base (Marsh *et al.* 2010). Evaluation of pyrosequencing results yielded Shannon diversity values for a microbial community that were double what was determined using T-RFLP (Pilloni *et al.* 2012).

Next generation sequencing is now being applied to analyze the diversity of cyanobacteria. Changes in taxa composition detected using such methods showed a peak in potential bloom-formers part way through growing seasons along a Korean river (Hur *et al.* 2013) and in two lakes in Wyoming, USA (Steven *et al.* 2012). Pyrosequencing also recently enabled the identification of genera responsible for toxin production (microcystin and cylindrospermopsin) in Antarctic cyanobacterial mats (Kleinteich *et al.* 2014).

1.3 Temporal and Spatial Trends in Cyanobacteria

Seasonal variation in cyanobacteria and other phytoplankton is well understood (Sommer *et al.* 1986) but year-to-year and decadal trends are not well established. A shift in composition in the dominance of cyanobacteria over algae, such as diatoms, exists along a temporal gradient in sediment cores (e.g. Kling *et al.* 2011), but has not been assessed to the same degree between genera of cyanobacteria. This is an important consideration given the concern of the effect of warmer air temperatures on the potential dominance of toxic cyanobacterial genera over non-toxin producers (Gallina 2011). Certain environmental drivers may stimulate the growth of particular strains of cyanobacteria rather than influence total cyanobacterial abundance, which would be missed if community composition is not considered (Cai and Kong 2013; Lu *et al.* 2013). Precise sequencing approaches applied to sediment cores could assist in elucidating this issue.

Similarly, the proportion of cyanobacteria within phytoplankton has been assessed spatially. The proportion of cyanobacteria was found to increase with temperature along a latitudinal transect along the Eastern coast of South America and extending through Northern Europe (Kosten *et al.* 2012). Diversity within cyanobacteria on a spatial scale has not been well assessed. There is evidence of certain species of cyanobacteria originally considered subtropical expanding to northern temperate lakes (Fastner *et al.* 2003; Hamilton 2005). The apparent proliferation of *Cylindrospermopsis raciborskii* has been attributed to its broad range of temperature tolerance combined with increasingly warmer temperate northern lakes (Briand *et al.* 2004). Sequencing approaches could be of use in confirming the biogeography and spatial differences of such species.

Phylogenetic analysis of *C. raciborskii* based on genetic markers showed clustering of sequences in relation to geographic location (Moreira *et al.* 2014).

Northern Canada (both Arctic and sub-Arctic) is particularly sensitive to climate warming (Ruhland *et al.* 2005; Prowse *et al.* 2009; Royer *et al.* 2013). Lakes in such remote areas are not easily accessed and are thought to be less perturbed by human activity than lakes further south. Cyanobacterial blooms have not been historically in these regions though benthic populations and microbial mats composed of cyanobacteria are common and vital to northern ecosystems (Varin *et al.* 2012; Vincent and Quesada 2012). Cyanobacteria are an important component of microbial mats in polar regions and changes in diversity could lead to loss of ecosystem function in these regions (e.g. Jungblut *et al.* 2010; Kleinteich *et al.* 2012).

1.4 Thesis objectives

Perceived temporal changes in the abundance, assemblages, and toxigenic potential of cyanobacteria in theory could be corroborated through a paleolimnological approach. The goal of this thesis was to use this approach to determine whether reports of cyanobacterial dominance and increased toxin production are in fact recent phenomena. The secondary goal was to evaluate the use of molecular markers as proxies of cyanobacterial abundance and diversity in sediments.

The second chapter of this thesis compares different storage conditions of sediment on the preservation potential of DNA. The objective was to determine the implication of storing sediment over several months and at different temperatures on DNA yield and quality as well as effects on downstream molecular analyses.

The third chapter involves tracking cyanobacterial abundance through the sediment record in five lakes in and adjacent to a protected area. I predicted that climate warming (post-1980) is responsible for recent observations of cyanobacterial blooms (LeBlanc *et al.* 2008). I tracked changes in cyanobacterial abundance in cores taken from lakes within the protected area and compared these findings to cyanobacterial growth patterns in lakes outside of the area, where human activity has altered the landscape.

The fourth chapter focuses on toxigenic cyanobacteria by detecting a gene involved in microcystin synthesis (*mcyD*) in cores taken from two temperate lakes. One of the objectives was to compare these results to concentrations of microcystin measured chemically in the same cores in order to evaluate the potential for using the *mcyD* gene as a molecular proxy for microcystin. I predicted that toxigenic blooms are related to patterns of eutrophication in these lakes. I further hypothesized that cyanobacterial community composition would be shifting toward toxigenic taxa over time. This was investigated by pyrosequencing the cyanobacterial 16S rRNA gene.

The fifth chapter of the thesis examined temporal changes in cyanobacterial diversity using a pyrosequencing approach on top and bottom samples of sediment cores taken from six northern temperate and Arctic lakes selected along a latitudinal transect in Northern Canada. I tested the hypothesis that cyanobacteria assemblages have changed through time in a region, in theory, considered too cold for planktonic bloom proliferation (Reynolds and Petersen 2000). I hypothesized a shift in community composition, even in northern areas, toward potential bloom-forming genera in relation to changes in climate regime.

Table 1.1: Examples of organisms identified through extracted DNA in sediment cores.

Taxa	**Location**	**Core length(s) (cm)**	**Source**
Algae	Antarctica	150	(Coolen *et al.* 2004)
Bacteria	Antarctica	60	(Xiao *et al.* 2005)
Bacteria/Archaea	Tibet	900	(Jiang *et al.* 2007)
Bacteria	New Zealand	300	(Matisoo-Smith *et al.* 2008)
Rotifers	Kenya	30	(Epp *et al.* 2010)
Virus	Black Sea	116	(Coolen 2011)
Cyanobacteria	Antarctica	165-245	(Fernandez-Carazo *et al.* 2013)
Methanotrophs (bacteria)	France	93-146	(Belle *et al.* 2014)
Cyanobacteria and algae	China	500	(Hou *et al.* 2014)
Cyanobacteria	Norway	27-45	(Kyle *et al.* 2015)

CHAPTER 2: THE EFFECT OF TEMPERATURE AND DRYING ON LONG-TERM STORAGE OF LAKE SEDIMENT INTENDED FOR MOLECULAR ANALYSES

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Statement of Author Contributions

I performed the experiments and wrote the manuscript. AP helped develop the project design and FP supervised the project.

2.1 Abstract

The quality of DNA extracted from environmental matrices has increased substantially in recent years due to advances in laboratory methods and commercial kits. Lake sediment is one such matrix that is of particular importance to paleolimnological research. The potential of various methods and kits to extract DNA from sediment has been evaluated based on yield, integrity, and purity. However, effects of storage conditions of sediment on DNA quality have not been properly assessed. We compared the quality and PCR potential of extracted DNA from sediment stored at different temperatures (4°C, -20°, and -80°C) for 3 and 6 months relative to freshly extracted DNA. The potential use of DNA from freeze-dried and oven-dried sediment was also evaluated. We found overall that DNA concentrations and quality were mostly consistent at the storage temperatures considered but that DNA quality and concentration from freeze-dried sediment significantly decreased after both 3 and 6 months. Storing sediment at 4°C over 3 or 6 months led to changes in the chemotrophic but not phototrophic bacterial community as evidenced by an increase in gene copies of the eubacterial gene *glnA* (for glutamine synthetase) but not for a16S rRNA gene fragment specific to cyanobacteria. We recommend (1) to extract DNA as soon as possible after retrieving sediment from the field or storing sediment at -20° or -80°C once collected and proceeding with DNA extraction as soon as possible. If sediment is to be freeze-dried, we recommend extracting DNA directly after freeze-drying. Oven-drying sediment for use in analyses of nucleic acids is not recommended.

2.2 Introduction

In recent years, extracting DNA from environmental matrices has become more efficient and has produced higher yields (Bohmann *et al.* 2014; Orgiazzi *et al.* 2015). Methods for quantification and high throughput sequencing have also advanced substantially, further progressing the field of environmental microbiology (Shokralla *et al.* 2012). One application of these methods has been the analysis of DNA from lake and marine sediment cores to reconstruct historical microbial community structures (e.g. Coolen *et al.* 2004; Jiang *et al.* 2007; Coolen 2011). DNA from surface sediment also has applications to the ecology of benthic lake communities (Tsuboi 2013) and the overwintering capabilities of certain bacteria (Misson *et al.* 2012).

To extract DNA directly from sediment, physical lysis methods such as bead mill homogenization (More *et al.* 1994) and freeze-thawing (Erb and Wagner-Dobler 1993) are commonly used. Physical lysis is typically followed by chemical and enzymatic lyses involving a detergent such as sodium dodecyl sulfate (SDS) and an enzyme such as proteinase K (Alain *et al.* 2011). These lysis steps are typical of extracting DNA from a variety of matrices. Extractions have been modified to address sediment-specific concerns (reviewed by Roose-Amsaleg *et al.* 2001) and have been compared in terms of quantity and quality of DNA extracted (Miller *et al.* 1999). Soil and sediment extraction processes usually involve specific steps to remove humic acids which inhibit subsequent amplification of DNA (Tsai 1992; Juniper *et al.* 2001).

Commercially available kits designed specifically for soil and sediment have gained popularity due to their ease and efficiency of use, though the total cost is typically higher than using solvent-based extraction methods such as phenol/chloroform (Miller *et al.* 1999). Most comparative studies of kits and other extraction methods have focused on DNA yields and quality. Few have evaluated the effect of storage conditions, prior to DNA extraction, on the yield, quality and integrity of DNA. Those who have taken storage conditions into account have typically done so over a short time period. For example, after 14 days of storage at 20, 4, -20, and -80°C, there was no significant difference in community diversity of terrestrial soil samples, based on pyrosequencing results (Lauber *et al.* 2010). In another study, sediment was collected from a model aquifer and stored at -80°C for one month. Bacterial 16S rRNA gene copies were amplified from the sediment and found similar to what was amplified from freshly extracted DNA (Brow *et al.* 2010). Similar studies have been conducted on the storage of other types of samples such as water (Gilpin *et al.* 2013), phytoplankton (Miller and Scholin 2000), leaf tissue (Johnson *et al.* 2003), and human gut (Wu *et al.* 2010) prior to nucleic acid extraction. To our knowledge, the effect of storage conditions and temperature of sediments kept for long-term (>3 months) on the extraction and amplification potential of DNA has not been well assessed.

Sediment is often freeze-dried prior to pigment analysis (Leavitt and Hodgson 2001; Hagerthey *et al.* 2006), radioisotope dating (Appleby 2001), or quantifying concentrations of organic compounds such as chlorins and alkenones (McClymont *et al.* 2007). Samples may also be oven-dried for the analysis of sulphur or sulphate ions (Tabatabai 1982), metals (Iqbal *et al.* 2013), mercury (Canário *et al.* 2007), and pesticides (Wasswa *et al.* 2011). Should the same samples be used for molecular work in conjunction with these other analyses, evaluating the effects these sample treatments have on downstream molecular analyses could be informative. If samples can be given the same initial treatment or stored the same way, laboratory analyses could be conducted more efficiently. This would also be conducive toward performing multiple analyses on the same initial sample.

Our objective was to determine whether conditions of sediment storage prior to DNA extraction would have a significant effect on (1) the amount and quality of DNA extracted and (2) downstream applications such as polymerase chain reaction (PCR) and quantitative PCR. We evaluated differences in storage temperature and sediment drying processes over a period of up to 6 months. Evaluating preservation methods could lead to a more standardized protocol of DNA extraction from sediment and reduce inter-laboratory variation.

2.3 Methods

Lake sediment was collected from mesotrophic Lake Heney near Gracefield, Quebec (46.02245°N, 75. 92872°W) at the maximum water depth (34 m) using a mini gravity corer. A 20-cm length core was collected and kept at 4°C overnight. The core was then extruded and sediment was homogenized prior to separating for different storage conditions. One set of extractions was done on fresh sediment (n=5). To prepare for freeze-drying, approximately 5 g of sediment was placed in standard 50 mL Falcon™ conical centrifuge tubes (n=5). These tubes were kept at -20°C before freeze-drying (which took approximately 48 hours). To prepare for oven drying, 5 g of sediment was placed in separate glass Petri dishes (Pyrex) and placed in an oven at 60ºC (n=5). Sediment was also divided up to be kept at 4°C and -20°C and -80° over the course of 3 and 6 months (n=5 for each treatment). All extractions were performed using the PowerSoil DNA Isolation kit (MoBio Laboratories, Inc) according to the manufacturer’s instructions except for the final step. The final DNA elution was done using 20 µL of elution buffer rather than the 100 µL elution recommended by the manufacturer. This was done as preliminary testing showed low downstream yields from PCR and qPCR from concentrations achieved from eluting in 100 µL of buffer. Preliminary tests also showed that after eluting the DNA in 20 µL of buffer there was minimal DNA left on the spin filter, verified by using a NanoDrop Spectrophotometer (Thermo Scientific). Extracted DNA was stored at -20°C until used - standard lab procedure (Knebelsberger and Stöger 2012) and as recommended by the extraction kit protocol (MoBio Laboratories Inc 2013).

Sediment DNA concentrations were determined fluorometrically using the Quant-IT DNA assay kit (Invitrogen) according to the manufacturer's protocol. The quality of the DNA was estimated based on the ratio of absorption at 260 nm to 280 nm using the NanoDrop Spectrophotometer. At a A_{260}/A_{280} ratio of ~1.8, DNA is typically accepted as "pure" and a ratio below 1.7 indicates protein contamination (Tien *et al.* 1999; Thermo Scientific 2011). Total genomic DNA was visualized on a standard 1% (w.v.) agarose gel stained with ethidium bromide using the Lambda DNA *Hind* III Digest ladder (Sigma-Aldrich).

PCR and qPCR conditions

PCR and qPCR are typical downstream applications that follow DNA extraction and were therefore used as measures of quality of extracted DNA. To assess the amplification potential of extracted DNA, primers for bacterial and cyanobacterial genes were used. The first set amplified a section of the single-copy glutamine synthetase gene (*glnA*) and has previously been used to amplify bacterial DNA from sediment (Hurt *et al.* 2001). The glutamine synthetase enzyme is essential for nitrogen metabolism in bacteria; making *glnA* a suitable housekeeping gene for bacterial activity and a marker for overall microbial abundance (Stoeva *et al.* 2014). The forward and reverse primer sequences were GS1β, 5'- GAT-GCC-GCC-GAT-GTA-GTA-3' and GS2γ, 5'- AAG-ACC-GCG-ACC-TTP-ATG-CC-3' and yielded a product of 153-156 bp gene fragment. The second primer set used was a probe for cyanobacteria, amplifying the region of the 16S rRNA gene conserved through all cyanobacteria. The forward and reverser primers were respectively, CYAN 108F, 5'-ACG-GGT-GAG-TAA-CRC-GTR-A-3' and CYAN 377R, 5'-CCA-TGG-CGG-AAA-ATT-CCC-C-3'. These primers were modified (Rinta-Kanto *et al.* 2005), from those originally designed (Urbach *et al.* 1992; Nübel *et al.* 1997), and were also previously used by Ye *et al.* (2011). Standard curves for absolute quantification for both sets of primers were developed by cloning amplified gene fragments into plasmids (Appendix A; Figure A.1). The qPCR conditions for both primer sets were as follows: preheating at 95°C for 3 min followed by 35 amplification cycles of 10 s at 95°C and 10 s at 55°C. This was followed by a melt curve from 60°C to 95°C at 0.5°C increments with 5 s holds. The optimal annealing temperature for both primer sets was found to be 60°C. DNA samples were pooled together and serially diluted between the lowest C_q (as determined during melt temperature optimization) and a C_q of 35, which is often considered the detection limit of qPCR (Bustin *et al.* 2009). The serial dilution was performed to establish the dilution factor needed to lessen the effect of PCR inhibitors. A dilution factor of 10 was deemed appropriate based on serial dilutions using both primer sets. This dilution fell within an efficiency range of 90 to 110% (which is considered free of inhibitors). Melting temperature (T_m), which is dependent on the nucleotide sequence of each amplicon were found to be similar between amplified samples and the diluted plasmid used for the standard curve (Appendix A; Figure A.2).

Statistical Analyses

To evaluate the precision of amplification (qPCR), the coefficients of variation from the mean were calculated for the copy numbers of both genes of interest from freshly extracted DNA. To compare mean DNA concentration and mean DNA purity, analysis of variance (ANOVA) test was used. When data was not normally distributed (as assessed using the Shapiro-Wilk test), it was normalized through a logarithmic or square (^2) transformation. A p-value of 0.05 was considered significant for all analyses. All statistical analyses were performed using the software JMP version 11.

2.4 Results

DNA concentrations and quality of extract

DNA was successfully extracted from all sediment treatments. Mean DNA concentrations (adjusted for w.w. sediment) are shown in Fig. 2.1. After 3 months, DNA extracted from sediment stored at -80°C was significantly less concentrated than DNA extracted fresh ($p=0.008$) though after 6 months, there was no observed effect on DNA concentration from freezing (at -20°C or -80°C) or refrigeration (4°C). Freeze-dried samples were significantly less concentrated after both 3 and 6 months ($p<0.0001$). Temperature of storage appeared to affect DNA concentrations more than the length of storage time.

After 3 months of storage, overall quality of DNA extracted from untreated sediments was very similar among different temperature storage based on the A_{260}/A_{280} ratio (all > 1.77). The mean ratio was significantly lower when sediment was stored at 4°C ($p=0.005$), -20°C and -

80°C ($p<0.001$) when compared to freshly extracted DNA (Table 2.1). After 6 months, the means of DNA purity kept at 4°C, -20°C, and -80°C were all significantly lower ($p<0.05$) than the mean purity of fresh DNA but were again very close to what is considered pure (all > 1.79). DNA quality extracted from freeze-dried sediment was significantly reduced after both 3 months and 6 months with values less than 1.7.

Genomic DNA was run through gel electrophoresis to visualize DNA fragment size, as smaller fragments migrate further along the gel. Sediment kept at 4°C for 3 or 6 months (lanes 1-3 middle and bottom gels) showed larger DNA fragments than fresh DNA (10 kb). After both 3 and 6 months, DNA from sediment kept frozen at -20°C (lanes 4-6; middle and bottom gels) or -80°C (lanes 7-9; middle and bottom gels) did not extract to the same quality as fresh DNA, as the DNA fragments were smaller and fainter. Though DNA extracted after freeze-drying gave a strong signal initially (lanes 4-6; top), after 3 and 6 months of storage, the degradation of the DNA was apparent due to the fainter signal (lanes 10-12; middle and bottom gel). DNA extracted directly after oven drying was barely visible (Fig. 2.2, lanes 7-9; top).

Gene copy numbers from qPCR

Gene copies of freshly extracted DNA (n=5) showed coefficients of variation from the mean of 17% (cyanobacterial 16S rRNA) and 18% (*glnA*). Gene copy numbers of the *glnA* and cyanobacterial 16S rRNA genes in DNA extracted from wet sediment stored at 4°C, -20°C, and -80°C and freeze-dried sediment stored at room temperature for 3 and 6 months were compared to copy numbers in freshly extracted DNA expressed per gram of sediment (Figure 2.3) and per ng of extracted DNA. After 3 months of storage, gene copies of *glnA* showed a significant increase in sediment stored at 4°C (p<0.0001) and significant decreases in sediment stored at -20°C (p=0.0004) and -80°C (p=0.0011). After 6 months of storage, *glnA* gene copies were also significantly higher in sediment stored at 4°C (p<0.0001) and significantly lower in sediment stored at -20°C (p=0.011) and -80°C (p=0.0008). Interestingly, the cyanobacterial 16S gene copies showed no significant change compared to fresh DNA when amplified from sediment stored at 4°C, for 3 months or 6 months (Fig. 2.3B and C). There was a significant decrease in copy numbers of cyanobacterial 16S rRNA when stored at -20°C or -80°C after 3 months, but not after 6 months. Both primer sets showed that amplification of DNA extracted from freeze-dried sediment stored for 3 months and 6 was much lower than sediment stored frozen.

2.5 Discussion

Concentrations and quality of DNA

To our knowledge, this is the first long-term study (>3 months) conducted to assess changes in DNA yield, quality, and amplification potential from sediment stored at different temperatures and conditions. Overall, we found that DNA concentration and quality was relatively constant after both 3 and 6 months. We found that the quality of DNA was not significantly affected by storage temperature and extracting DNA directly after freeze-drying sediment also had no effect on DNA quality. However, freeze-dried sediment stored in the dark for 3 and 6 months at room temperature showed significant deterioration in quality and integrity (based on qualitative fragmentation results). Oven-dried sediment yielded good quality DNA but at low concentrations. The quality of extracted DNA from the environment influences whether PCR amplification can occur (Bonot 2010). In fact, Pan *et al.* (2010) found a strong correlation between quality of DNA extracted from rice field soil and gene copy numbers representative of methanotrophic bacteria.

DNA fragment size and downstream application

When sediment was stored at 4°C for 3 or 6 months, the resulting DNA fragments extracted were larger than even freshly extracted DNA fragments suggesting an active microbial community thriving in the stored sediments. This was reflected in the qPCR results where after 3 and 6 months, there were 9-fold and 6-fold increases in copy numbers of the *glnA* gene, compared to the fresh extraction. These increases in bacterial abundance are likely not reflective of all bacteria present, as increases in the cyanobacterial 16S rRNA gene, indicative of phototrophic cyanobacteria, were not observed; significant phototrophic growth was not expected in sediments stored in the dark. Chemotrophic bacterial growth during sample storage has been reported previously, where sediment oxidation during storage changed the abundance of certain bacterial lipids detected in marine sediments (Lin *et al.* 2010). Furthermore, microbial populations in marine sediments were shown to remain metabolically active if stored at 4°C for 3 months, evidenced by pyrosequence analysis of cDNA of the small subunit RNA gene (SSU rRNA; Mills *et al.* 2012). After 3 months, Mills *et al.* (2012) found a decrease in bacterial taxa related to fermentative sequences. They also found that 45% of the sequences obtained at this time were related to aerobes, whereas none of the sequences obtained from samples stored at -80°C were related to aerobes. This demonstrates significant bacterial activity over the 3 months, with regards to fermentation and potential revival of dormant species based on storage conditions. Lanoil *et al.* (2009) found that storing sediments retrieved from an Antarctic ice stream at 4°C for 15 months may have induced growth of bacteria able to adapt to such a temperature as evidenced by higher than expected cell counts. Different storage temperatures (4°C, -20°C, and -80°C) have also been compared using both oxic and anoxic sediment. Though there was no significant effect on DNA yield after 1 month at -20°C and -80°C, there was a significant decrease of DNA yield from anoxic sediment stored at 4°C after this time, likely due to oxygen exposure (Rissanen *et al.* 2010).

Molecular methodology in conjunction with other analyses

Due to advances in molecular ecology, methods such as qPCR and next generation sequencing can now be used to analyze sedimentary DNA in conjunction with analyses of other markers of interest. Understanding the effect of long-term storage of sediment at different temperatures and conditions may assist researchers interested in using their collected samples for multiple applications (including molecular analyses) in choosing appropriate storage protocols. Researchers often separate sediment samples intended for different analyses to store at specific temperatures and conditions. For example, Guibert *et al.* (2012) stored sediments at -80°C for the analysis of genes associated with biodegradation of hydrocarbons and at -20°C for hydrocarbon analysis. Parducci *et al.* (2013) combined DNA barcoding and pollen taxonomy to identify Holocene plant composition in lake sediments. Sediment samples were stored at 5°C for pollen analysis and at -20°C for molecular analysis. Stoof-Leichsenring *et al.* (2014) compared genetic and microscopic techniques in evaluating diatom assemblages in sediment cores. Retrieved samples were stored at 7°C for microscopic analyses and in a buffer at 10°C for molecular analysis. These examples illustrate the potential nuisance of separating sediment core sections prior to sample storage. If performing molecular analyses, choosing between -20°C and -80°C (for example), may not be necessary. These studies also illustrate the variance in what is considered ideal conditions for sediment stored for future molecular work. Often collected sediment cores are kept in dark cold rooms (4°C) before assessing classic paleolimnological algal markers such as diatom fossils (e.g. Bouchard *et al.* 2013) or cyanobacterial akinetes (e.g. Eilers *et al.* 2004). If such analyses are to be done in conjunction with or in comparison to molecular analyses, the potential for community change at 4°C will lead to important biases in the interpretation of the data.

As molecular assays become common complements to other analyses in paleoecology, it is useful to know, assuming no changes in microbial community structure and hence excluding all samples stored at 4C, what the sediment can be subjected to prior to molecular work and still yield DNA of workable quality and concentration. Our results show that oven-drying sediment degrades the quality of the DNA and decreases the DNA yield and purity. We therefore do not recommend oven drying sediment for molecular work. If other samples require this step, they should be sub-sampled beforehand. For example, Belle *et al.* (2014) used oven-dried sediment (60°C) for stable isotope analysis but used wet sediment for their DNA extractions and analysis.

Freeze-drying sediment is another popular preparatory step for certain analyses, as mentioned earlier. Fernandez-Carazo (2013) kept sediment meant for molecular analyses at -20°C and only freeze-dried sediment for pigment extraction. We found that extracting DNA right after freeze-drying, had no effect on the quality of the DNA. We also found that the concentrations (expressed per µL) were higher than DNA extracted from wet sediment. However, once concentrations were expressed per gram of sediment, the concentrations of DNA extracted from freshly retrieved sediment were found to be higher. Therefore, depending on the downstream applications of DNA, extracting from freeze-dried sediment is acceptable. Due to significant degradation of DNA in freeze-dried sediment, we recommend freeze-drying for molecular work only if the extractions are expected to be done directly after drying. Freeze-dried samples are exposed to more oxygen due to the powdery texture (Leavitt and Hodgson 2001) which is one manner by which DNA in these samples likely degrades over time.

Various storage solutions have also been assessed on sediment and soil including ethanol (Harry *et al.* 2000), DMSO-EDTA-salt solution (DESS; Tatangelo *et al.* 2014), and commercial products such as RNAlater® (Rissanen *et al.* 2010), and LifeGuard™ (Tatangelo *et al.* 2014). Rissanen *et al.* (2010) found that ethanol was ineffective as a storage solution, potentially causing humic acids to become fixed onto nucleic acids during the extraction process. Tatangelo *et al.* (2014) found that storing soil in LifeGuard™ decreased the number of terminal restriction fragments (representative of microbial strains) detected. These studies recommend careful evaluation of storage solutions before use (depending on downstream applications of DNA).

2.6 Conclusions

We recommend lake sediment samples meant for molecular analyses be stored frozen at -20°C or -80°C following collection, if extraction of DNA within several weeks is not possible. Storing sediment at 4°C for community analyses is not advised for molecular work, as it will alter microbial community structure. If molecular work performed on sediments aims only to detect (presence or absence) a targeted gene or taxonomic group, then 4°C may be used if no other storage options are available though no quantitative data can be derived from such DNA. We also only recommend freeze-drying sediment, if it is to be used for DNA extractions immediately afterward. DNA is best preserved in anoxic, cold conditions. Storage potential of samples may vary depending on the type of sediment collected, due to differences in chemistry, organic matter content, and lake water temperature.

Table 2.1: Mean (±SE) A_{260}/A_{280} ratio of extracted DNA subjected to each treatment (n=5). Note that "no treatment" indicates DNA extracted from freshly retrieved sediment.

	No treatment	4°C	-20°C	-80°C	Freeze-dried	Oven-dried
Fresh	1.85 (±0.01)	-	-	-	1.85 (±0.02)	1.75 (±0.03)
3 mths	-	1.80 (±0.01)	1.77(±0.01)	1.78(±0.02)	1.73(±0.02)	-
6 mths	-	1.81 (±0.01)	1.80(±0.01)	1.79(±0.01)	1.67 (±0.01)	-

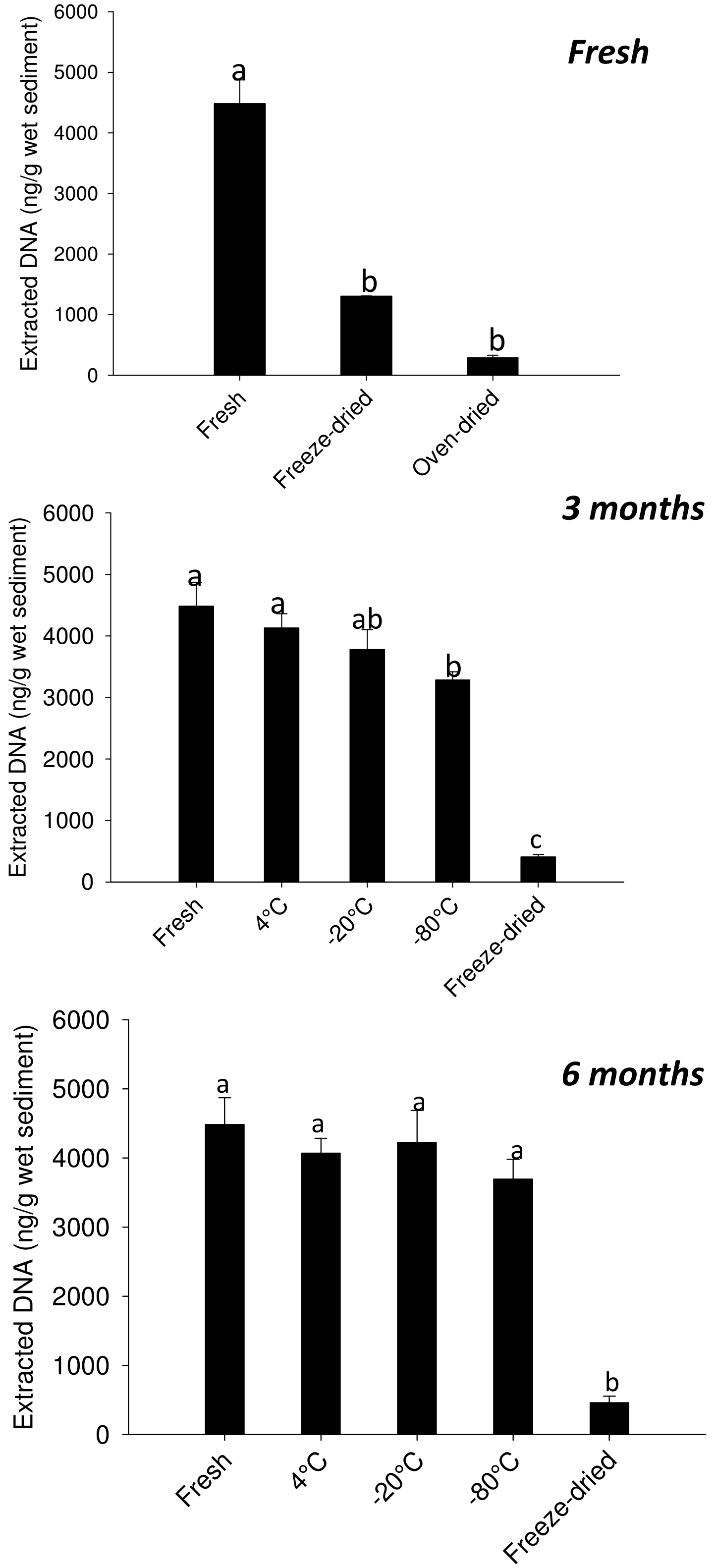

Figure 2.1: Mean (±SE) DNA concentrations expressed per gram of wet sediment (n=5 per group). Top: extractions performed day after sediment core was retrieved from lake (fresh) and performed directly after freeze-drying and oven-drying sediments. Middle: Extractions performed after storing sediment for 3 months. Bottom: Extractions performed after storing sediment for 6 months. Significantly different means ($p<0.05$) are indicated by different letters.

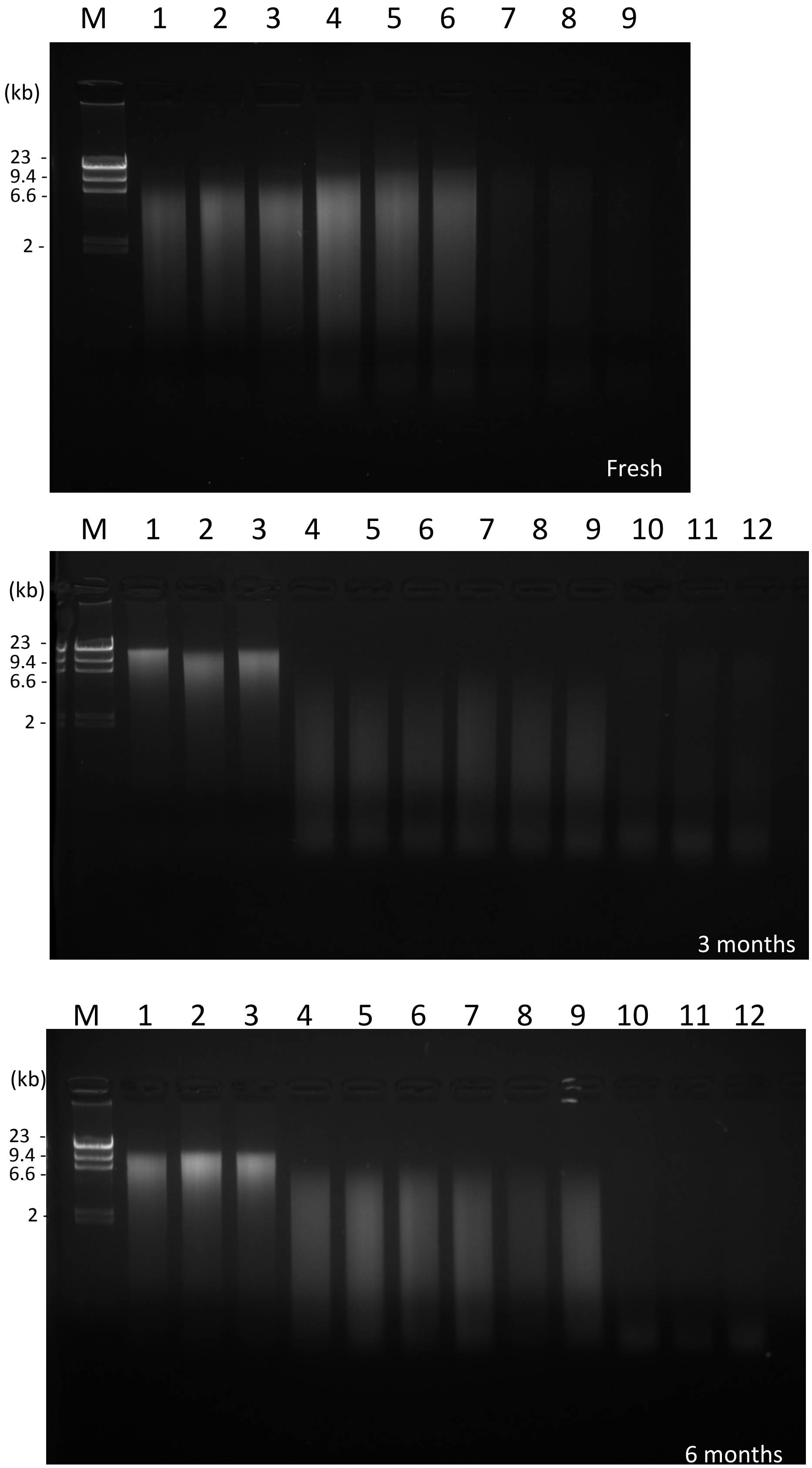

Figure 2.2: Genomic DNA on 1% agarose gel (w.v.) stained with ethidium bromide. Top: Lanes 1-3: freshly extracted DNA from wet sediment; Lanes 4-6: freeze-dried DNA extracted immediately after drying, Lanes 7-9: oven-dried DNA extracted immediately after drying. Middle (3 months) and Bottom (6 months): Lanes 1-3: kept at 4°C, Lanes 4-6: kept at -20°C, Lanes 7-9: kept at -80°, Lanes 10-12: freeze-dried DNA kept at room temperature. M=genomic ladder.

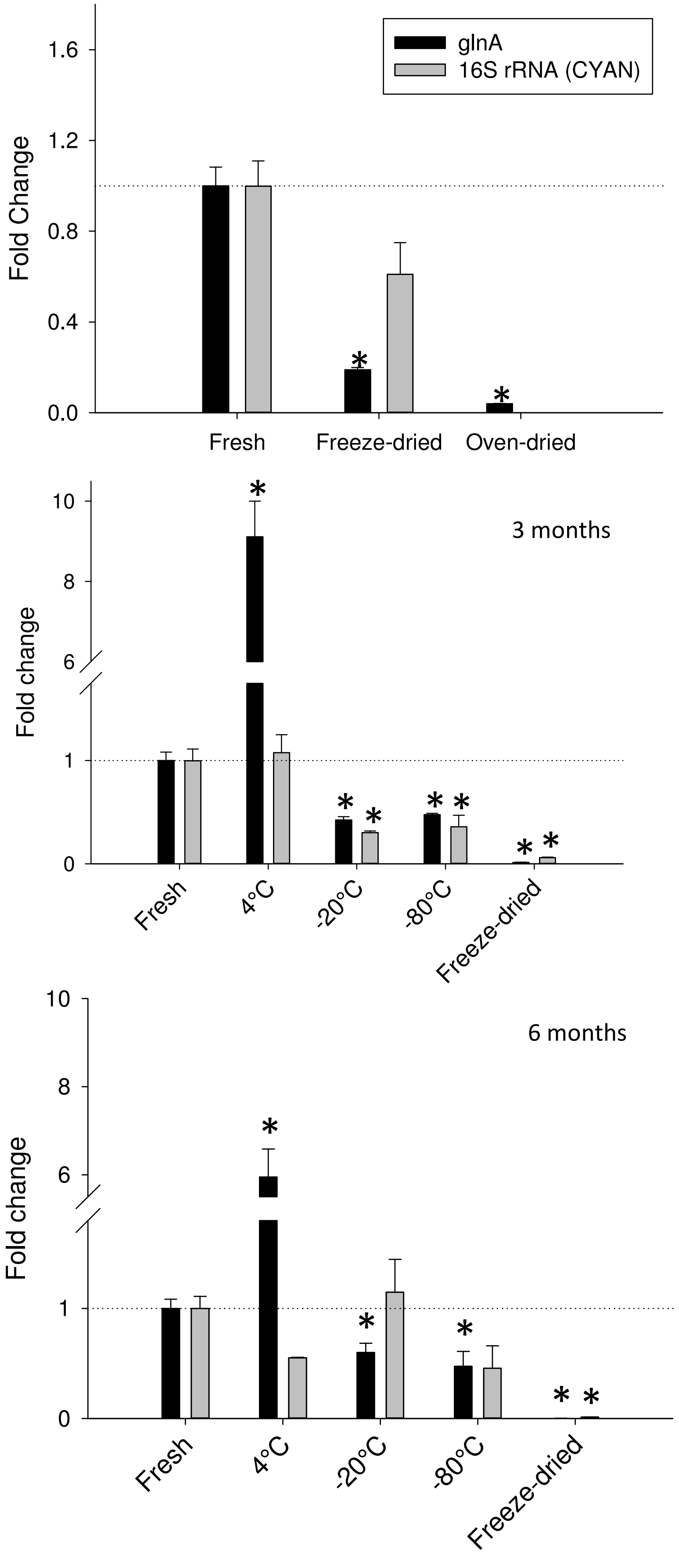

Figure 2.3: Mean fold change (±SE) of gene copies/g in wet sediment compared to freshly extracted DNA. Per treatment, n=2 except for fresh DNA (n=5). Results of qPCR of DNA extracted from sediment freshly retrieved as well as dried directly after retrieval of sediment are presented in the top panel. Note that gene copies of 16S rRNA were not measured for oven-dried DNA. Treatments that are significantly different ($p<0.05$) than the fresh extraction are indicated with asterisks (*).

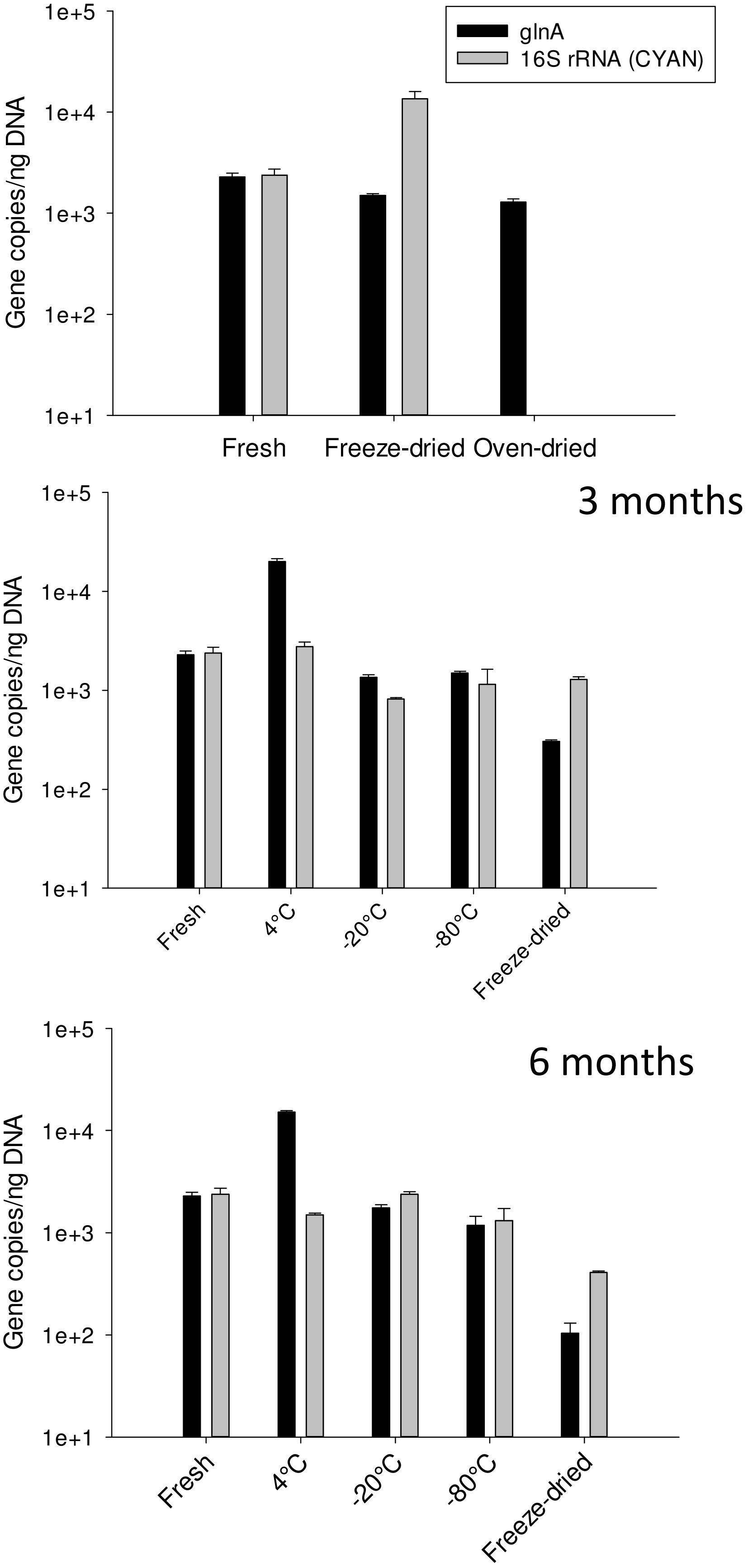

Figure 2.4: Mean (±SE) gene copies/ng of total extracted DNA on a logarithmic scale. Per treatment, n=2 except for fresh DNA (n=5). Results of qPCR of DNA extracted from freshly retrieved sediment and sediment freeze-dried and oven-dried directly after retrieval of sediment are presented in the top panel. Gene copies of 16S rRNA were not measured for oven-dried DNA.

CHAPTER 3: TEMPORAL TRENDS IN CYANOBACTERIA REVEALED THROUGH DNA AND PIGMENT ANALYSES OF LAKE SEDIMENT CORES

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Statement of Author Contributions

I collected the sediment cores, performed molecular analyses, and wrote the manuscript. I prepared samples for lead-210 dating. IG-E supervised the pigment analyses performed. FP supervised the project. Special thanks to Linda Kimpe for analytical assistance with the lead-210 dating and Leen Stephan for performing pigment analyses.

3.1 Abstract

Reports of cyanobacterial blooms in temperate lakes have been increasing over the last few decades. If blooms are indeed becoming more frequent or intense, this poses a problem for water and ecosystem management as blooms can be toxic to wildlife and humans. Here we used a paleolimnological approach to determine whether cyanobacteria have been increasing in Western Quebec, a region with thousands of lakes and a lack of historical surface water monitoring data. We compared lakes within and outside of Gatineau Park, a protected conservation area since 1938. Sediment cores dating back to pre-European settlement of the region were analyzed for temporal trends in cyanobacteria, in order to assess the effect of land use change and/or climate change before and after the Park's creation. We extracted sediment DNA and analyzed for the 16S rRNA gene specific to cyanobacteria based on a qPCR assay for absolute gene copy numbers. These results were compared with analyses of the carotenoid pigments zeaxanthin and echinenone, specific to cyanobacteria, along with analyses of diatoxanthin and β-carotene, representative of diatoms and all algae respectively. Overall, gene copy numbers of cyanobacterial 16S rRNA pointed to a significant increase in cyanobacteria in all five lakes over the past 30 years and also since the Park's creation, when compared to the historical average (past 150 years). In contrast, qPCR analyses of eubacterial gene copies for glutamine synthesis indicated that total microbial abundance exhibited a relatively smaller change over the same time periods. No significant difference in the percent increase of cyanobacteria, quantified by both gene copies and carotenoid pigments, was observed between lakes within and outside of the Park. This would suggest that other factors, such as a warming climate documented in this region, may be driving the increase in cyanobacteria. Sediment DNA has the potential to corroborate more classical fossil remains and provide novel information on microbial structure and function of past ecosystems.

3.2 Introduction

Cyanobacterial blooms are of concern for ecological and human health reasons, especially when a bloom releases toxins. Generally, high nutrient concentrations (Pick and Lean 1987; Downing et al. 2001) and water temperatures (Liu et al. 2011; Kosten et al. 2012; Beaulieu et al. 2013) are positively correlated with cyanobacterial biomass in lakes. Based on monitoring records (spanning the last 15 to 20 years), blooms seem to be increasing in temperate lakes (Wiedner et al. 2007; LeBlanc et al. 2008; Winter et al. 2011). However, relative to the number of lakes, there are few long-term records of algal assemblages, from either monitoring or the sediment record. This poses a clear difficulty when assessing whether the frequency and prevalence of cyanobacterial blooms has truly increased in recent years or whether this trend is simply due to improved monitoring.

Traditionally, microfossils and pigment analyses have been used to reconstruct historical trends in cyanobacterial assemblages in sediment cores. Some cyanobacteria produce resting cysts (akinetes) with thick cells walls or colony remnants, and thus they have a high potential for preservation (Adams and Duggan 1999) and are suitable for paleolimnological analyses (van Geel et al. 1994; Eilers et al. 2004; Bunting et al. 2007). However, many taxa do not preserve well in sediments and therefore cannot be differentiated through microscopy. For this reason, various carotenoid pigments have been used as proxies for the abundance of cyanobacteria and/or algae (Leavitt and Findlay 1994). The carotenoid β-carotene is often used as an indicator of total algal biomass while zeaxanthin and echinenone are pigments considered indicative of cyanobacteria. Other pigments often used as indicators of algal groups include diatoxanthin (diatoms), alloxanthin (cryptophytes), fucoxanthin (diatoms, chrysophytes, and dinoflagellates), and lutein (chlorophytes, Leavitt and Hodgson 2001).

Over the past decade, DNA has been successfully extracted from marine and lake sediment cores thus providing a novel way of reconstructing past community structure and the abundance of specific genes (Xiao et al. 2005; Coolen et al. 2006; Gregory-Eaves and Domaizon 2014). Some studies have shown successful extraction and subsequent quantification of “ancient” DNA dating at least as far back as 17,000 years (Jiang et al. 2007). Organisms studied span the realm of viruses and bacteria (Coolen et al. 2006; Li et al. 2006), to microscopic organisms (Coolen et al. 2004; Coolen et al. 2006; Matisoo-Smith et al. 2008; Coolen et al. 2013), to vertebrates (Giguet-Covex et al. 2014). DNA from cyanobacteria has been quantified from surface sediment of the Great Lakes (Rinta-Kanto et al. 2009) and only a few dated cores: China (Ye et al. 2011), New Zealand (Wood et al. 2009), and France (Savichtcheva et al. 2011; Domaizon et al. 2013). In these studies, primers were designed for segments of the 16S rRNA gene conserved through the cyanobacterial phylum (Nübel et al. 1997) or the internal transcribed spacer (ITS) region (Wood et al. 2009).

Given the demonstrated potential of molecular markers when applied to sediment records, we designed a study to determine the coherence of these tools and classical pigment approaches to evaluate how differences in land use histories could alter cyanobacterial dynamics. Specifically, we conducted a comparative analysis of lakes inside and outside of Gatineau Park (Québec, Canada). Gatineau Park, under the authority of the National Capital Commission (NCC) of Canada, was first created in 1938. The Gatineau region provides an ideal setting to compare lakes located in the same climate and geographical area with similar underlying geology, but either subjected to anthropogenic influence (outside the Park) or considered relatively pristine (inside the Park). Outside the Park boundary, cottages, permanent residences and small, recreational farms or logging operations surround lake perimeters, which potentially subject these lakes to increased nutrient loading due to human development (Clerk et al. 2000). In recent years, unusual fall cyanobacterial blooms have been recorded in lakes both inside and outside the Park and have been associated with regional warming rather than increases in nutrient loading (LeBlanc et al. 2008). The dominant bloom-forming species in these lakes, as observed by LeBlanc et al. (2008) have included *Anabaena smithii* among other *Anabaena* species and *Aphanizomenon flos-aquae* both inside and outside the park. We have observed blooms as late as the beginning of December, which is unusual for this region, yet also recently reported in Ontario lakes (Winter et al. 2011). Our study was designed to: (1) compare historical trends in cyanobacteria of lakes inside verses outside the Park (2) determine whether climate warming is affecting cyanobacterial abundances as inferred from sediment records using DNA markers and pigments, and (3) quantify the coherence between cyanobacterial 16S rRNA gene copies and the cyanobacterial pigments zeaxanthin and echinenone in sediment cores, which are two different methods of tracking the historical record of cyanobacteria.

3.3 Methods

Study sites and sampling

Gatineau Park is located within the Boreal Shield and encompasses an area of about 360 km^2 (National Capital Commission 2005), just north of Ottawa, Canada (Fig. 3.1). Sediment cores were obtained from three lakes within the Gatineau Park boundaries (Lac Meech [45°31’N, 75°52’W], Lac Philippe [45°35’N, 75°59’W], and Lac La Pêche [45°36’N, 76°11’W]). All three are open for recreational activities such as swimming and non-motorized boating during the summer. The watersheds of Lac Meech and Lac Philippe are within the limits of the Park while the watershed of La Pêche extends outside of the Park. Two lakes near the Park boundary (Lac des Loups [45°40'N, 76°12'W] and Lac Gauvreau [45°39'N, 75°59'W]) with watersheds entirely outside of the Park were also sampled (Fig. 3.1). Average surface water measurements of total phosphorus (TP; 2002-2007), indicate that the lakes are mostly oligotrophic to mesotrophic (5-36 μg L^{-1}), with a tendency for lakes inside the park to have lower TP values (5-17 μg L^{-1}) than those outside the park (7-36 μg L^{-1}; LeBlanc et al. 2008). The TP ranges of individual lakes are presented in Table 3.1. All sampled lakes are deep enough to stratify (maximum depth ranging from 8 m to 20 m; Table 3.1) although the water column of Lac Gauvreau does not mix vertically every spring due to high surrounding hills that provide protection from wind exposure.

^{210}Pb dating

From one of the cores retrieved from each lake, 16 to 17 depth sections were chosen for dating through measurement of ^{210}Pb using an Ortec High Purity Germanium Gamma Spectrometer (Oak Ridge, TN, USA). We also measured ^{137}Cs to evaluate the performance of the ^{210}Pb-derived age models. Certified Reference Materials obtained from the International Atomic Energy Agency (IAEA; Vienna, Austria) were used for efficiency corrections, and age models were developed using ScienTissiME (Barry's Bay, ON, Canada). For each core, data were first fitted with three different models based on the ^{210}Pb activity: Constant Rate of Supply (CRS), Constant Initial Concentration (CIC), and Constant Flux Constant Sedimentation (CFCS; Binford 1990; Appleby 2008). The model of best fit in all cases was the CRS model, with the exception of the core from Lac des Loups. For this core, the CFCS model was chosen because of a better exponential curve fit. To approximate the date of samples at depths below the point at which unsupported ^{210}Pb reached background levels, we estimated sedimentation rates based on a polynomial curve fitted through the core. The age of the depths was then calculated from the measured cumulative dry mass and the estimated sedimentation rate. Subsamples from the dated core were used for molecular and pigment analyses to ensure consistency.

DNA extraction and qPCR

Subsamples of sediment were taken out of Whirl-Pak bags at approximately each cm through the core's depth and placed in a 2-mL snap-cap microcentrifuge tube (Eppendorf). These tubes were centrifuged at 10,000 x g for 5 min. Excess water was pipetted out of the tube. The DNA was extracted from approximately 0.25 g of the centrifuged sediment (exact mass noted) using a Powersoil DNA extraction kit (MoBio Laboratories Inc.) with a final volume of 20 µL in elution buffer rather than the recommended 100 µL. This was done to create a more concentrated template for downstream applications of PCR and Q-PCR, and preliminary analyses demonstrated that minimal DNA was left on the spin filter. The DNA extractions and subsequent analyses were performed in a laboratory designated for molecular assays, separate from where initial sediment sub-sampling was done. The extracted DNA concentrations were quantified using the Quant-IT DNA assay kit (Invitrogen). The purity of the DNA was verified using a NanoDrop Spectrophotometer (Thermo Scientific). Purity was estimated based on the absorbance of the DNA at 260 nm and 280 nm.

The forward and reverser primers used to quantify total cyanobacteria were respectively CYAN 108F, 5'-ACG-GGT-GAG-TAA-CRC-GTR-A-3' and CYAN 377R, 5'-CCA-TGG-CGG-AAA-ATT-CCC-C-3'. These primers were modified by Rinta-Kanto et al. (2005) to quantify blooms in Lake Erie, from those originally designed (Urbach et al. 1992; Nübel et al.

1997). The modified primers were also recently used by Ye et al. (2011). The primers were validated on Primer Blast (using Primer3 software) to ensure they were not detecting non-cyanobacterial 16S rRNA (i.e. algal DNA). To further ensure specificity and to serve as a negative control, the primers were tested against DNA extracted from the green alga *Pseudokirchneriella subcapitata*, and no amplification was detected.

Primers representative of eubacteria, which include cyanobacteria, were also used in conjunction with the cyanobacterial 16S rRNA primers, as a marker of overall microbial abundance. In particular, we applied a primer set that amplifies part of the single-copy glutamine synthetase gene (*glnA*) and has been previously used to amplify bacterial DNA from sediment (Hurt et al. 2001). The glutamine synthetase enzyme is essential in nitrogen metabolism in bacteria and therefore *glnA* is considered a housekeeping gene for core bacterial activity (Stoeva et al. 2014). These forward and reverse primer sequences were GS1β, 5'- GAT-GCC-GCC-GAT-GTA-GTA-3' and GS2γ, 5'- AAG-ACC-GCG-ACC-TTP-ATG-CC-3' and yielded a product of 153-156 bp gene fragment. Standard curves for absolute quantification for both sets of primers were developed by cloning amplified gene fragments into plasmids. For the cyanobacterial 16S rRNA primer set, DNA was extracted from a culture of *Microcystis aeruginosa* (CPCC 300; Fortin et al. 2010). The PCR product, generated using the cyanobacterial 16S rRNA primer set was gel-purified (QIAEX II gel extraction kit; Qiagen) and subsequently ligated into the pSC-A-amp/kan PCR cloning vector (StrataClone, Agilent Technologies). Competent cells (StrataClone) were then transformed to possess the gene of interest (16S rRNA or *glnA* gene fragment).

The qPCR conditions for both primer sets were as follows: preheating at 95 °C for 3 min followed by 35 amplification cycles of 10 s at 95 °C and 10 s at 55 °C. This was followed by a melt curve from 60 °C to 95 °C at 0.5 °C increments with 5 s holds. The annealing temperature was 60 °C (optimized from a pool of samples). Samples were also pooled from each core and serially diluted based on the lowest C_q as determined during melt temperature optimization and by calculating the fold dilution needed to end at a C_q of 35. A C_q of 35 is often considered the detection limit of this method (Bustin et al. 2009). The serial dilutions were performed to establish the dilution factor needed for samples from each core to lessen the effect of PCR inhibitors. A dilution factor between 10 and 40x was chosen for each core to ensure that we achieved an amplification efficiency range between 90 to 110% (which is considered free of inhibitors). The mean concentration of DNA extracted, amplification efficiencies, and the coefficients of determination (R^2) of the diluted DNA are presented in Table 3.2. All samples from each core were run at the same time (in triplicate) to avoid inter-assay variation. The mean (±SE) efficiency and coefficient of determination (R^2) of the standard curve (derived from the plasmid) were respectively 106.7% (±2.9) and 0.99 (±0.004).

Pigment analysis

All pigments were measured using High Performance Liquid Chromatography (HPLC) following previously described procedures (Zapata et al. 2000; Saulnier-Talbot et al. 2014). In brief, sediments were freeze-dried and pigments extracted in acetone (-20 °C for 24 hours). The acetone extract was centrifuged and the supernatant filtered (0.2 μm) and placed in vials for HPLC analysis (Waters, model 600/626 with a Waters Photodiode Array 2996, a Waters 2475 Multi λ Fluorescence detector and a refrigerated waters autosampler 717). Pigment data were expressed per gram of organic matter based on loss on ignition (LOI) at 550 °C of dried sediment. Cyanobacterial pigments analyzed included zeaxanthin and echinenone, although echinenone was only detected through one core (Lac des Loups). Algal pigments analyzed included chlorophyll-a, β-carotene, lutein, alloxanthin, fucoxanthin, pheophytin-*a*, and diatoxanthin.

Statistical analyses

All data were verified for normal distribution (Shapiro-Wilk test). Data that did not fit a normal distribution were log-transformed and re-verified. Pigment data were linearly interpolated to be on the same scale as the gene copy data when testing for correlations between pigments and gene copies of cyanobacterial 16S rRNA. Correlations were assessed using Pearson's Correlation coefficient (r). Results were considered significant when the p-value was less than 0.05.

Inter-lake comparison can be difficult due to inherent differences in physical structure such as depth, water clarity, and stratification that may lead to differences in preservation of fossil remains (Leavitt and Hodgson 2001). Therefore, we compared the mean percent change since 1980 and since 1938 in gene copies and pigments relative to the historical average (over the last 150 years) between lakes using Analysis of Variance (ANOVA). We selected 1980 as a cut-off for our most recent interval of interest because global air temperature has been warmer in the last three decades (1980 to 2010) compared to any other time since 1850 based on climate data normalized to the average between 1961 and 1990 (IPCC 2013). Regional mean annual air temperatures (Ottawa-Gatineau area) were also compared to historical climate data (Environment Canada 2014) and found to exhibit an overall increase since 1980 as well. Furthermore, the mean annual cooling degree days (>18 °C) over the past few decades have also mainly surpassed the historical norm (LeBlanc et al. 2008). The year 1938 was chosen as a second cut-off as it represents the creation of Gatineau Park as a protected area.

3.4 Results

^{210}Pb dating and sedimentation rates

Unsupported ^{210}Pb reached background levels of activity at depths from 16 to 20 cm in each core (Fig 3.2), which was reflected in the variable sedimentation rates across lakes (Fig. 3.3). Lac Gauvreau had a particularly variable sedimentation rate within the core, but comparison of the clear ^{137}Cs peak for this core (Fig 3.2) to the estimated age of this interval based on CRS modeling showed good correspondence. After extrapolating to the maximum depth of each core, the deposition record of all cores spanned at least 150 years before present (Table 3.1). The extrapolated points do not have horizontal error bars and can be thus distinguished from points plotted before unsupported ^{210}Pb reached the background level (Fig 3.3).

Cyanobacterial and eubacterial gene copies

DNA was successfully extracted and amplified with the primer sets for cyanobacterial 16S rRNA and *glnA* (for the bacterial glutamine synthetase gene) through each retrieved sediment core (Fig. 3.4). Most cores showed consistently “pure” DNA (Table 3.2). Only the DNA through the Gauvreau core had a mean 260:280 ratio less than 1.7, indicating potential contamination. The DNA from this core also had the lowest 260:230 ratio, suggesting a greater interference from humic acids than found in the other cores. This was consistent with the more highly coloured surface waters of Gauvreau. Mean wet weight DNA concentrations (Table 3.2) were also lowest in the Gauvreau core. In most cores, both cyanobacterial 16S rRNA and *glnA* genes showed an increase in copy number through time but the increases in 16S rRNA gene copies were generally higher, especially in des Loups and La Pêche (Fig. 3.4).

Total DNA concentrations were positively correlated to the *glnA* gene copy numbers in cores from La Pêche ($r=0.76$, $p<0.01$), Philippe ($r=0.78$, $p<0.01$), des Loups ($r=0.91$, $p<0.01$), and Gauvreau ($r=0.74$, $p<0.01$) but not significantly correlated in the Meech core ($r=0.45$, $p=0.12$). The gene copies of *glnA* showed less variation in the Meech core than in some of the other cores, which may explain the lack of correlation. The *glnA* gene copies were positively correlated to gene copies of cyanobacterial 16S rRNA through all cores (La Pêche $r=0.97$, $p<0.01$; Philippe $r=0.57$, $p<0.05$; des Loups $r=0.83$, $p<0.01$; Gauvreau $r=0.92$, $p<0.01$; and Meech $r=0.75$, $p<0.01$).

All cores showed higher mean percent increases of cyanobacterial 16S rRNA gene copies since 1980 compared to the time interval between 1938 and the present, when compared to the historical average (Fig. 3.5). Among lakes, there was no significant difference in the mean percent change in cyanobacterial 16S rRNA gene copies since 1980 ($F=0.72$, $p=0.60$) or 1938 ($F=0.64$, $p=0.64$). The Lac Gauvreau core showed the least amount of change in cyanobacterial 16S rRNA gene copies g^{-1} of sediment since 1938 (13% increase) whereas Lac des Loups showed the most change (76% increase). The same trend was observed after 1980, where the Gauvreau core showed a 32% increase and Lac des Loups showed a 322% increase. This overall trend was similar when cyanobacterial 16S rRNA gene copies were expressed per unit of DNA extracted rather than per unit sediment (wet weight).

Pigment concentrations

In the four lakes where pigments were analyzed, zeaxanthin, diatoxanthin, alloxanthin, and β-carotene were detected and quantified all through the cores. To represent total algae, β-carotene and pheophytin-*a* were chosen over chlorophyll-*a*, as they are considered more stable compounds (Leavitt and Findlay 1994). The β-carotene pigment was significantly positively correlated to pheophytin-*a* in cores from Pêche, Philippe, and Loups but not in Meech. Since 1938, β-carotene showed little deviation from the historical average (<10%) in all cores. However, the Lac des Loups core showed a mean (±SE) decrease of β-carotene of 39.5 (±10) % since 1980, which was the only significant decrease amongst cores analyzed (Fig. 3.6). Alloxanthin, representative of cryptophytes, showed relatively less change than other pigments at <10% change since 1938 and <50% increase since 1980. Zeaxanthin in the Lac des Loups core showed a mean (±SE) percent decrease of 21.8 (±8.7) % since 1938 and of 52.0 (±5.5) % since 1980, compared to the historical average. However, echinenone (only detected in the Lac des Loups core) showed a slight mean (±SE) increase of 4.6 (±12.5) % since 1938 and an increase of 29.1 (±19.4) % since 1980 in the same core. The other lakes analyzed for zeaxanthin (Meech, Pêche, and Philippe) had lower mean percent changes (<20%), based on both time points, and showed no significant difference in percent change between lakes. The diatom indicator pigment, diatoxanthin, showed an overall decrease in all lakes since both 1938 and 1980. Fucoxanthin was not detected in the Meech core or the Philippe core. This pigment tends to be more labile than other carotenoid pigments and may therefore have degraded below the detection limit in the aforementioned cores (Leavitt 1993). Lutein was not detected in the Philippe core either. There was less inter-lake variation in the percent change of the algal pigments alloxanthin, fucoxanthin, and lutein, where detected (Fig. 3.7).

The pigment β-carotene was significantly positively correlated to zeaxanthin in cores from Lac des Loups (r=0.84, p<0.01), Lac La Pêche (r=0.44, p<0.01), and Lac Philippe (r=0.68, p<0.01) but not significantly correlated in the Lac Meech core (r=0.13, p=0.53). Echinenone in the Lac des Loups core was significantly correlated to β-carotene (r=0.48, p<0.01).

Comparison between pigments and cyanobacterial genes

In the Meech and Philippe cores (Fig.3.4), we found significant positive correlations between cyanobacterial 16S rRNA gene copies and the zeaxanthin concentrations (*r*=0.59, p<0.01; *r*=0.61, p<0.01). The core from Lac des Loups (Fig. 3.4) showed a significant positive correlation between cyanobacterial 16S rRNA gene copy numbers and echinenone concentrations (*r*=0.59, p<0.05) but, conversely, the cyanobacterial 16S rRNA gene copies were negatively correlated to the zeaxanthin concentrations (*r*=-0.59, p<0.05). The core from Lac La Pêche had cyanobacterial 16S rRNA gene copies that did not correlate to zeaxanthin concentrations (*r*=-0.10, p=0.68).

Cyanobacterial genes were expressed per wet gram of sediment whereas the pigment data were expressed per microgram of organic matter. However, when the gene copies were expressed per gram of organic matter, there was no difference in the trends observed. This was likely due to the consistency in organic matter content through each core. The LOI in the Meech and Philippe core only varied ~3% through the length of the cores. The LOI in the La Pêche and des Loups cores showed more variation (14% and 10%) respectively. The LOI in the Gauvreau core was the most variable (27%) but also showed the most variation in sedimentation rate. Variation in the down-core profiles of LOI and sedimentation are both indicative of changes in input from erosion and runoff (Shuman 2003).

3.5 Discussion

Overall increase in cyanobacteria

Our comparison of molecular data to cyanobacterial pigments (zeaxanthin and echinenone) illustrates the potential benefits of using specific genes in the analysis of sediment cores for past ecosystem reconstruction. By quantifying molecular markers and carotenoid pigments we confirmed the presence of cyanobacteria throughout the core from each lake. Based on the cyanobacterial 16S rRNA gene copies, we observed a mean increase in the abundance of cyanobacteria since 1938 in all lakes, which further intensified since 1980. However, no significant differences in trends were observed between lakes within and outside the Park. The strong increase in cyanobacterial 16S rRNA gene copies since 1980, even within the Park, may be attributed to warmer temperatures and longer growing seasons which have arisen throughout the wider Great Lakes basin (Magnuson et al. 1997). Both of these changes are likely favorable to cyanobacteria since overall they tend to thrive at higher water temperatures but also under conditions of prolonged stratification because of their capacity for buoyancy control (Beaulieu et al. 2013; Carey et al. 2012).

Our zeaxanthin pigment results in cores from Meech and Philippe and the echinenone profile in the Lac des Loups core echo findings of other pigment studies that have reported increases in cyanobacteria over the last century. Similar results were found in Florida, where 5 out of 6 lakes studied showed an increased proliferation of cyanobacteria in recent decades based on sediment core profiles of the cyanobacterial pigments oscillaxanthin and myxoxanthophyll (Riedinger-Whitmore et al. 2005). Similarly, filamentous cyanobacteria showed an abrupt increase after 1987 in Windermere Lake, England, based on myxoxanthophyll profiles in sediment (McGowan et al. 2012). However, in the one core in our study (Lac des Loups) where we detected both cyanobacterial pigments, trends were not coherent and several explanations are possible. This may be due to differential degradation between pigments (Leavitt and Hodgson 2001; Sobiechowska et al. 2010). Differences in zeaxanthin and echinenone concentrations in Baltic Sea sediments have been attributed to the higher stability of zeaxanthin as well as higher sedimentation than echinenone (Bianchi et al. 2002). This could also explain why echinenone was only detected in one of the four cores analyzed for pigments in our study. The difference between the zeaxanthin and echinenone in the Lac des Loups core could also be due to a shift in the type of cyanobacteria to groups that contain relatively less zeaxanthin. For example, a shift away from picocyanobacteria could mean a decrease in zeaxanthin, given that picocyanobacteria contain more zeaxanthin than other cyanobacteria (Romero-Viana et al. 2010).

Across most of our lakes, the changes in cyanobacterial pigments tracked the shifts in the algal community as a whole, but divergent responses were apparent when cyanobacterial and diatom pigments were compared. For example, we detected positive correlations between zeaxanthin and β-carotene in the cores from Lac des Loups, Lac La Pêche, and Lac Philippe. Lac Meech was an exception as we failed to detect a significant correlation here between zeaxanthin and β-carotene, and the mean percent decrease in total algae since 1980 suggests that only cyanobacteria are increasing. Whereas there was an overall increase in cyanobacteria, the abundance of diatoms seemed to be decreasing, based on the diatoxanthin concentrations detected through all cores. The inverse relationship between diatom abundance and cyanobacteria has also been noted based on the pigments oscillaxanthin and myxoxanthophyll compared to diatom numbers in Lake Wabamun, Alberta (Hickman and Schweger 1991) and could be due to increases in the length of summer thermal stratification (Harris et al. 2006).

The mean percent increases of gene copies since 1980 were higher for all cores compared to percent changes in pigment levels. One explanation for this could be the presence of akinetes from species within the *Nostocales* order in the sediment. During akinete differentiation, pigment production is known to decrease (Sarma et al. 2000). Furthermore, akinetes have been shown to possess 15 times the chromosomal content of vegetative cells (Sukenik et al. 2012). Both of these factors could obscure the relationship between cyanobacterial gene copies and pigments.

Nutrient concentrations and/or shifts to warmer water temperatures are known drivers for cyanobacterial dominance. Certainly, the connection between cyanobacterial blooms and eutrophication is well established (Schindler 1974). A previous study of these lakes showed that Lac Gauvreau had the highest TP concentrations (18-36 $\mu g\ L^{-1}$) and summer - fall dominance of cyanobacteria in the plankton (LeBlanc et al. 2008). However, we found a much lower range of cyanobacterial 16S rRNA gene copies through the Lac Gauvreau core compared to the others. This was likely due to the presence of humic acids in Lac Gauvreau which could be inhibiting the PCR amplification (Tsai 1992). Unlike the other lakes, Gauvreau is tea- coloured with poor transparency, indicative of humic/fulvic acids. As mentioned earlier, the higher humic acid content in this core was suggested by the lower 260:230 ratio of absorbance. Since many temperate lakes, particularly in the boreal or taiga region, are coloured lakes (dystrophic), interference with humic materials is a challenge that will need to be overcome in future studies.

Using qPCR (gene copies) in conjunction with pigments

Our results show the potential of quantifying cyanobacterial 16S rRNA as a proxy for cyanobacterial abundance. In three out of four of the cores where both DNA and pigments were extracted, significant positive correlations were seen between the pigment data (either zeaxanthin or echinenone) and the cyanobacterial 16S rRNA gene. Although the cyanobacterial 16S rRNA gene was successfully amplified through each core, echinenone was only detected in one of the four cores. Generally, molecular methods are far more sensitive than chemical analyses but whether DNA tends to be also better preserved in sediments than pigments has not been established. In a recent study of two Antarctic lakes, cyanobacterial carotenoid pigments were extracted for HPLC analysis (Fernandez-Carazo et al. 2013) but were often not detected, though cyanobacterial DNA was successfully amplified at all depths of the sediment cores. Also, the down-core profile of DNA from green sulfur bacteria was found to be more stable than the profile of carotenoids in the same core (Boere et al. 2011).

To date, carotenoid pigments have been used widely as biomarkers to illustrate algal community composition in the historical record of lakes (Hickman and Schweger 1991; Itoh et al. 2003; Romero-Viana et al. 2010; Efting et al. 2011). However, a challenge of pigment analysis is the variable degradation between pigments at sites of different geology and hydrology (Cuddington and Leavitt 1999). Another challenge of using carotenoid pigments is that a marker thought to represent a particular algal group, may actually be present in more than one group. Zeaxanthin is generally considered indicative of cyanobacteria but is also found within rhodophytes (Takaichi 2011) and chlorophytes (Schlüter et al. 2006). Most species of rhodophytes are found in marine environments but chlorophytes are an important freshwater algal group (Graham et al. 2006). Zeaxanthin can also co-elute with lutein (a pigment found in chlorophytes and euglenophytes), but in most of our chromatograms we were able to distinguish these peaks.

Similar to carotenoid pigments, DNA is also subject to degradation in sediment (Fernandez-Carazo et al. 2013) although cold temperatures and anoxic conditions are thought to mitigate this (Coolen et al. 2004; Willerslev and Cooper 2005; Anderson-Carpenter et al. 2011). Two of the sites in this study (Lac La Pêche and Lac des Loups) are shallower and have more variable O_2 content in deeper waters with weaker stratification. However, both these lakes (and the others included in our study) exhibit lower O_2 content at the sediment-water interface in the late summer. The amount of DNA degradation may also differ among taxa. For instance in a sediment core representative of a 2700 year span, diatom DNA was found to degrade much less in comparison to dinoflagellate DNA (Boere et al. 2011). There is also the potential that DNA of vegetative cells degrades more relative to DNA in akinetes of Nostocales taxa (Wood et al. 2009). Amplification could also be dependent on gene fragment size, as smaller gene fragments are better preserved in sediment. In this study, we found that the cyanobacterial 16S rRNA and *glnA* gene copies were significantly positively correlated through all cores. Fragment size should not be a factor between these two genes as they are both <300 bp in length. However, the mean percent increase in *glnA* copies was lower in all cores after 1980 compared to the change in cyanobacterial 16S rRNA gene copies. These results suggest that the increases seen in cyanobacterial gene copies closer to the tops of the cores were not likely due to cyanobacterial DNA degradation with depth.

Another factor to consider when interpreting the results from molecular markers relates to interpreting abundance from the number of gene copies. The 16S rRNA gene is present in multiple copies in each cell whereas the *glnA* gene is a single copy eubacterial functional gene (Stoeva et al. 2014). In cyanobacteria, there are one to four copies of the rRNA operon per cell, depending on the species (Schirrmeister et al. 2012). The significant positive correlation observed between cyanobacterial 16S rRNA and *glnA* gene copy numbers strongly suggests that the patterns seen in the cyanobacterial 16S rRNA gene copy numbers are indicative of changes in overall cyanobacterial abundance and not changes in copy numbers per cell of rRNA operons. Variation in the number of ribosomal operons is lower within cyanobacteria than in other bacteria, though 16S rRNA copy numbers show low variation in general compared to other genes (Schirrmeister et al. 2012).

The primers for the cyanobacterial 16S rRNA gene used in our study were originally designed for a study of sediment cores derived from Lake Erie (Rinta-Kanto et al. 2009). Rinta-Kanto et al. found cyanobacterial 16S rRNA gene copy numbers ranging from 9.5×10^4 to 6.2×10^7 g^{-1} of wet sediment. Using the same primers, Ye et al. (2011) reported a range of 2.05×10^5 to 2.20×10^6 gene copies g^{-1} in sediment cores taken from the eutrophic Lake Taihu, China. Our results covered a wider range of values (1.01×10^2 to 1.91×10^8 gene copies g^{-1} of wet sediment). The values reported by both previous studies were from one lake each whereas our results are from five different lakes, exhibiting a wider range in chemical conditions.

A final consideration that is apparent in all paleolimnological studies is the potential for bioturbation of sediments. Bioturbation is known to recruit viable cyanobacteria in resting stages within the sediment back to the water (Adámek and Maršálek 2013). For example, the presence of crayfish was found to be a significant variable in the recruitment of bloom-forming cyanobacteria (including *Microcystis* and *Anabaena*) from sediment during the growing season in ponds (Yamamoto 2010). The saw-tooth pattern evident in the unsupported ^{210}Pb profiles of several of our cores over the first few centimetres may be indicative of surface sediment mixing but could also be explained by recent increases in autochthonous productivity which can dilute atmospherically derived inputs of ^{210}Pb (Binford 1986).

3.6 Conclusion

The DNA analyses of lake cores suggest that cyanobacteria have increased since European settlement in Western Quebec, particularly since substantial warming circa 1980. We observed no significant difference in percent increase of cyanobacteria of lakes within the protected area compared to those outside, indicating that factors affecting cyanobacterial dominance and growth may be in part regional and beyond control of the Park's boundary. As there was general corroboration between the molecular and pigment data sets, we argue that the integration of molecular techniques into analyses of sediment cores has the potential to yield more comprehensive and sensitive descriptions of past ecological conditions in temperate lakes.

Table 3.1: Depositional record and sedimentation rates for lake cores from Western Quebec. Age models from Meech, Philippe, la Pêche, and Gauvreau lake cores were developed by applying the CRS model whereas the CFCS model was applied to the des Loups core. Meech, Philippe, and la Pêche are located within Gatineau Park whereas des Loups and Gauvreau are located outside the Park boundary

Lake	Water depth at core site (m)	Core length (cm)	Estimated age at maximum core depth (yr CE)	Range in sedimentation rates (g cm^2 /y)	TP seasonal range (µg/L)[1]
Meech	20	26.5	1730	0.009 – 0.037	5-12
Philippe	15	24.5	1800	0.005 – 0.021	6-16
la Pêche	9	40	1578	0.001 – 0.009	5-17
des Loups	8	34.5	1682	0.0093	7-32
Gauvreau	20	32.5	1778	0.008 – 0.174	18-36

[1] LeBlanc *et al.* 2008. The data represent 2002-2007.

Table 3.2: Mean (±SE) total DNA concentrations and purity (A_{260}/A_{280} and A_{260}/A_{230} ratios; n=11 to 18). The qPCR efficiency and correlation coefficients (R^2) are based on the C_q values from the serial dilution of pooled samples from each core

Core	DNA (ng/g wet sediment)	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio	Dilution factor (for qPCR)	CYAN 16S rRNA		*glnA*	
					Efficiency (%)	R^2	Efficiency (%)	R^2
Meech	5623 (±502.1)	1.83 (±0.01)	1.70 (±0.08)	30x	89.9	0.97	105.1	0.99
Philippe	6118 (±761.7)	1.83 (±0.01)	1.95 (±0.03)	32x	105.9	0.96	97.7	0.99
La Pêche	2752 (±263.6)	1.74 (±0.05)	1.36 (±0.12)	25x	99.8	0.93	91.2	0.99
des Loups	2202 (±466.4)	1.93 (±0.02)	1.39 (±0.11)	43x	101.5	0.94	92.1	0.99
Gauvreau	2122 (±265.3)	1.69 (±0.02)	1.21 (±0.06)	10x	93.5	0.97	94.5	0.99

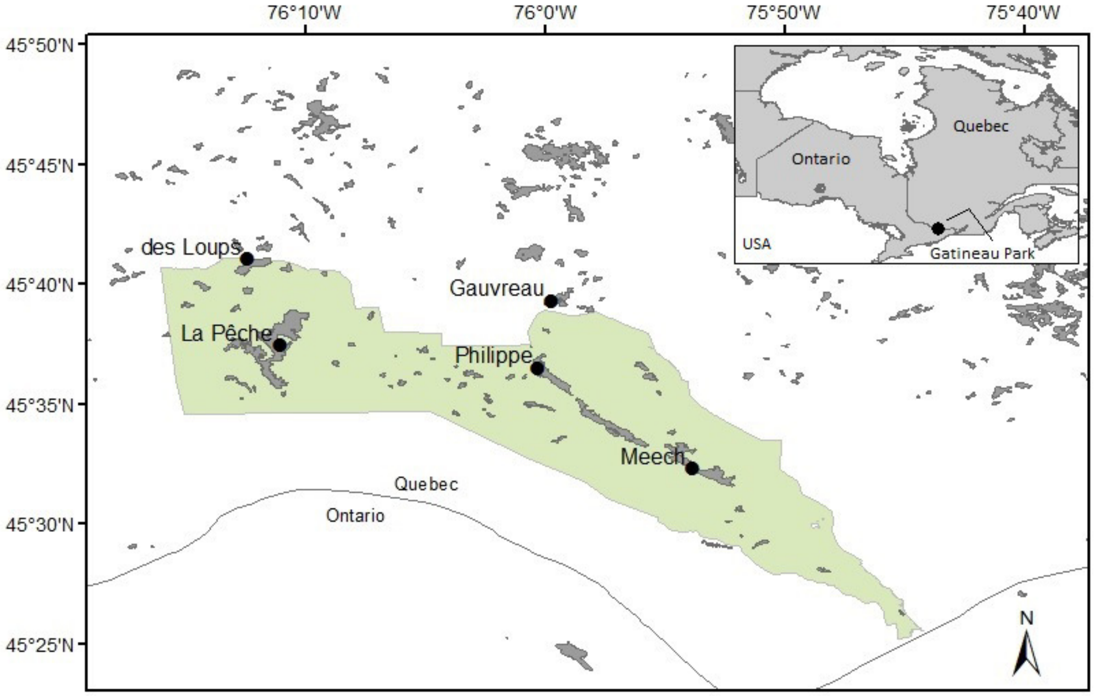

Figure 3.1: Map of Western Quebec showing location of the 5 sampling sites with the outline of Gatineau Park indicated as the shaded area. Inset is of the Canadian provinces Ontario and Quebec.

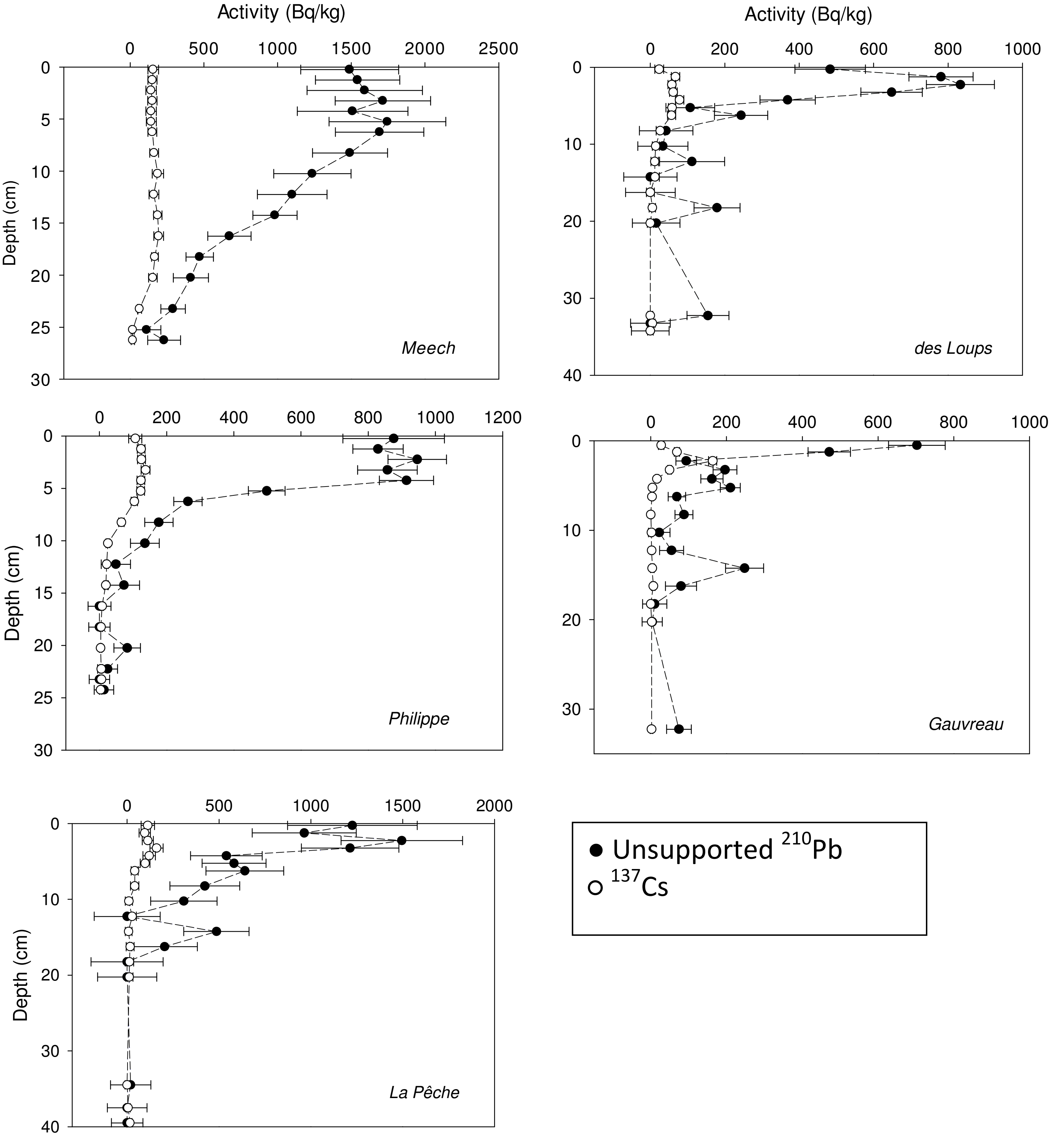

Figure 3.2: Unsupported ^{210}Pb and ^{137}Cs (Bq/kg, ±error) in depth profiles of sediment cores taken from Gatineau Park and surrounding area.

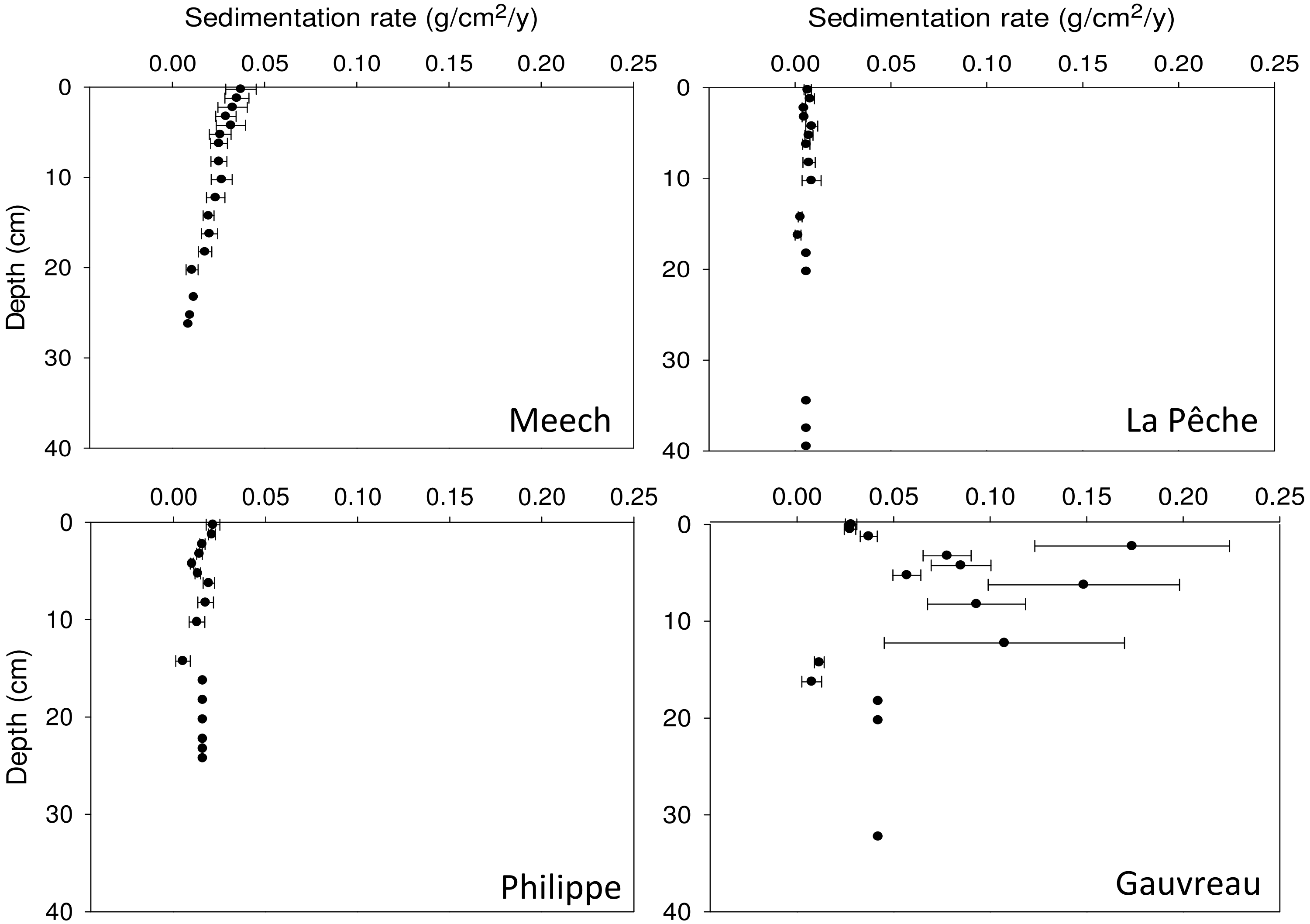

Figure 3.3: Sedimentation rate (±error) determined through the Constant Rate of Supply (CRS) model through each core taken in the Gatineau Park area: The Lac des Loups sedimentation rate (±SE) was assumed constant at 0.0093 (±0.0031) g/cm^2/y as the Constant Flux Constant Sedimentation (CFCS) model was used to fit sedimentation data and was therefore not plotted.

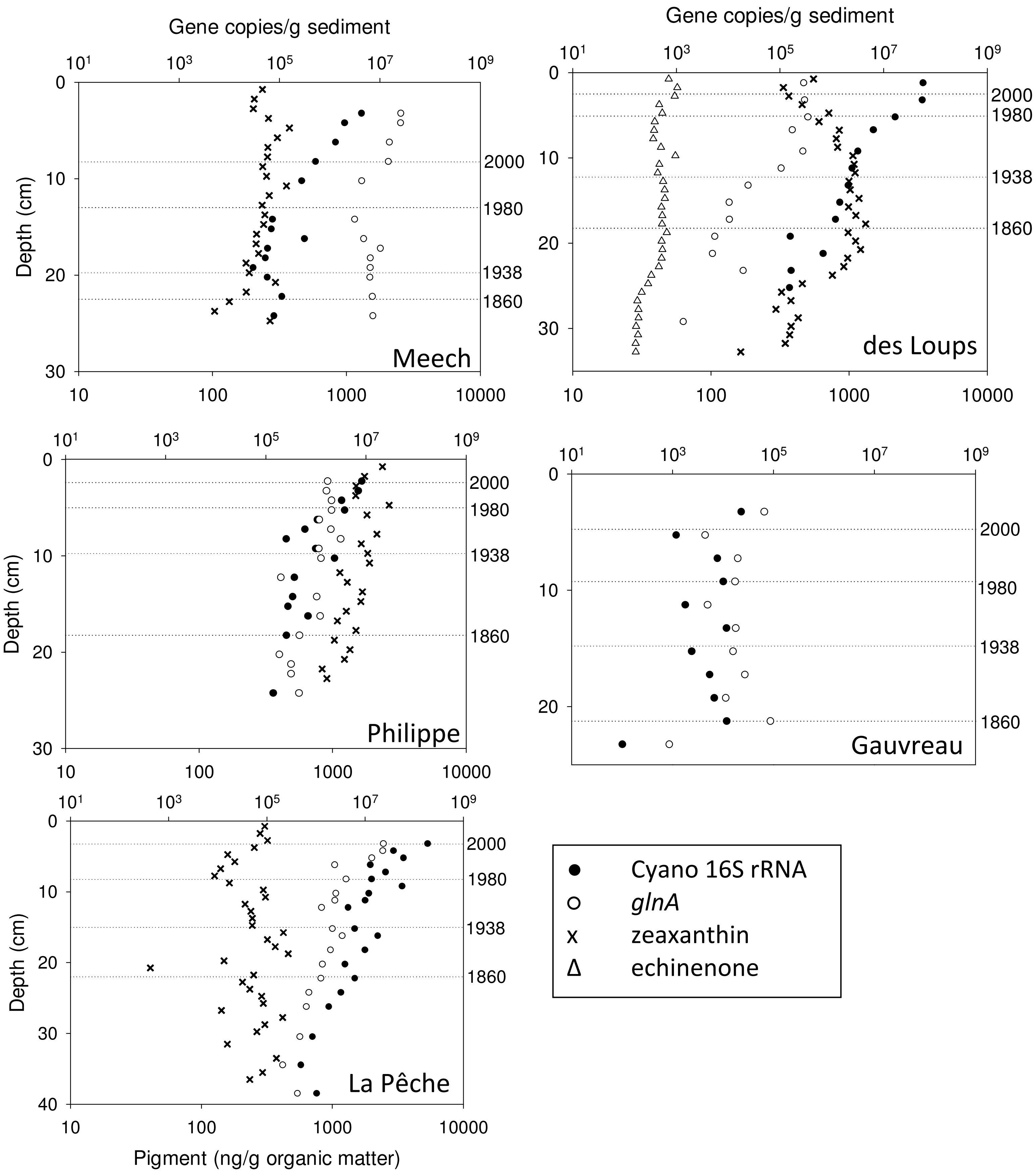

Figure 3.4: Depth profiles of cyanobacterial 16S rRNA and *glnA* gene copies/g of wet sediment along with the carotenoid pigments zeaxanthin (where included in analysis) and echinenone (where detected)/µg of organic matter presented on a logarithmic scale. Relevant dates: 1938 represents the creation of Gatineau Park and 1980 represents the start of significant increases in global air temperature.

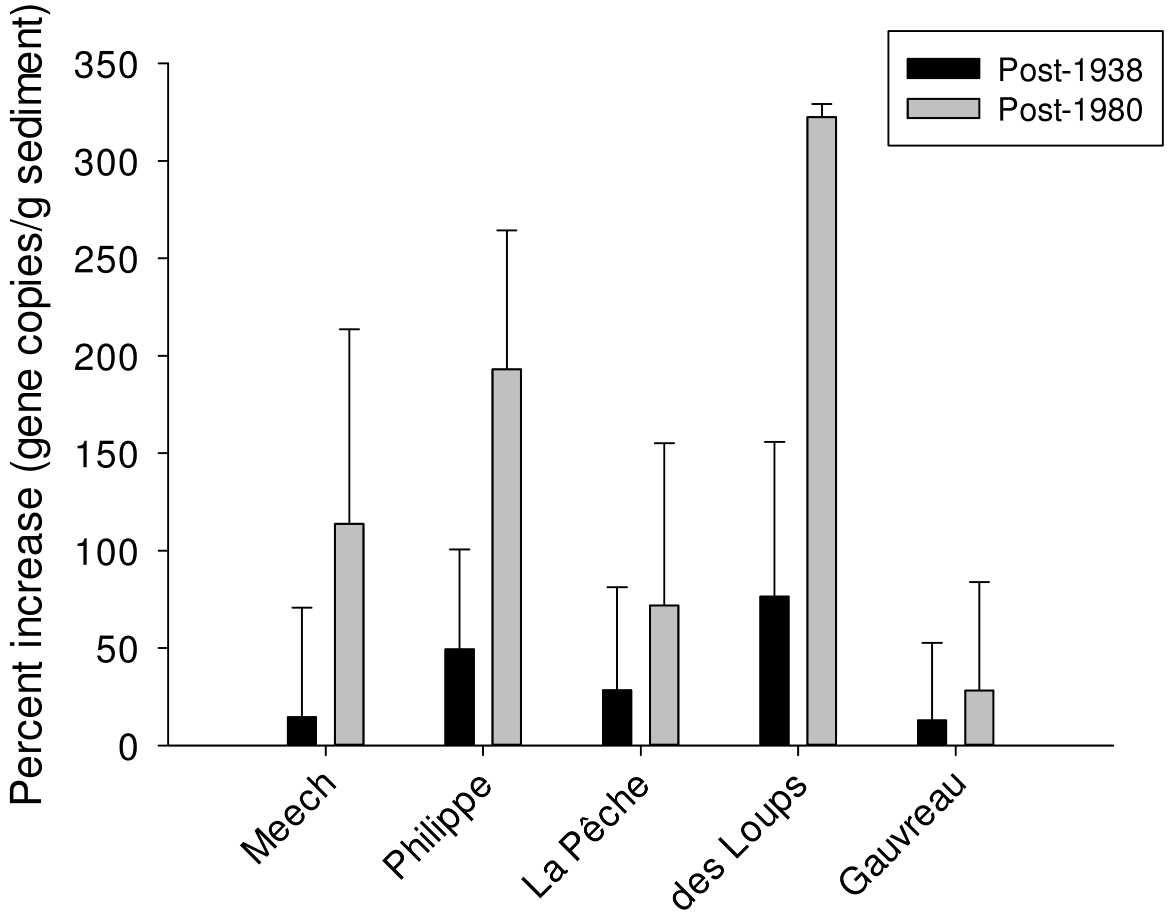

Figure 3.5: Mean percent increase (±SE) of gene copies/g of cyanobacterial 16S rRNA in wet sediment since 1938 and 1980 compared to the historical average (past 150 years) through each core.

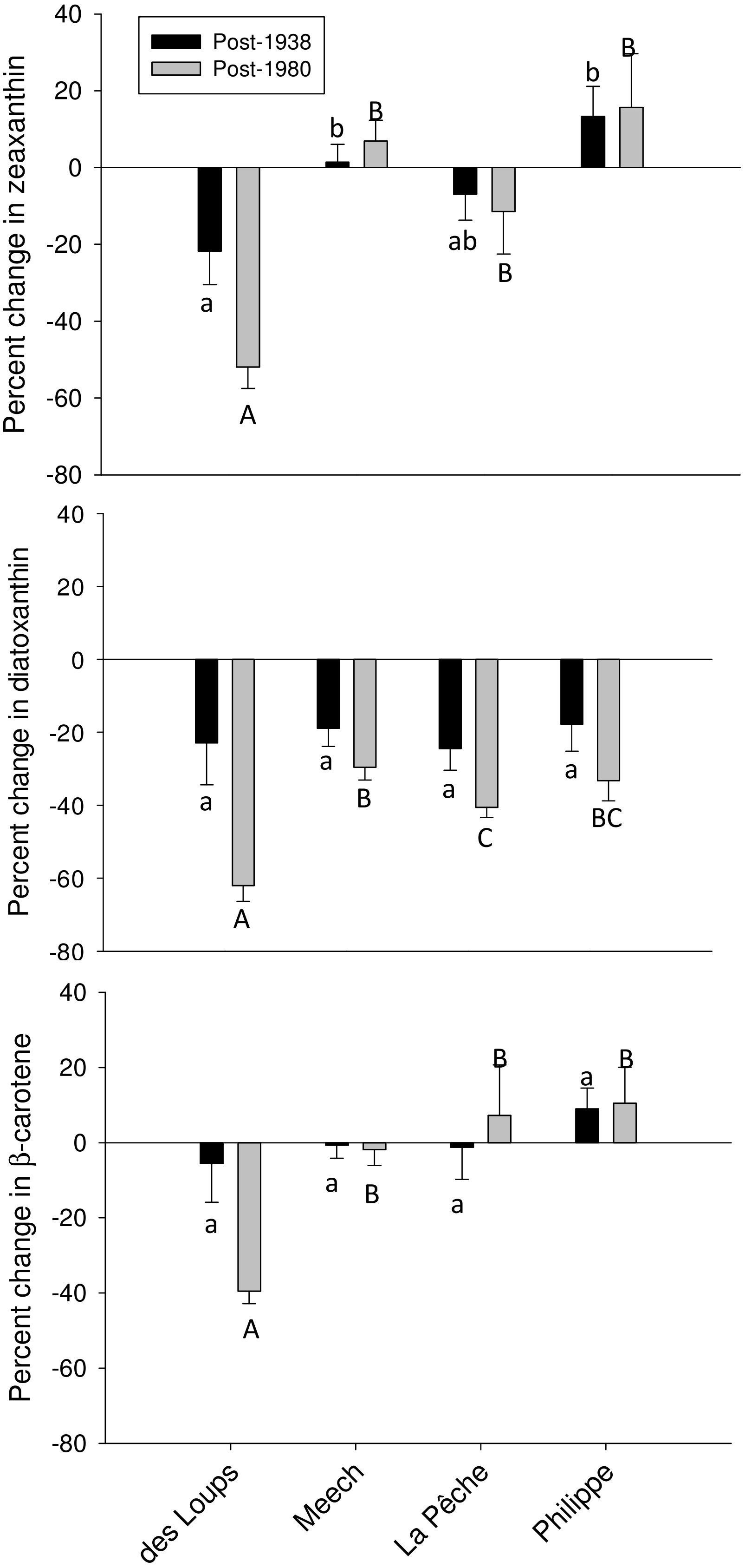

Figure 3.6: Mean percent change (±SE) of the carotenoid pigments/µg of organic matter: zeaxanthin (top), diatoxanthin (middle) and β-carotene (bottom) since 1938 and 1980 compared to the historical average (past 150 years) through each core where pigments were analyzed.

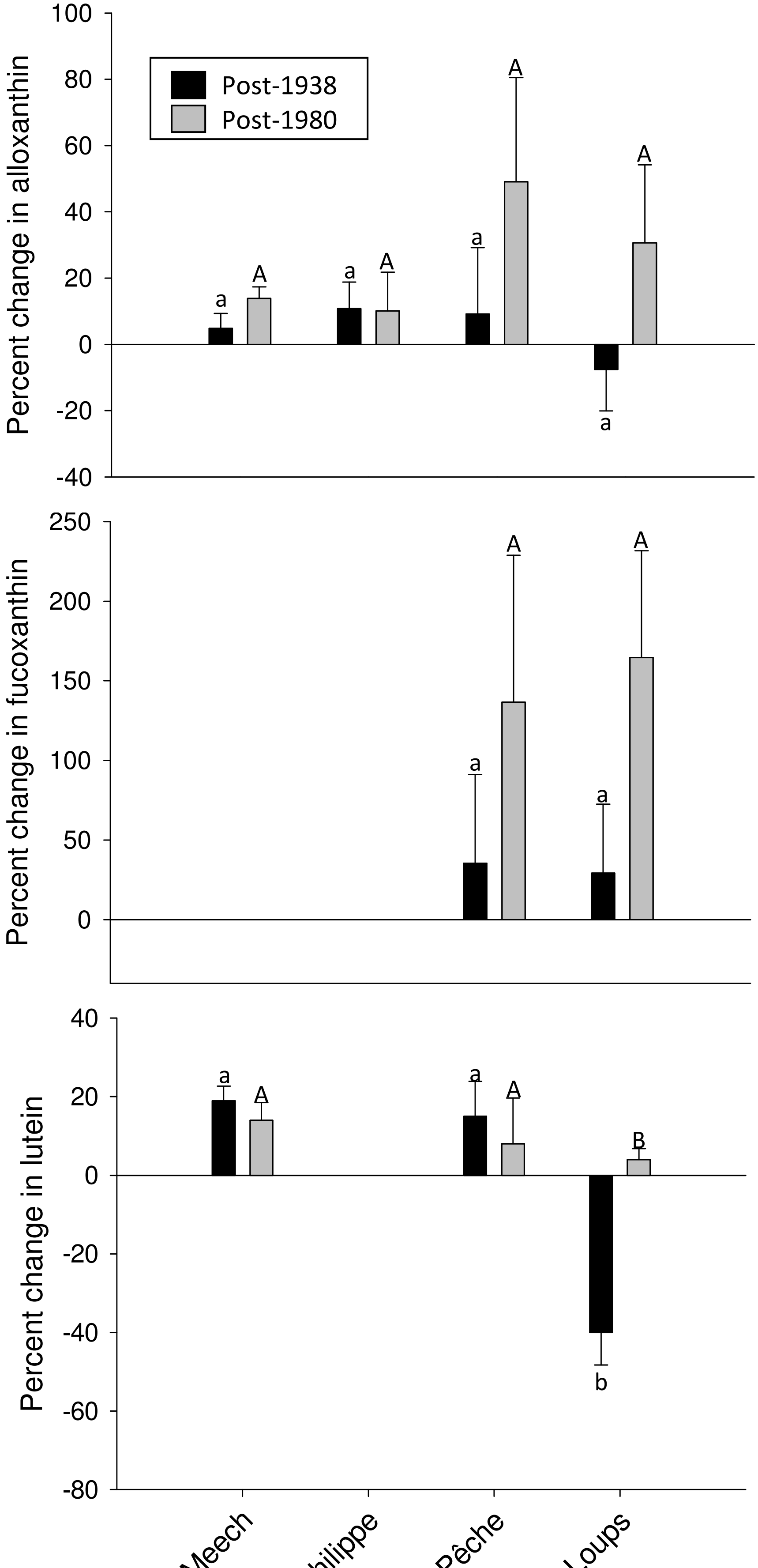

Figure 3.7: Mean percent change (±SE) of the carotenoid pigments/μg of organic matter: alloxanthin (top), fucoxanthin (middle) and lutein (bottom) since 1938 and 1980 compared to the historical average (past 150 years) through each core where pigments were analyzed.

CHAPTER 4: THE HISTORY OF TOXIC CYANOBACTERIA – THROUGH THE ANALYSIS OF LAKE SEDIMENT DNA

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Statement of Author Contributions

I conducted the molecular analyses with assistance from MR. I performed the statistical analyses and wrote the manuscript. Detection of microcystins was done by AZ. Cores were collected by ZT. Pigments were analyzed by ZT and IG-E. AP provided expertise in bioinformatics. FP supervised the project.

4.1 Abstract

Paleolimnological analyses have enabled determination of historical occurrences of cyanobacterial blooms using pigment data and microscopy. The increasingly efficient and accurate detection of archived sediment DNA could complement and enhance such analyses. Combining analysis of gene copy numbers of the microcystin-synthesizing gene (*mcyD*) and concentrations of sediment microcystin concentrations, the past incidences of toxic blooms in a trans-boundary lake experiencing recent eutrophication (Lake of the Woods, Manitoba-Ontario-Minnesota) and a naturally eutrophic lake (Baptise, Alberta) were determined. In Lake of the Woods, these proxies of toxic cyanobacteria were only detected beginning in the early 1980s (*mcyD*) and 2000s (microcystin) and were significantly correlated with one another. In Baptiste Lake, both *mcyD* and microcystins could be detected throughout the entire core as far back as 1830. Microcystins in this system were not significantly correlated with the *mcyD* gene but did correlate to 16S rRNA gene copies representative of the genus *Microcystis*, a known toxin-producer. Additionally, pyrosequencing of genes specific to cyanobacteria in the Baptiste core suggested that cyanobacterial diversity has progressively decreased in recent times, although total abundance has increased based on both the cyanobacterial pigment echinenone and 16S rRNA gene copies representative of cyanobacteria. Overall, our results imply the emergence and increased abundance of toxigenic cyanobacteria (Lake of the Woods) and a recent decrease in diversity (Baptiste Lake). Increased monitoring and mitigation efforts are advisable for these lakes to ensure the temporal trends in toxic cyanobacteria observed from the sediment record are kept in check.

4.2 Introduction

Cyanobacterial blooms are known to occur in lakes with high nutrient levels and warm water temperatures and they appear to be increasing in intensity and frequency, according to monitoring reports (Zhang and Prepas 1996; Paerl and Huisman 2008; Liu *et al.* 2011; Winter *et al.* 2011; Beaulieu *et al.* 2013). Many blooms include or are dominated by cyanobacterial strains that produce toxins linked to acute and chronic toxicity (Chorus and Bertram 1999; Funari and Testai 2008). However, the environmental conditions and potential physiological cues that cause a bloom to be toxic remain unclear. The hepatotoxic microcystin is one of the most ubiquitous cyanotoxins found in freshwater systems and has been monitored in lakes of Western Canada for some time now (Kotak and Zurawell 2007). Exposure to microcystins has lead to mortality in biota such as fish and sea otters (Malbrouck and Kestemont 2006; Miller *et al.* 2010), as well as humans (Yuan *et al.* 2006).

There are few long-term monitoring programs of algae and cyanobacteria and it is therefore difficult to determine whether blooms are indeed increasing and whether they are indeed more toxic. As a result, dated sediment cores are often used to track algal communities using various proxies. The fossil remains of some species lend well to identification using light microscopy, such as the silica frustules of diatoms and scales of some chrysophytes (Smol 2008). Some cyanobacterial taxa enter a resting stage (akinetes) and can therefore be detected in a similar way (van Geel *et al.* 1994; Räsänen *et al.* 2006). Others produce characteristic pigments that can be extracted from sediments. Carotenoid pigments representative of cyanobacteria include echinenone and zeaxanthin (Leavitt and Hodgson 2001).

Taxa that do not leave behind fossil remains or produce pigments common to multiple groups can be challenging to identify through the sediment record. Over the last decade, molecular tools have been developed to serve as markers of particular species or groups in sediment cores both qualitatively (through conventional PCR) and through quantitative PCR. A number of studies have reconstructed historical conditions of certain bacteria by using gene fragments specific to the species of interest (Coolen *et al.* 2004; Li *et al.* 2006; Jiang *et al.* 2007; Brazeau *et al.* 2013; Stoeva *et al.* 2014). Few studies report the use of molecular proxies for cyanobacteria in dated sediment cores, but preliminary studies show promise (Rinta-Kanto *et al.* 2009; Ye *et al.* 2011; Kyle *et al.* 2015). In the last few years, advances in next generation sequencing of genes amplified from environmental DNA have additionally made it possible to reconstruct community diversity (Coulon *et al.* 2012; Shokralla *et al.* 2012; Lejzerowicz *et al.* 2013).

Microcystin is synthesized by a 55-kb gene cluster (Tillett *et al.* 2000) composed of a non-ribosomal peptide synthetase (NPRS) coupled with a polyketide synthase (PKS; Dittmann *et al.* 2001). The gene cluster responsibly for its synthesis (*mcy*) is present in all toxic strains of cyanobacteria that are known producers of microcystin (Neilan *et al.* 1999). To our knowledge, the *mcy* gene cluster has not been traced through sediment cores. Part of the cluster (*mcyD*) is involved in producing the Adda moiety of microcystin which is mainly responsible for the toxicity of the compound (Jaiswal *et al.* 2008) and is a highly conserved region of the gene cluster. In this study, the *mcyD* gene was used to trace the pattern of toxic blooms in sediment cores taken from two temperate lakes of different trophic states and was compared to microcystin concentrations determined in the same cores. To interpret toxic blooms in the context of total cyanobacterial abundance and diversity, we sequenced a 16S rRNA gene region specific to cyanobacteria. Our objectives were to first determine whether the *mcyD* gene fragment could successfully be quantified using DNA extracted from sediment cores and whether when quantified, it could serve as a suitable proxy for microcystin-producing cyanobacteria and/or correlate to microcystin concentrations. Our second objective was to assess the value of a pyrosequencing approach to quantify changes in cyanobacterial community structure through time to and relate these changes to bloom toxicity.

4.3 Methods

Study sites and Sampling

Two lakes in western Canada were chosen based on reports of microcystin-producing blooms in both. Lake of the Woods is a freshwater body that lies in the provinces of Ontario and Manitoba along with the state of Minnesota, USA, covering a total area of 3850 km^2 with a watershed of 14,864 km^2 (Gartner Lee Limited 2007). The phosphorus concentrations in Lake of the Woods have varied over time and across different bays of the lake (DeSellas *et al.* 2009). Baptiste Lake in Alberta is much smaller in comparison (roughly 10 km^2) and is a naturally eutrophic water body with a watershed that covers an area of 310 km^2 (Mitchell and Prepas 1990).

Two sediment cores were collected from Hay Island of Lake of the Woods (49° 9' 25.6", 94° 7' 23.2”) and the south basin of Baptiste Lake, Alberta (54° 44' 19.7", 113° 33' 7.4") in 2010 (Fig. 4.1). With the aid of bathymetric maps, the deepest part of each lake was located. To retrieve cores, a Glew gravity corer was used. The cores used in this study were 38.5 and 46 cm in length from Lake of the Woods and Baptiste Lake respectively. At the shore, cores were extruded out of the tubes and packaged in Whirl-pak bags (Fisher Scientific, Canada) in 0.5 cm intervals. The samples were kept in a cooler on ice and promptly transferred to a -20°C freezer upon return to the laboratory.

^{210}Pb dating

Samples were processed for dating as indicated in Chapter 3. For both cores, three models of chronology were constructed (Constant Rate of Supply, Constant Initial Concentration, and Constant Flux Constant Sedimentation) and fitted for ^{137}Cs data and ^{210}Pb data (Benoit and Rozan 2001). Based on the fits, the Constant Rate of Supply model was chosen for both cores. Further details on sampling and core dating have been outlined previously (Zastepa 2014).

Microcystin and pigment quantification

Microcystins were analyzed through LC-MS (liquid chromatography-mass spectrometry) following the method developed by Zastepa *et al.* (2014). Sediments were first filtered out of pore water and then extracted using Dionex accelerated solvent extraction (ASE). Microcystins were then quantified using High Performance Liquid Chromatography (HP series 1100 HPLC-PDA fitted with a Zorbax Eclipse XDB-C18 column) against certified standards. Total microcystin concentrations reported here are based on combined values of the dominant congeners microcystin-LR, -^{7dm}LR, -RR, -YR, -WR, -LA, -LF, -LY, -LW with method detection limits ranging between 0.3 and 2.5 ng g^{-1} dry weight. Samples that needed more detailed analyses were run through Liquid Chromatography-Mass Spectrometry (LC-MS/MS). The LC (Agilent 1200 series) was coupled with a triple-quadropole mass spectrometer (AB Sciex 3200) equipped with a turbo electrospray ionization source.

The cyanobacterial pigments zeaxanthin and echinenone were measured using High Performance Liquid Chromatography (HPLC) following previously described procedures (Zapata *et al.* 2000; Saulnier-Talbot *et al.* 2014). Zeaxanthin and echinenone are both cyanobacterial pigments (Lami *et al.* 1992) and are thus used as markers of cyanobacterial abundance, although zeaxanthin is known to co-elute with lutein. To remain consistent with previous analyses of this core, echinenone was used as a proxy for cyanobacterial abundance instead of zeaxanthin (Taranu 2014). In brief, sediments were freeze-dried and pigments extracted in acetone (-20°C for 24 hours). The acetone was centrifuged and the supernatant filtered (0.2μm) and placed in vials for HPLC analysis (Waters, model 600/626 with a Waters Photodiode Array 2996, a Waters 2475 Multi λ Fluorescence detector and a refrigerated waters autosampler 717). Pigment data were expressed per gram of organic matter by calculating the loss on ignition (LOI) at 550°C of dried sediment. To see if there was a relationship between cyanobacterial abundance and diversity in the Baptiste core, we used echinenone and cyanobacterial 16S rRNA gene copies as proxies of abundance.

DNA extraction

DNA was extracted using the Powersoil DNA extraction kit as described in Chapter 3. All DNA was quantified using the Quant-iT DNA assay kit (Invitrogen), following the kit protocol. For positive controls (PCR and gel electrophoresis) and in order to create standard curves for qPCR, DNA was extracted from pure cultures of the toxin-producing *Microcystis aeruginosa* strain CPCC 300 (Canadian Phycological Culture Centre) using a phenol-chloroform extraction (Hisbergues *et al.* 2003; Fortin *et al.* 2010). Strain CPCC 300 is a known toxin-producer (Renaud *et al.* 2011).

Quantitative PCR amplification

The sequences, amplicon length, and annealing temperature of all primers used in this study are presented in Table 4.1. The primers used to quantify the *mcy* gene cluster targeted the *mcyD* gene and were originally designed by Fortin *et al.* (2010), based on all *mcyD* sequences available in GenBank in March 2006, using the β-ketoacyl synthase region of the gene (product of 107 bp; $mcyD_{ks}$). To complement *mcyD* results, total abundance of cyanobacteria was also quantified using a region of the 16S rRNA gene specific to cyanobacteria (Nübel *et al.* 1997; Rinta-Kanto *et al.* 2005). To further understand changes in cyanobacterial toxicity through time, the 16S rRNA gene fragment specific to the genus *Microcystis* was also amplified (Rinta-Kanto *et al.* 2005). Because the 16S rRNA is present in multiple copies, dependent on the bacterial group (Sun *et al.* 2013), the mono-copy gene for glutamine synthetase (*glnA*; Hurt *et al*. 2001), present in all bacteria, was used to ensure changes seen in the other genes were not simply reflective of the amount of DNA extracted or based on changes in gene copy numbers. After basic PCR amplification and gel electrophoresis of culture and sediment samples, bands representative of each gene fragment were sequenced to ensure they represented the correct gene. A culture known to not produce microcystin (CPCC 632) was used as a negative control for the *mcyD* primers and similarly, a green algae culture (*Pseudokirchneriella subcapitata*) was used to verify the cyanobacterial 16S rRNA primers.

To create a standard curve for absolute quantification, each gene used was cloned into a plasmid before creating a standard curve to use for subsequent quantitative PCR (qPCR) using the Strataclone kit (Agilent Technologies) according to the manufacturer's instructions as described in Chapter 3. All qPCR amplifications were performed using the Bio-rad CFX96 Real-Time PCR Detection Module and all results were analyzed using Bio-Rad CFX Manager Version 3.0. Each PCR mixture contained 1 μL of template, 7.5 μL of SsoFast EvaGreen Supermix (Bio-Rad), and 0.1 μM of each primer (forward and reverse) brought up to a final volume of 15 μL. All samples were loaded in 0.2 mL 8-strip tubes (Bio-Rad) in triplicate. The qPCR conditions were as follows: preheating at 95°C for 3 min followed by 35 amplification cycles of 10 s at 95°C and 10 s at 55°C. This was followed by a melt curve from 60°C to 95°C at 0.5°C increments with 5 s holds. The annealing temperature for each primer set (Table 4.1) was first optimized using a thermal gradient surrounding the primer melting temperatures. In order to test for amplification inhibitors in the extracted DNA, samples from each sediment core were first pooled into a mix and then diluted by a factor based on the lowest C_q from the thermal gradient results for each set of primers to a C_q of 35 (limit of detection). The efficiency (E) scores and r^2 values from these dilution tests and the plasmid DNA dilutions used to create standard curves were consistently between the recommended guidelines of 90-110% (E) for the slope, and >0.98 (r^2) for the regression line (Taylor *et al.* 2010). The mean efficiencies based on the serial dilutions of plasmid DNA used to generate each standard curve are presented in Table 4.2.

Pyrosequencing

Samples were sequenced by Molecular Research DNA (MR DNA, Austin, Texas) as originally described by Dowd *et al.* (2008) using 16S rRNA cyanobacterial primers targeting the variable V3 and V4 regions of the gene (Table 4.1; Nübel *et al.* 1997; Jungblut *et al.* 2005). Initially, samples from 6 core sections at midpoint depths of 0.25 (top), 11.25, 21.25, 31.25, 41.25, and 45.75 cm (the bottom) were used for pyrosequencing analysis. After analyzing these results, a better resolution of the first 10 cm was deemed appropriate and therefore the midpoint depths of 2.25, 5.75, and 7.75 cm were added to the pyrosequencing analysis. To validate the addition, DNA from the 0.25 and 11.25 core sections was re-sequenced on the second run. We found that although there was a difference in total number of sequences obtained, there was no difference between the first and second run with respect to cyanobacterial orders represented by the sequences once grouped into OTUs.

Initial data trimming was performed using the program MOTHUR (v 1.33.3; Schloss *et al.* 2009). The quality file was used to eliminate sequences with an average Phred quality score below 25. Low-quality sequences (length less than 100 and over 1000 bp) were removed as well as sequences with ambiguous bases. Remaining sequences were then aligned using Silva reference files and chimeras were subsequently removed using Chimera Slayer (Haas *et al.* 2011). Chimeras are hybrid sequences often present after PCR amplification that may be falsely interpreted as novel sequences. Sequences representative of cyanobacteria were identified using the online RDP classifier, which is specifically designed for classifying bacterial 16S rRNA sequences (Wang *et al.* 2007; Cole 2014). Sequences not identified as cyanobacterial by the RDP classifier were removed from further analyses. Previous studies have found non-cyanobacterial species amplified using cyanobacterial-specific primers and have similarly filtered them out of downstream analyses (Kleinteich *et al.* 2014).

Cyanobacterial sequences were imported into the Quantitative Insights into Microbial Ecology (QIIME) program (v.1.8.0; Caporaso *et al.* 2010) . The sequences were again passed through initial screening processes thus removing any sequences with a homopolymer of 6 bases or more (other QIIME trimming conditions having already been met using MOTHUR). Sequences were then grouped according to operational taxonomic unit (OTU) and a representative sequence was chosen for each OTU at 97% similarity. Rarefaction curves were constructed using the number of OTUs observed based on sampling depth (i.e. number of sequences sampled). The maximum sampling depth was set to the sample with the lowest number of sequences, which corresponded to 1137 sequences. Both weighted and unweighted UniFrac analyses were performed to assess the relationship between samples using Jackknife support (maximum sequences sampled set to 1137) to establish whether clustering was of statistical significance (Lozupone *et al.* 2011). Weighted UniFrac analysis is a quantitative measure of β-diversity, suitable for use even if the overall groups of organisms remain the same (Lozupone *et al.* 2007).

Data Analysis

Pearson's correlation analyses of gene copy numbers, microcystin and echinenone concentrations, and Shannon diversity index values were conducted using JMP version 11. Variables that did not fit a normal distribution, as identified by the Shapiro-Wilk test, were transformed to meet assumptions of correlation analyses. Gene copy numbers which were past the method detection limit were replaced by half the detection limit. The limit of detection of the starting quantity (SQ) of DNA, which was used to calculate the number of gene copies, was determined by using the linear equation derived from the standard curve (C_q=slope(log(SQ) + y-intercept) where the limit of detection (C_q) is assumed as 35 cycles (Bustin *et al.* 2009).

4.4 Results

Gene copy numbers in comparison to microcystin

Based on the chronology established from ^{210}Pb dating, the Lake of the Woods core dated from 1858 to 2010 and the Baptiste Lake core covered a timeframe from 1824 to 2010. DNA was successfully extracted from all sections sampled from each core. The Lake of the Woods core had a mean (±SE) DNA concentration of 10.9 (±1.7) ng/µL with a range of 1.7 ng/µL to 37.4 ng/µL. The *mcyD* gene was not detected in the Lake of the Woods core below a depth of 13 cm of sediment (corresponding to 1983) and the *Microcystis* 16S rRNA gene was not detected below a depth of 16 cm (corresponding to 1971; Fig. 4.2A, B). Nevertheless, the *mcyD* gene and *Microcystis* 16S rRNA were positively correlated in the Lake of the Woods core ($p<0.05$; Table 4.3). In contrast to the toxin-synthesizing gene and *Microcystis* gene copies, the cyanobacterial 16S rRNA gene was detected all through the Lake of the Woods core (Fig. 4.2C).

The mean (±SE) concentration of DNA through the Baptiste core was 13.4 (±1.3) ng/µL with a range of 3.2 to 33 ng/µL. The gene copy numbers of both *mcyD* and *Microcystis* 16S rRNA fluctuated throughout the core (Fig. 4.3A, B) and were significantly correlated ($p<0.05$; Table 4.4). The cyanobacterial 16S rRNA gene copies in the Baptiste core increased over time, to a greater extent than what was seen in the Lake of the Woods core (Fig. 4.3C). Gene copies representative of total cyanobacteria were significantly positively correlated to *mcyD* gene copies and to *Microcystis* 16S rRNA gene copies ($p < 0.05$).

Although certain primer sets were unable to detect gene copies at certain core depths, the *glnA* primers yielded a positive signal at all sections analyzed through both cores (Fig. 4.2D; 4.3D). This result validated the down-core patterns seen using the other primer sets, meaning the depths at which certain cyanobacterial genes were not detected was not simply a consequence of poor DNA yield or quality. The *glnA* gene copies were significantly positively correlated to cyanobacterial 16S rRNA gene copies in both cores (Table 4.3; 4.4). This indicated that the trends in cyanobacterial 16S rRNA were not a result of changes in the number of 16S rRNA gene copies per cell which can lead to misinterpretation of 16S rRNA results for prokaryotes (Sun *et al.* 2013; Stoeva *et al.* 2014).

The microcystin concentrations (Appendix B; Figure B.1) significantly correlated to *mcyD* gene copies in the in the Lake of the Woods core, though the gene copies representative of the genus *Microcystis* did not ($p<0.05$; Fig. 4.4A). Through the Baptiste Lake core, the microcystin concentrations (Appendix B; Figure B.2) were positively correlated to the gene copies of *Microcystis* 16S rRNA ($p<0.01$) but not to gene copies of *mcyD* (Fig. 4.4B) or cyanobacterial 16S rRNA (Table 4.4).

Pyrosequencing through the Baptiste Lake core

Because of the presence of both *mcyD* gene and microcystin concentrations throughout the Baptiste Lake core, total cyanobacterial diversity based on the cyanobacterial 16S rRNA gene was determined through pyrosequencing to determine if community change had occurred despite temporal uniformity in toxic cyanobacteria. From the 9 samples used to represent the Baptiste Lake core, between 5219 and 25457 sequences were obtained through pyrosequencing based on each sample (Table 4.5). After initial data trimming (based on conditions mentioned earlier) between 1137 and 11168 sequences remained that were classified as cyanobacterial using the RDP classifier (Cole 2014). To assess whether changes in diversity affected the toxicity of blooms, both microcystin and the gene copies of *mcyD* were compared to the down core diversity, as represented by the Shannon Index and total OTUs. We found no correlations between *mcyD* gene copies or microcystin to the Shannon Index or the total number of OTUs.

Down core community diversity in Baptiste Lake

The rarefaction curves did not plateau (Fig. 4.5), indicating that pyrosequencing did not reach a sequence depth fully representative of the cyanobacterial community. However, the rarefaction curves did show that species richness was highest at the bottom of the core. With the exception of the very top section (0 cm), the samples representative of more recent times exhibited less species richness. Cyanobacterial diversity, according to the Shannon index, also seemed to have decreased over time (Table 4.5). Our results show that Nostocales was the dominant cyanobacterial order (>75% of total cyanobacteria) through the whole core (Fig. 4.6a). On further analysis, the order Chroococcales (which included the genus *Microcystis*) accounted for less than 24% of the total cyanobacteria through the core (Fig. 4.6b). Conversely, within Nostocales, the genus *Dolichospermum* (previously *Anabaena*) consistently accounted for more than 85% of Nostocales (Fig. 4.6c). To test whether there was a down-core pattern in community composition, samples were clustered using a jackknifed approach for UniFrac analysis. The core sections with a significant portion of Chroococcales or specifically, *Microcystis* (Fig. 4.7) which included 0.25, 31.25, and 45.75 cm, formed a monophyletic group with strong confidence (>75% jackknife support, weighted UniFrac). The other sections that composed another monophyletic group were composed mostly of the order Nostocales. The core section representing a midpoint depth of 41.25 cm is separated from the others in this cluster, potentially due to a low presence of Chroococcales and Oscillatoriales (<5%) at this depth. Though both weighted and unweighted UniFrac analyses were initially performed, only results of the weighted analysis are presented. The samples all came from the same core and hence the weighted analysis was chosen so that potential sample cross-contamination from when the core was extruded would not affect results.

We found a significant negative relationship between echinenone and the Shannon Index of diversity (p=0.037) in the Baptiste Lake core. Though a negative trend is observed between the cyanobacterial 16S rRNA gene copies and the Shannon Index, this is not statistically informative due to a small samples size (n=5).

4.5 Discussion

Gene copies as proxies of toxin concentrations

Our unique set of data allowed us to evaluate the relationship between cyanobacterial genes quantities, diversity, and toxins over a temporal scale. We found significant positive correlations between genes representative of toxin producers and concentrations of total microcystins through both cores. In the Lake of the Woods, the *mcyD* gene was detected further down in the core (13 cm, 1983) than microcystin, which was not detected past 7 cm, corresponding to 2001. This suggests that molecular analyses are more sensitive than analytical methods and can thus provide information on bloom toxicity further back in time than can be determined by evaluating the toxin only.

In Baptiste Lake, though gene copies representative of microcystin (*mcyD*) were not significantly correlated to microcystins, gene copies of *Microcystis* 16S rRNA showed strong correlation to microcystin concentrations. Through this core, the microcystin concentrations ranged over one order of magnitude except for the top 2 cm of the core, where an increase of another two magnitudes was observed (Appendix, Fig. B.2). Microcystin concentrations and *mcyD* were both highest in 2008 (3340 ng/g; microcystin, 1.72 x 10^5 gene copies/g). The much smaller concentration range in microcystin compared to the Lake of the Woods core could explain the lack of correlation to *mcyD* gene copies in the Baptiste Core (Fig. 4.4). The large difference in concentrations at the top of the Baptiste Lake core could be due to differential degradation of microcystin congeners at this site (Zastepa *et al.* 2014). Also, the primers used to quantify the *mcy* gene were a result of aligning the 50 *mcyD* sequences available on GenBank at the time of primer design and were designed specifically to match toxic strains of *Microcystis aeruginosa* (Fortin *et al.* 2010). It is possible that the primers did not target toxin-producing strains of the genus *Anabaena* as efficiently as *Microcystis*.

Phosphorus levels and chlorophyll *a* (as a proxy for cyanobacterial abundance) can predict microcystin concentrations in water samples (Rantala *et al.* 2006; Rogalus and Watzin 2008; Otten *et al.* 2012). However, we found no relationship between total cyanobacteria (represented by cyanobacterial 16S rRNA) and microcystin concentrations in either core although microcystin was correlated to total cyanobacteria when represented by echinenone in the Baptiste core (Zastepa 2014). This illustrates the value of combining multiple potential indicators of microcystin. Although the molecular data did not always complement pigment or toxin data, both data sets independently showed a concurrent increase of both cyanobacteria and toxicity. The sharp increases in microcystin and to a lesser extent, *mcyD*, near the surface of the core, followed a trend seen in diatom-inferred TP and TKN in the same core (Adams *et al.* 2014). In comparison to the Lake of the Woods core, there was less overall historical variation in both cyanobacterial abundance and toxicity. The *mcyD* gene copies were significantly positively correlated to microcystin levels in the Lake of the Woods core. Such correlations have previously been observed in the plankton over the growing season in lakes (Ha *et al.* 2009; Fortin *et al.* 2010; Pimental and Giani 2013). Similarly, positive relationships between other cyanotoxins (nodularin and cylindrospermopsin) and the genes that synthesize them have also been reported in water bodies (Koskenniemi *et al.* 2007; Zhang *et al.* 2014).

Changes in cyanobacterial diversity

We found no correlation between *Microcystis* and total cyanobacteria using 16S rRNA primers in either core suggesting that the cyanobacterial community composition has been changing without affecting total cyanobacterial abundance. In the Baptiste core, where we analyzed certain sections using a pyrosequencing approach, a general decrease in diversity in cyanobacterial assemblages was observed over time. We also compared the abundance of *Microcystis* to levels of *mcyD* to examine the contribution of this genus to microcystin production in relation to other cyanobacterial genera known to produce microcystin (primarily *Anabaena* and *Planktothrix* (Chorus and Bertram 1999). Although *Microcystis* gene copies were significantly correlated to *mcyD* gene copies in both cores, gene copy numbers of *mcyD* consistently exceeded those of *Microcystis* 16S rRNA suggesting that other genera were responsible for producing the bulk of the toxin. These results were corroborated by our pyrosequencing dataset in the Baptiste Lake core. We found that *Dolichospermum* (previously *Anabaena*) composed the majority of the cyanobacteria seen all through the core. As *Anabaena* is a known microcystin producer, the high *mcyD* levels seen through the core are most likely a reflection of the presence of *Anabaena* and not *Microcystis*.

Toxic cyanobacteria in relation to nutrients and warming

Lake of the Woods is a complex water system with a number of bays and islands. Paleolimnological studies have shown shifts in algal community structure and water quality due to damming and logging at the onset of the 1900s and warmer air temperatures over the past few decades, recorded through the adjacent climate station of Kenora, Ontario (Rühland *et al.* 2010; Summers *et al.* 2012). Results from this study were based on cores taken from 4 locations within the Ontario part of the lake. Recent overall temperature increases were found to be exacerbating the effect of high phosphorus levels by making conditions more conducive to cyanobacterial dominance (Rühland *et al.* 2010). The increase in air temperature recorded in the area could explain the emergence of toxic blooms to Lake of the Woods which were not detected using molecular or analytical techniques before the 1970s in our sediment core. Data collected from surface water samples have shown a stronger relationship between water surface temperature and *mcy* gene copies than with total cyanobacteria, as determined by 16S rRNA gene copies; (Conradie and Barnard 2012), meaning that temperature may influence bloom toxicity more than total cyanobacterial abundance. In a subtropical lagoon in Uruguay the *sxtU* gene, which synthesizes saxitoxin, was recently quantified in a sediment core but only detected in samples from the year 2000 forward (Martínez De La Escalera *et al.* 2014).

Baptiste Lake has been consistently eutrophic over the past ~150 years (Adams *et al.* 2014). Paleolimnological evidence suggests the lake is becoming increasingly eutrophic since the watershed was subjected to human influence such as land-clearing activities beginning in the early 1900s (Hickman *et al.* 1990; Manning *et al.* 1999). Particularly, total Kjeldahl nitrogen (TKN) has increased by ~50% since the 1990s, based on water monitoring data (Adams *et al.* 2014). This is corroborated by the presence of *mcyD*, microcystin, and the *Microcystis* 16S gene lower down in the core in comparison to Lake of the Woods. The cyanobacterial 16S rRNA gene was not evaluated below 30 cm in this core because extracted DNA was expended in other analyses. We presume the signal would continue to the end of the core, given the other primer sets reveal continuous down core signals.

The enhancement of molecular approaches in environmental biology could help track cyanobacteria in the water column and in sediment cores, better developing potential links between certain environmental factors and potential toxicity. For instance, the proportion of the harmful genus *Microcystis* to picocyanobacteria (*Synechococcus*) was found to be positively correlated with total nitrogen and organic matter in a sediment core from Lake Taihu, China (Ye *et al.* 2011). Down-core diversity within picocyanobacteria was found to be related to diatom-inferred phosphorus levels in sediments from Lake Bourget (Domaizon *et al.* 2013). Nitrogen and phosphorus levels (Kotak *et al.* 2000; Graham *et al.* 2004; Dolman *et al.* 2012) and temperature (Wu *et al.* 2006) have been related to microcystin synthesis independent of the total abundance of cyanobacteria but the relative importance of these physiochemical factors is inconclusive due to their potentially additive or synergistic effects.

Pyrosequencing of the 16S rRNA gene has been used to identify cyanobacteria down to the taxonomic rank of genus. This is useful when assessing changes in cyanobacterial composition through the course of a bloom (e.g. Hur *et al.* 2013) or as shown here, over time. Recently, the pyrosequencing approach was used to distinguish between toxin and non-toxin producing cyanobacterial taxa in cyanobacterial mats (Kleinteich *et al.* 2014) and to explore the relationship between bacterial species richness and cyanotoxin production (Wilhelm *et al.* 2011; Bai *et al.* 2013). As high throughput sequencing becomes more cost-effective, efficient, and accurate it is likely to be incorporated into lake management programs.

4.6 Conclusion

Historical information on toxic blooms, which are a growing global concern, could help make present-day and future blooms easier to predict and mitigate. We report a historical presence of toxic cyanobacteria in Baptiste Lake, in contrast with a recent emergence of toxin-synthesizing genes in Lake of the Woods. We also show a decrease in overall cyanobacterial diversity in Baptiste Lake over time. The *mcy* gene and other cyanobacterial DNA can be successfully extracted, amplified, and sequenced from freshwater lake sediments. Given the positive correlations between certain genes and microcystin, this could be a potentially cost effective and rapid way of tracing cyanobacterial composition and diversity and potential toxin production through time.

Table 4.1: Primer sequences (for qPCR and pyrosequencing), amplicon size, and annealing temperatures (T_a).

Target	**Primers**	**Sequence (5' – 3')**	**Size (bp)**	**T_a (°C)**	**Reference**
Mcy gene	mcyD_{ks}F	TGG GGA TGG ACT CTC TCA CTT C	107	55	(Fortin *et al.* 2010)
	mcyD_{ks}R	GGC TTC AAC ATT CGG AAA ACG			
Microcystis 16S rRNA	MICR F	GCC GCR AGG TGA AAM CTA A	247	54.6	(Rinta-Kanto *et al.* 2005)
	MICR R	AAT CCA AAR ACC TTC CTC CC			
Cyanobacteria 16S rRNA (qPCR)	CYAN 108F	ACG GGT GAG TAA CRC GTR A	269	60	(Rinta-Kanto *et al.* 2005)
	CYAN 377R	CCA TGG CGG AAA ATT CCC C			
Cyanobacteria 16S rRNA (pyrosequencing)	CYAN 359F	GGG GAA TYT TCC GCA ATG GG	450	53	(Nübel *et al.* 1997; Jungblut *et al.* 2005)
	CYAN 809R	GCT TCG GCA CGG CTC GGG TCG ATA			
glnA	GS1β	GAT GCC GCC GAT GTA GTA	153-156	60	(Hurt *et al.* 2001)
	GS2γ	AAG ACC GCG ACC TTY ATG CC			

Table 4.2: Mean efficiencies (± SE) and r-squares (±SE) of qPCR results based on serially diluted plasmid DNA for each primer set

	Efficiency (%)	**r^2**
mcyD	104.1 (±10.6)	0.995 (±0.002)
MICR 16S rRNA	98.6 (±15.6)	0.959(±0.033)
CYAN 16S rRNA (108/377)	102.9 (±0.7)	0.988 (±0.003)
GS1β/GS2γ	100.6 (±11.4)	0.972 (±0.007)

Table 4.3: Pearson's correlations (*r*) of normalized molecular data (per wet gram of sediment) and microcystin content in the Baptiste Lake core

	MC (ng/g)	*mcyD*	16S MICR	16S CYA	*glnA*	DNA
MC (ng/g)	1.00					
mcyD	0.193	1.00				
16S MICR	0.500*	0.555*	1.00			
16S CYA	0.371	0.751*	0.492*	1.00		
glnA	-0.026	-0.179	0.044	-0.244	1.00	
DNA	0.158	0.163	0.055	0.658*	0.433	1.00

*$p<0.05$

Table 4.4: Pearson's correlations (*r*) of normalized molecular data (as per gram of wet sediment) and microcystin content in the Lake of the Woods core

	MC (ng/g)	*mcyD*	16S MICR	16S CYA	*glnA*	DNA
MC (ng/g)	1.00					
mcyD	0.620*	1.00				
16S MICR	0.754*	0.674*	1.00			
16S CYA	0.358	0.864*	-0.639	1.00		
glnA	0.230	0.750*	-0.282	0.779*	1.00	
DNA	-0.155	0.273	-0.235	0.583*	0.483*	1.00

*p<0.05

Table 4.5: The OTUs (based on 97% sequence similarity) and Shannon diversity Index through the Baptiste Lake core. Sequences were trimmed using MOTHUR and OTUs were determined using QIIME.

	No. of Sequences			**Species Diversity**	
Depth (cm)	**Total original sequences**	**Post-trimming, total sequences**	**Only cyanobacterial sequences**	**No. of OTUs**	**Shannon Index**
0	14154	7788	6100	301	5.60
2	6689	5846	1383	115	4.54
5.5	6797	5772	1634	139	4.93
7.5	5219	4374	1137	94	4.23
11	25457	13983	11168	299	4.75
21	14733	5994	5518	297	5.06
31	13751	5543	4689	276	4.88
41	9005	4136	3192	251	5.51
45.5	11433	5039	1192	200	5.81

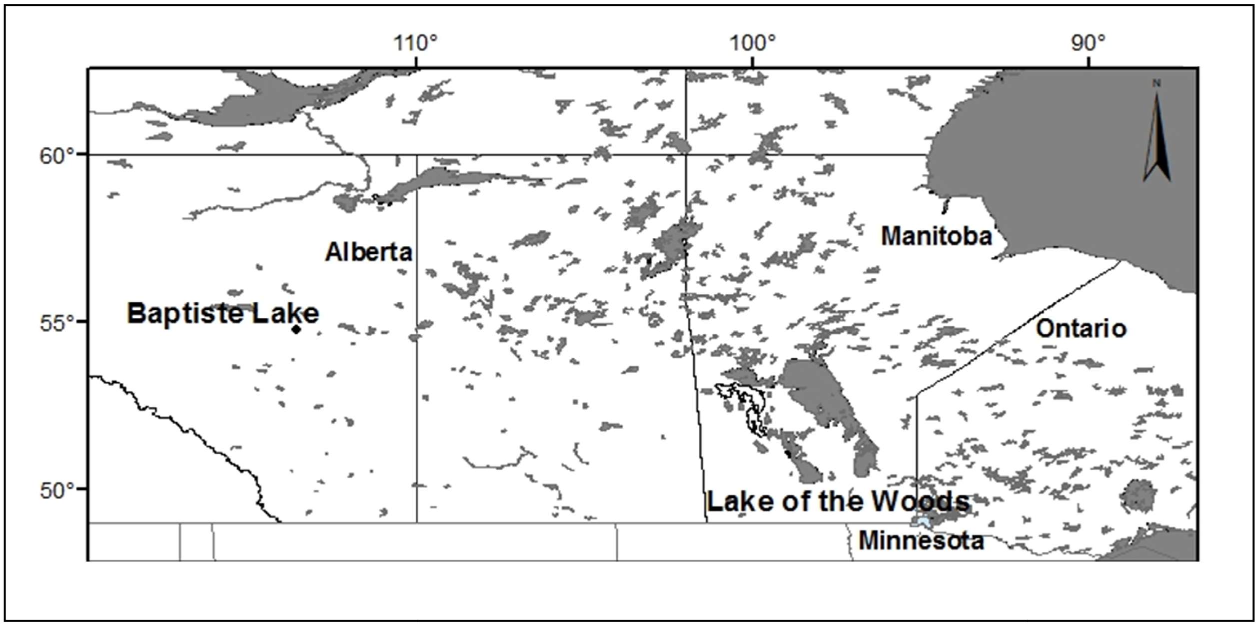

Figure 4.1: Location of sampling sites where cores were retrieved. Baptiste Lake is located in Alberta, Canada and Lake of the Woods straddles the borders of Manitoba and Ontario, Canada as well as the state of Minnesota, USA.

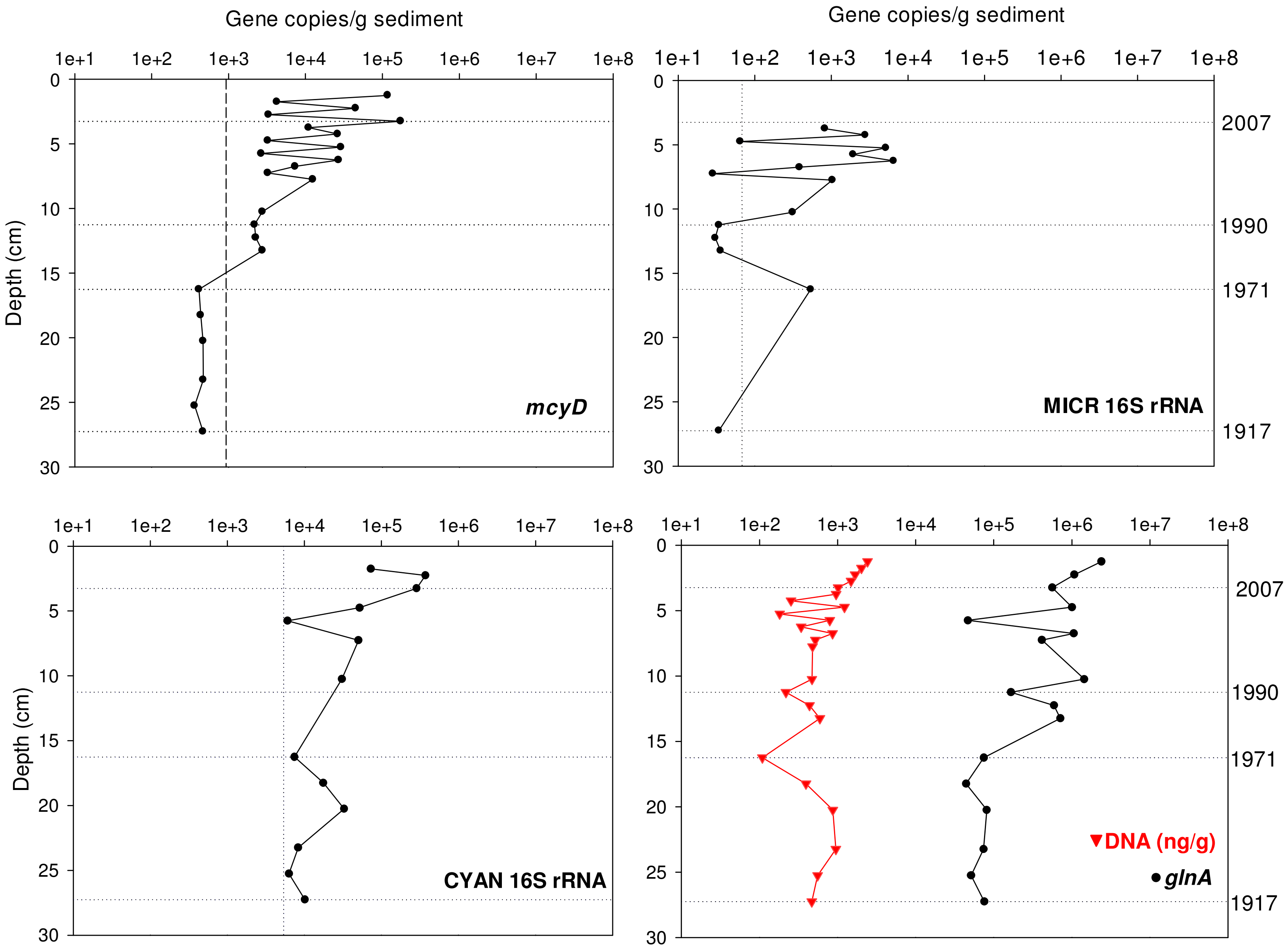

Figure 4.2: Gene copy numbers through the depth of the Lake of the Woods core as determined through quantitative PCR for *mcyD*, *Microcystis* 16S rRNA, cyanobacterial 16S rRNA, and *glnA* presented on a logarithmic scale. Total DNA is indicated in the *glnA* panel. The vertical dashed line in each panel represents the detection limit. A timeline (as determined through Pb^{210} dating) is provided on the right of the graphs.

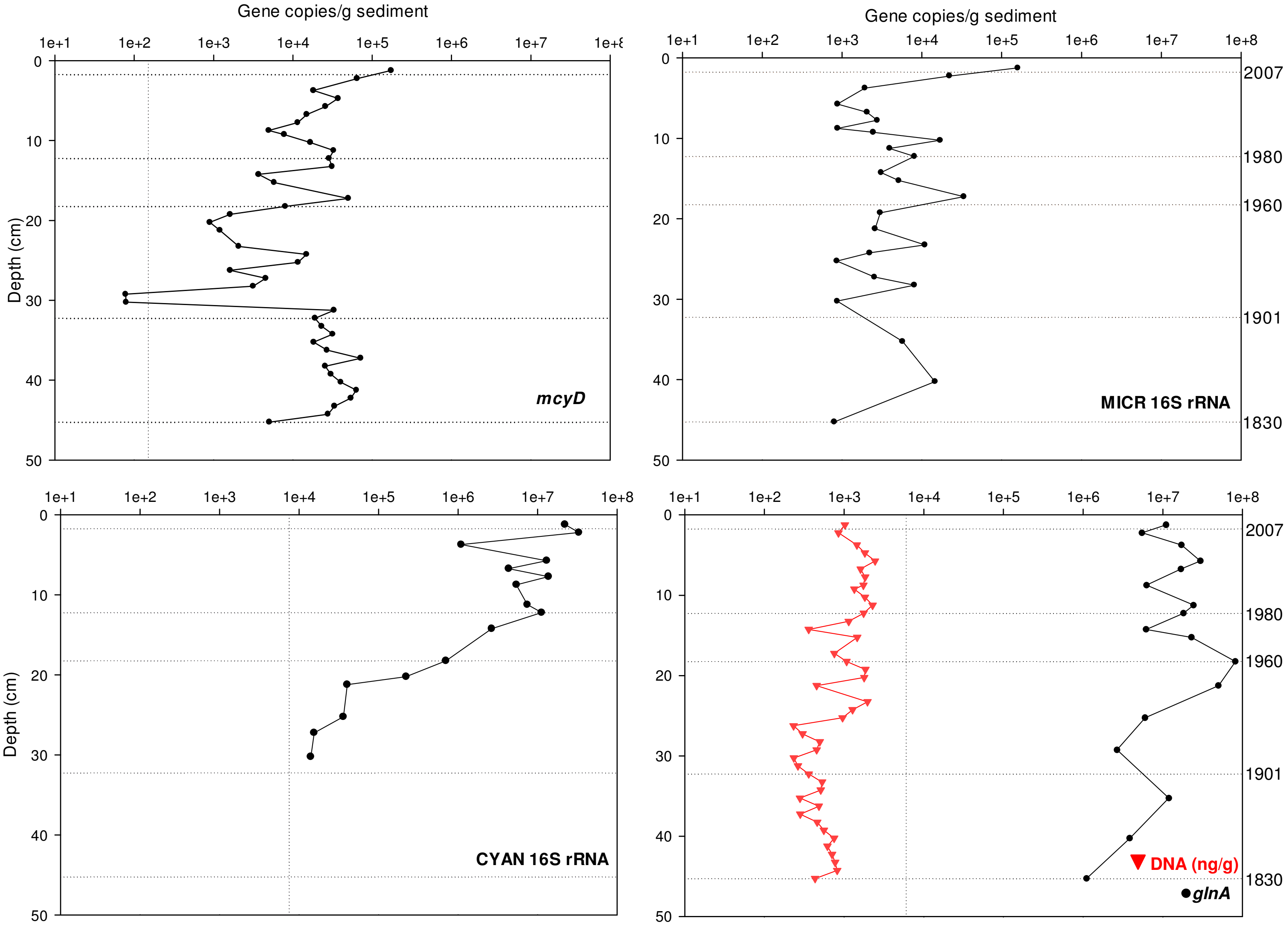

Figure 4.3: Gene copy numbers through the depth of the Baptiste Lake core as determined through quantitative PCR for *mcyD*, *Microcystis* 16S rRNA, cyanobacterial 16S rRNA, and *glnA* presented on a logarithmic scale. Total DNA is indicated in the *glnA* panel. The vertical dashed line in each panel represents the detection limit. A timeline (as determined through Pb^{210} dating) is provided on the right of the plots.

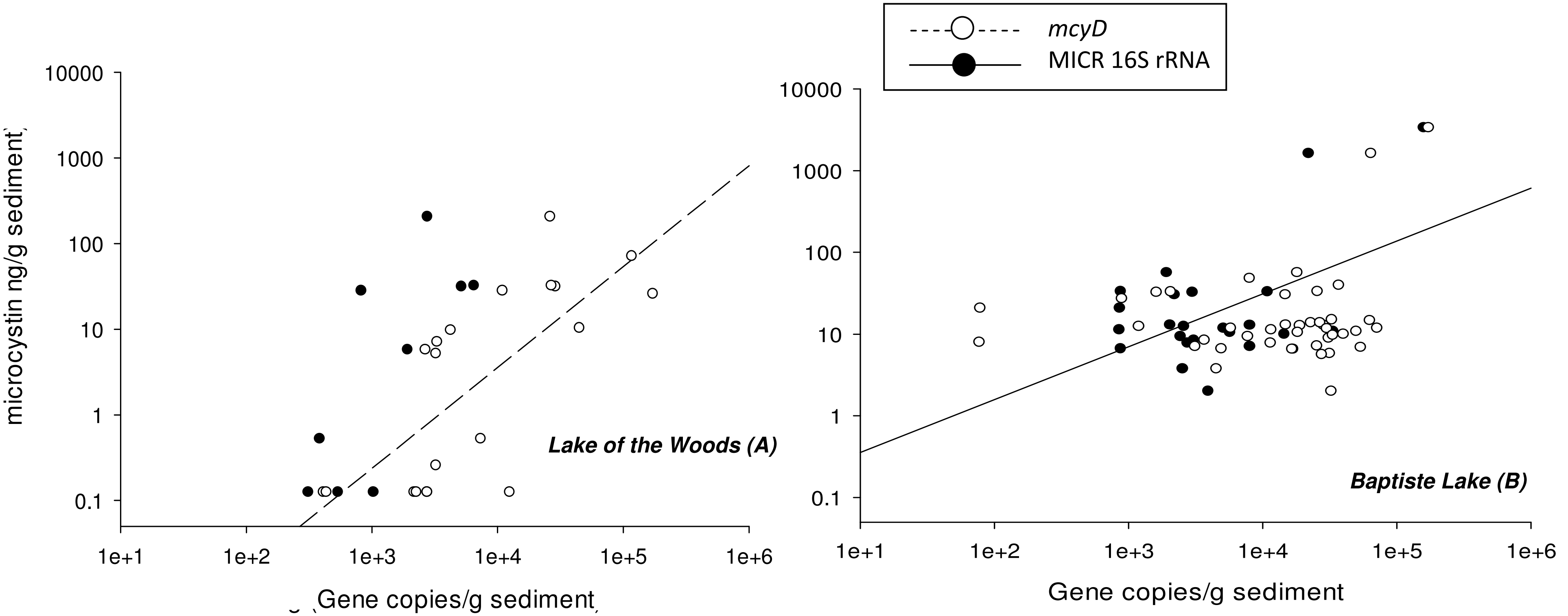

Figure 4.4: The relationship between microcystin (ng/g dry sediment) and total gene copies of *mcyD* and *Microcystis* 16S rRNA as found in Lake of the Woods (A) and Baptiste Lake (B) presented on logarithmic scales. Regression lines represent significant relationships ($p<0.05$) between gene copies and microcystin.

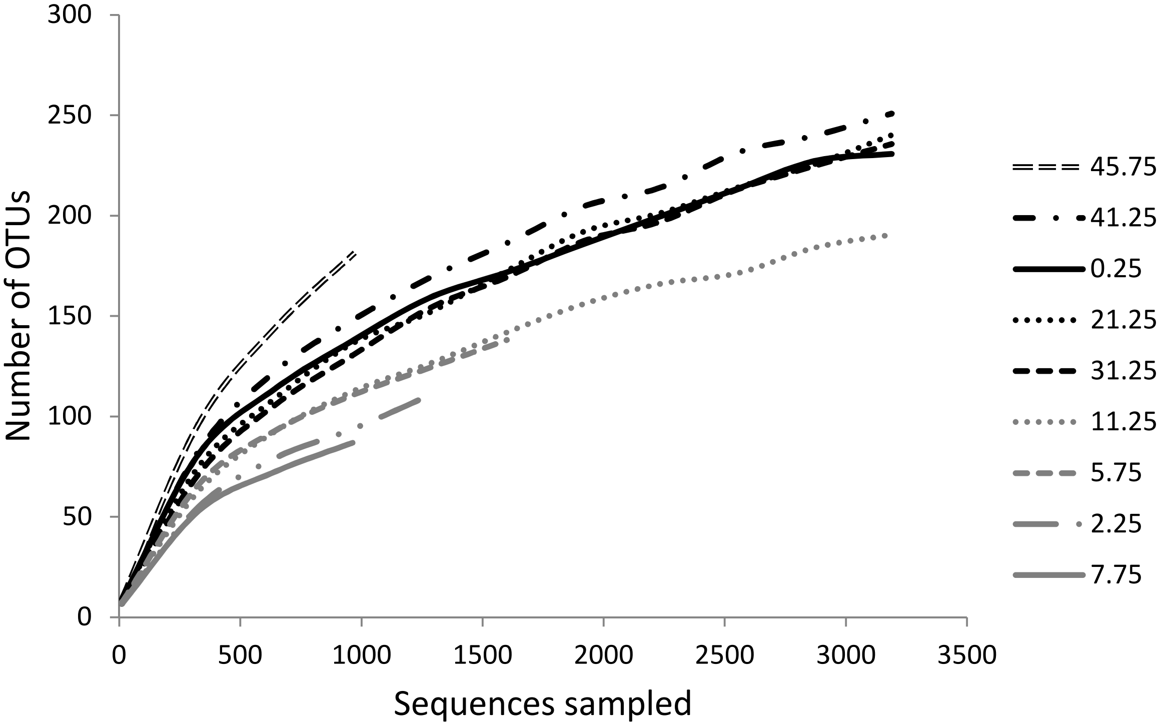

Figure 4.5: Rarefaction curves based on a sequence depth of 1137 cyanobacterial 16S rRNA sequences (minimum number of sequences in each samples) along a depth profile of samples taken from the Baptiste Lake sediment core.

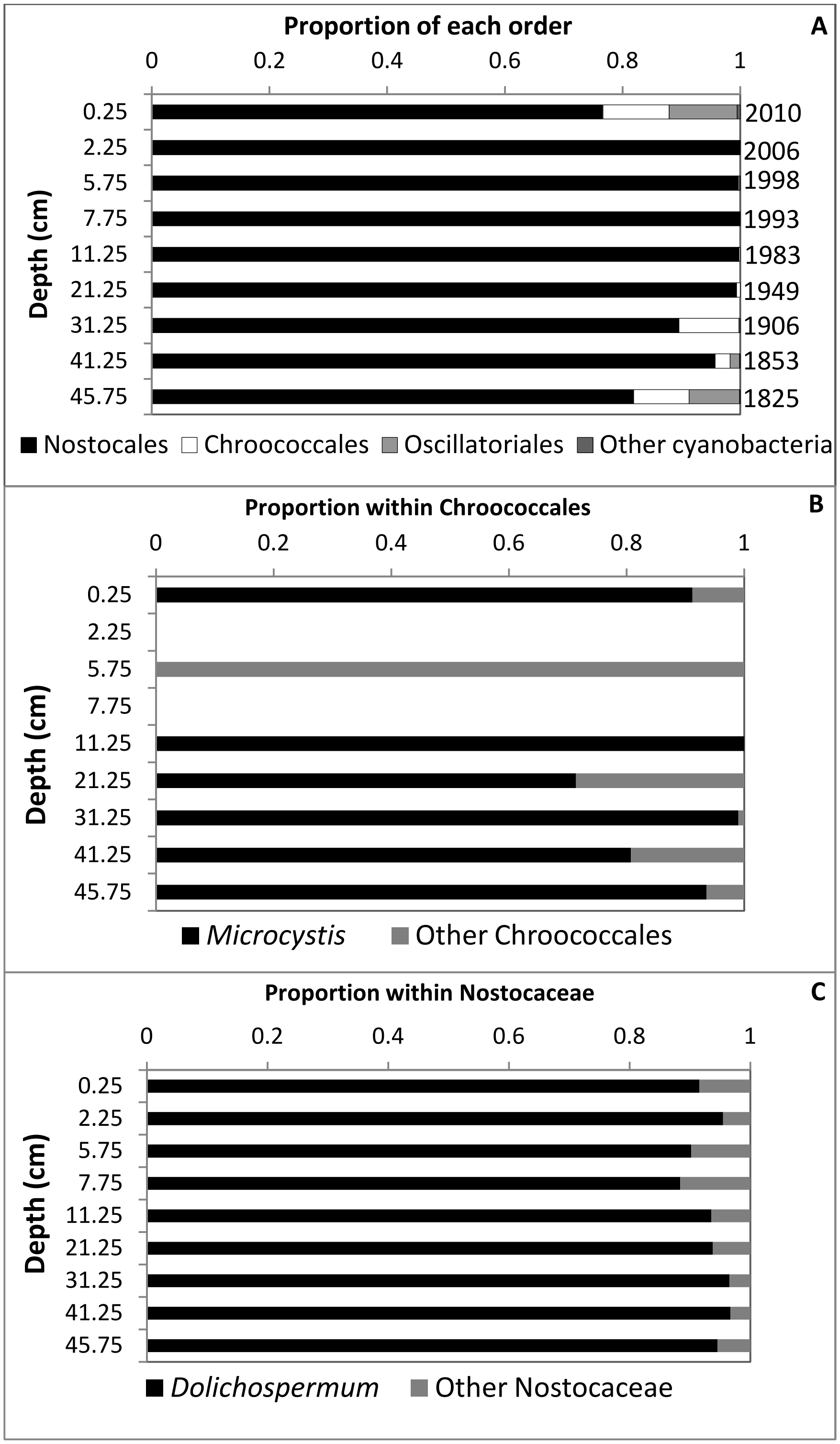

Figure 4.6: Taxa summaries through the Baptiste Lake core determined from pyrosequencing results of the cyanobacterial 16S rRNA gene. Proportion of main taxa are presented at the level of order (A) and broken down to illustrate genera potentially responsible for microcystin synthesis and blooms within Chroococcales (B) and Nostocales (C).

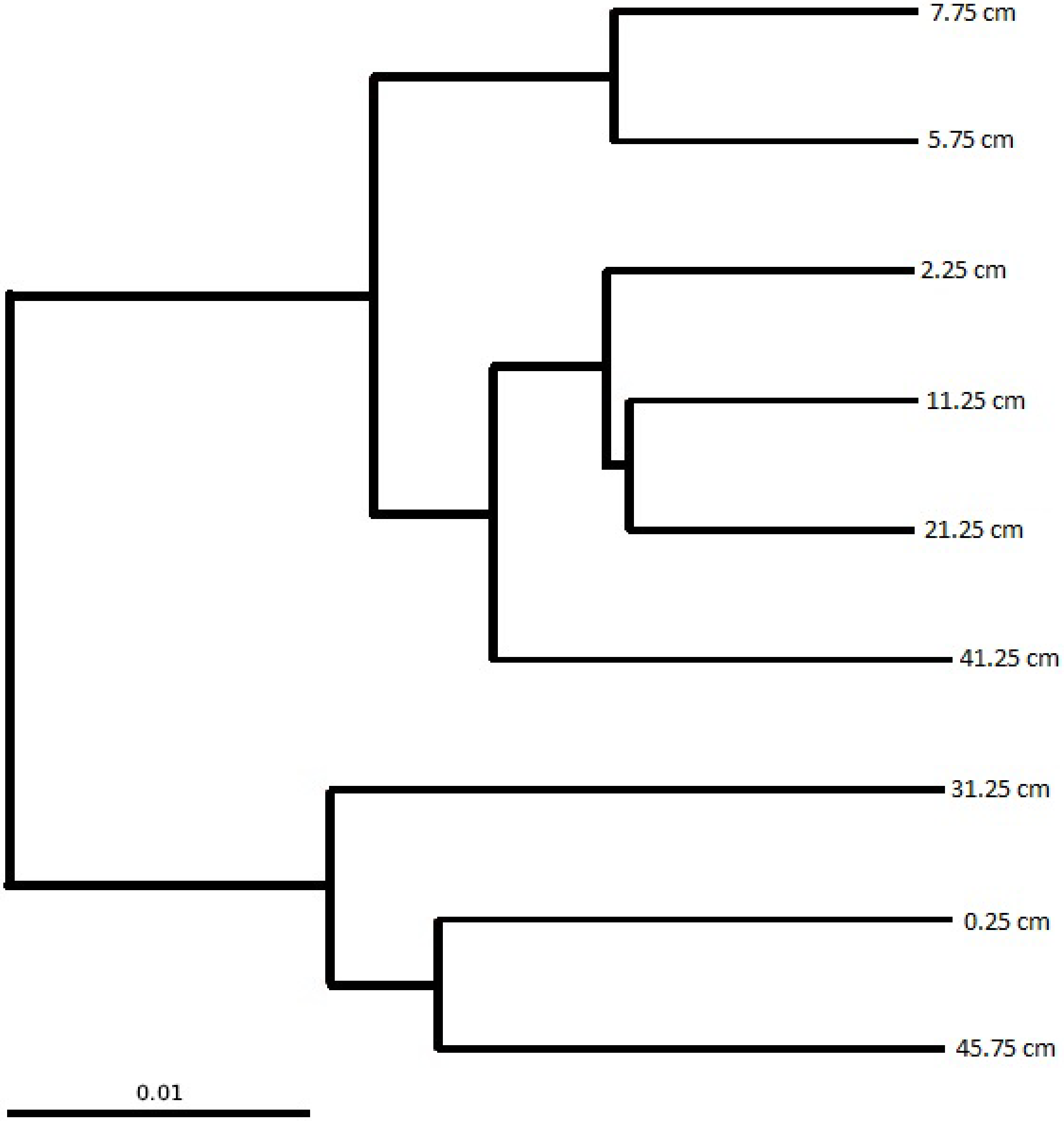

Figure 4.7: Weighted UniFrac cluster analysis of cyanobacterial 16S rRNA sequences amplified from samples taken from the Baptiste Lake core, as analyzed with QIIME. All branches show >75% jackknife diversity support.

CHAPTER 5: TEMPORAL AND LATITUDINAL TRENDS IN CYANOBACTERIA OF NORTHERN CANADA IDENTIFIED USING PYROSEQUENCING

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Statement of Author Contributions

I performed all the molecular data analyses and wrote the manuscript. Samples from Great Slave Lake and northern Quebec were collected by JC and RP respectively. AP provided samples from Char Lake and also expertise in data analysis of pyrosequencing results. FP supervised the project.

5.1 Abstract

In northern lakes, typically not prone to bloom-forming cyanobacteria, there have been sporadic reports of blooms over the last two decades. Increases in frequency and intensity of cyanobacterial blooms may partially be an effect of warmer temperatures. International climate reports have noted higher global air temperatures and other changes in climate regime in recent decades. This is especially true in northern Canada, recognized as an area sensitive to climate warming. We used a top/bottom approach to analyze sediment cores from six temperate freshwater lakes in the sub-Arctic to Arctic Canada. Pyrosequencing of the cyanobacterial 16S rRNA gene amplified from sediment DNA yielded between 11 and 355 operational taxonomic units (OTUs) in each sample taken from surface sediments, based on 97% sequence similarity. In the bottom samples, between 0 and 76 OTUs were detected per sample. Overall, a temporal trend of more cyanobacteria was seen at the tops of the cores as well as shifts toward bloom-forming and potentially toxigenic genera (e.g. *Anabaena*). Additionally, a decrease in cyanobacterial diversity was observed along a latitudinal gradient going north. Significant differences in species assemblages (>75% jackknife support) were seen between study sites based on UniFrac analyses. Diversity based on pyrosequencing was also compared to total cyanobacterial abundance, as determined through quantitative PCR of the cyanobacterial 16S rRNA gene. Expanding available reference genes of 16S rRNA in current public databases could assist in assigning more specific taxonomic rankings to OTUs representative of cyanobacteria. The strong temporal differences seen in both cyanobacterial diversity and abundance warrant the monitoring of blooms and cyanotoxins in northern Canada.

5.2 Introduction

Warmer temperatures and increased nutrient loading are thought to be the main reasons cyanobacterial blooms have become more frequent and intense in recent years (Paerl and Huisman 2008; O'Neil *et al.* 2012). Of particular concern is the potential increase in toxic blooms, as there is evidence that global warming may specifically lead to more toxigenic cyanobacteria (Davis *et al.* 2009; Gehringer and Wannicke 2014; Gkelis *et al.* 2014). Over the past few decades, substantial warming in Northern Canada and the Arctic has been reported based on several lines of evidence (Viau and Gajewski 2009; IPCC 2013). Concurrently, blooms have been reported in temperate lakes further north than previously found, potentially due to warmer temperatures (Winter *et al.* 2011). In high Arctic lakes of Canada, cyanobacteria are not known to form blooms but are present as benthic populations (Vincent and Quesada 2012). Yet, exposing cultured samples from this region to higher temperatures has been shown to increase toxin production and reduce cyanobacterial diversity (Kleinteich *et al.* 2013).

Sediment cores are used as proxies for the history of lakes beyond modern monitoring records and depending on the core length, may extend back before anthropogenic perturbation (Smol 2008). Given the recent climate warming of northern regions, we predicted increases in cyanobacteria in surface sediments compared to sediments representative of the historical record. Cyanobacterial abundance and composition have been tracked in sediment cores by identifying dormant cyanobacteria (akinetes) morphologically as well as by extracting characteristic carotenoid pigments (van Geel *et al.* 1994; Leavitt and Hodgson 2001).

Cyanobacteria have been detected in water samples (Rinta-Kanto *et al.* 2005) and more recently through sediment cores as well, by targeting specific genes (Savichtcheva *et al.* 2011;

Domaizon *et al.* 2013; Kyle *et al.* 2015). Using genes copy numbers as proxies of species abundance is relatively new to the field of paleoecology but has become possible with more efficient and precise DNA extraction methods (Anderson-Carpenter *et al.* 2011). In sediment, cyanobacterial community diversity has not been analyzed using molecular means very much and has mostly been limited to somewhat qualitative methods such as denaturing gradient gel electrophoresis (DGGE; Ye *et al.* 2011). Development of high throughput methods has enabled the quantification of species diversity using extracted DNA down to the level of species, depending on the library of sequencing data available. Methods such as pyrosequencing and Illumina have yielded results at a remarkable resolution using environmental DNA extracted from water (Shokralla *et al.* 2012). For example, pyrosequencing of the 16S rRNA gene revealed quantitative changes in cyanobacterial composition through the growing season in a Korean river (Hur *et al.* 2013). The same approach enabled distinction between toxic and non-toxic taxa in cyanobacterial mats (Kleinteich *et al.* 2014) and helped assess the relationship between bacterial species richness and cyanotoxin production (Wilhelm *et al.* 2011; Bai *et al.* 2013). Accurate identification of cyanobacterial genera through pyrosequencing could be more informative than the abundance of total cyanobacteria, if the objective is to detect toxigenic genera. For example, genera of interest as potential producers of microcystin include *Microcystis*, *Anabaena*, and *Planktothrix* (Chorus and Bertram 1999) and may therefore be more worthwhile to monitor than other genera.

Sequence data could complement typical proxies measured in sediment cores if used in conjunction. For example, Illumina sequencing was used to detect non-fossilized protist taxa in marine sediment cores that the microfossil record could not capture (Lejzerowicz *et al.* 2013). Though cyanobacterial probes have only been used in water samples for pyrosequencing, other bacteria and archaea have been tracked down sediment cores (Carr *et al.* 2013; Lin *et al.* 2014; Wasmund *et al.* 2014). For example, this approach was recently used through sediment cores taken from an Australian river to characterize the diversity of methanogens (Beckmann and Manefield 2014). Analysis of an entire core can yield a comprehensive history of a particular lake but may not be feasible or efficient when studying multiple lakes. In such circumstances, the "top/bottom" approach (Smol 2008) can be used to compare present-day conditions (top of the core) to what is considered pre-anthropogenic (bottom of the core).

Here, pyrosequencing data was used to compare cyanobacterial community composition and abundance in sediment cores taken from six lakes in Northern Canada. The first objective was to determine temporal changes in the cyanobacterial diversity and community composition by comparing present-day conditions (in surface sediment) to samples representative of each lake prior to significant anthropogenic nutrient enrichment and global warming. The second objective was to determine present-day cyanobacterial diversity (using surface sediments) along a latitudinal transect to further relate temperature and lengthened growing seasons to potential bloom-formers.

5.3 Methods

Study sites

Study lakes ranged from the 51st to 74th parallel north (Fig. 5.1). From Quebec, cores were taken from two lakes adjacent to the newly constructed James Bay road (Q9; 51°32'24" N, 77°26'24" W and Q21; 52°48'36" N, 77°18'00" W). Cores taken from these lakes were 20 - 25 cm in length. These lakes are located in the Boreal Forest region of Northern Quebec. The cores taken from Northern Ontario were from lakes within a remote First Nations reserve (Angling Lake; 53°50’29 N, 89°31’51 W and Weir Lake; 53°51’29 N, 89°34’36 W) on the other side of James Bay. Due to the short length of retrieved cores, only two top samples (the first 2 cm) were used from these cores. Total phosphorus (TP) measurements, taken at the same time as sediment, identified both lakes as oligotrophic (6 µg/L; Angling Lake and 15 µg/L; Weir Lake). Cores were retrieved from three locations in Great Slave Lake (Fig. 5.2) near Yellowknife, Northwest Territories (core 1: 62°17’56” N, 114°17’07” W; core 2: 62°25’10” N, 114°20’09” W; and core 3: 62°17’27” N, 114°18’42” W). Core 2 from Great Slave Lake was taken from the south end of Yellowknife Bay near the First Nations community of Dettah. These cores were dated using measurements of ^{210}Pb and fitted to the Constant Rate of Supply (CRS) model (Appleby 2008). The top samples were found to represent the time around when the cores were taken (2012-2013) whereas the bottom samples (24-25 cm in depth) spanned from 1896-1915. One core was taken from Char Lake, within the Arctic Circle (74°42’21 N, 94°53’48 W) near Resolute Bay, Nunavut (Stoeva *et al.* 2014). Cores were sectioned at site and top and bottom samples were kept on ice until stored in a -20°C freezer prior to DNA extraction.

DNA extraction and quantitative PCR

The top and bottom-most samples of each core were sub-sampled out of Whirl-Pak bags and DNA was extracted using the Powersoil DNA extraction kit (MoBio Laboratories Inc.) as described in Chapter 3. The extracted DNA concentrations were determined using a NanoDrop Spectrophotometer (ThermoFisher Scientific). DNA quality was estimated based on the absorbance of the DNA at 260 nm and 280 nm. The concentrations and A_{260}/A_{280} ratios of DNA extracted are presented in Table 5.1. Two extractions of 20 µL were performed from each sediment sample. One was used for quantitative PCR (qPCR) and the other was sent for pyrosequencing (20 µL of DNA extract was requested for pyrosequencing).

The forward and reverser primers used to quantify total cyanobacteria were respectively CYAN 108F, 5'-ACG-GGT-GAG-TAA-CRC-GTR-A-3' and CYAN 377R, 5'-CCA-TGG-CGG-AAA-ATT-CCC-C-3' and yielded a 269 bp product, which is ideal for qPCR . These primers were used to quantify total cyanobacteria in water samples from Lake Erie (Rinta-Kanto *et al.* 2005) and in sediment from Lake Taihu (Rinta-Kanto *et al.* 2005; Ye *et al.* 2011), modified from those originally designed (Urbach *et al.* 1992; Nübel *et al.* 1997). The standard curve for qPCR was developed as described in Chapter 2.

The qPCR conditions were as follows: preheating at 95°C for 3 min followed by 35 amplification cycles of 10 s at 95°C and 10 s at 55°C. This was followed by a melt curve from 60°C to 95°C at 0.5°C increments with 5 s holds. The annealing temperature was 60°C (optimized from a pool of samples). Samples were first pooled from each lake and serially diluted based on the lowest C_q as determined during melt temperature optimization and by calculating the fold dilution needed to end at a C_q of 35. A C_q of 35 is often considered the detection limit of this method (Bustin *et al.* 2009). The serial dilutions were performed to establish the dilution factor needed for samples from each site to decrease the effect of PCR inhibitors. A dilution factor between 4 and 16x was chosen for each site to ensure that an amplification efficiency range between 90 to 110% was obtained (considered free of inhibitors if less than 100). All samples were run together to avoid inter-assay variation. The mean (±SE) efficiency and coefficient of determination (r^2) of the standard curve (derived from the plasmid) were respectively 103.7% and 0.984. The limit of detection of the starting quantity (SQ) of

DNA, which was used to calculate the number of gene copies, was determined by using the linear equation derived from the standard curve (C_q=slope(log(SQ) + y-intercept) where the limit of detection (C_q) is assumed as 35 cycles (Bustin *et al.* 2009). The limit of detection of this qPCR assay was determined to be 8.7 gene copies/µL of DNA.

Pyrosequencing and data processing

Samples were sequenced by Molecular Research DNA (MR DNA) in Austin, Texas as originally described by Dowd *et al.* (2008). The 16S rRNA cyanobacterial primers used targeted the variable regions V3 and V4 of the gene. The sequences were CYA359-F 5'-GGG-GAA-TYT-TCC-GCA-ATG-GG-3' and CYA809-R 5'-GCT-TCG-GCA-CGG-CTC-GGG-TCG-ATA-3' (Nübel *et al.* 1997; Jungblut *et al.* 2005) for the forward and reverse primers respectively.

Initial data trimming performed using the program MOTHUR (v 1.33.3; Schloss *et al.* 2009) and identification of cyanobacterial sequences performed using the RDP classifier (Wang *et al.* 2007; Cole 2014) were done as described in Chapter 4. The cyanobacterial sequences identified using RDP were again passed through initial screening processes to remove any sequences with a homopolymer of six bases or more (other QIIME trimming conditions had already been met using MOTHUR). Using QIIME, sequences were grouped according to operational taxonomic units (OTU) and a representative sequence was chosen for each OTU (Caporaso *et al.* 2010). OTUs were defined at 97% similarity. Rarefaction and UniFrac analyses were performed next. There were five samples that had less than 100 sequences each and were therefore left out of the rarefaction curve and jackknifed UniFrac analyses. These included the bottom sample from the Q9 core, all bottom samples from Great Slave Lake and the bottom sample from the Char Lake core. This left the bottom section of Q21 being the sample with the lowest number of sequences (333) and thus the sampling depth for the rarefaction curves and beta diversity was set at 333. Rarefaction curves were built from the sequences of each sample to determine whether the pyrosequencing results were representative of cyanobacterial species richness. UniFrac analyses were performed to assess the relationship between samples using jackknife support to establish whether clustering was of statistical significance (Lozupone *et al.* 2011).

5.4 Results

Quantitative PCR

DNA was successfully extracted from all samples with concentrations ranging from 1.8 to 130.1 ng/µL and the A_{260}/A_{280} ratio ranging from 1.47 to 1.90. Generally, extractions from top samples of sediment cores yielded DNA of better quality and higher concentration. However, the cyanobacterial 16S rRNA gene was amplified through qPCR in almost all samples. In the bottoms samples of the Q9 core and core 1 of Great Slave Lake, the gene copy numbers were below the limit of detection (<8.7 gene copies/µL). In most cases, a higher abundance of cyanobacteria was seen in the top samples of the cores. The core from Lake Q21 was an exception where the total amount of DNA extracted was actually higher in the bottom sample than in the top sample and the gene copy numbers between top and bottom showed little difference (Fig. 5.3). The difference in gene copy numbers between top and bottom samples of each core exceeded the amount of expected variation in duplicate samples, as estimated through the coefficient of variation determined in Chapter 2 of 17%.

Amplified Sequences and OTUs

From the 16 samples used for analyses, 51 711 sequences were initially obtained with an average of 3232 sequences per sample. From these sequences, 11% were removed with initial trimming. Another 30% of sequences were removed after running the Chimera Slayer. Finally, the RDP classifier identified 53% (or 16 011 of the remaining sequences) as cyanobacterial, leading to the removal of the other bacterial sequences from the data set. The initial processing using the conditions set by QIIME removed another 6003 sequences from the dataset. After this, OTUs were chosen based on 97% similarity of sequences. Numbers of sequences and OTUs from each sample are presented in Table 5.2.

Cyanobacterial Diversity

The number of cyanobacterial OTUs ranged from 0 to 355 per sample. There were two cores for which no cyanobacterial sequences were obtained from the bottom samples post-trimming (one from core 3 of Great Slave Lake and the Char Lake core). From cores where both top and bottom sections were analyzed, there were substantially more cyanobacterial OTUs at the top compared to the bottom. Comparatively, there were more OTUs found in the surface sediment of cores from Northern Ontario and Quebec (173 to 355) than in surface sediment samples of Great Slave Lake or Char Lake (11 to 73). Rarefaction curves (Fig. 5.4) revealed that the number of OTUs per sample at the sampling depth chosen, were not enough to reach a plateau, meaning that the analysis likely did not capture all cyanobacterial diversity. Due to low numbers of detected sequences, Char Lake samples, the bottoms of the Great Slave Lake cores, and the bottom of Quebec core 9 were not included in this analysis or the jackknife diversity analysis. Although none of the curves reached a plateau, the rarefaction curves of the Great Slave Lake samples were the closest to plateau, indicating the lower diversity in these samples when compared to the surface sediment of the other lakes included in the rarefaction curve analysis.

The rarefaction curve results were corroborated with diversity based on the Shannon Index of OTUs found in surface samples. In Northern Ontario and Quebec cores the Shannon Index was higher (4.96 – 6.70) than in the surface samples of cores from lakes further north (Table 5.2). Great Slave Lake top sediment samples had similar diversity indices (2.91 – 3.19) and were higher than the top of the Char Lake core (2.86). By contrast, the bottoms of the Great Slave Lake cores showed little diversity (where any cyanobacterial OTUs were identified). A clear decrease of cyanobacterial diversity in surface sediment was seen along an increasing latitude, based on the Shannon Index (R^2=0.68, $p<0.05$) and the number of OTUs (R^2=0.78, $p<0.05$).

Samples where an appropriate number of cyanobacterial sequences were amplified were analyzed using weighted and unweighted jackknife UniFrac analyses and found to cluster together according to sampling location based on both analyses. The separation of Great Slave Lake and Quebec lakes had between 50 and 75% jackknife support in the unweighted analysis (Fig. 5.5). All sites clustered separately with more than 75% support in the weighted analysis (Fig. 5.6).

Community Composition

In both lakes from northern Ontario, a dominance of the order Chroococcales (a very small portion of which was represented by the genus *Microcystis*) was observed in the surface sediment (Fig. 5.7). The bottom samples of both Quebec cores showed mainly non-bloom forming picocyanobacteria (Synechococcales) and benthic *Leptolyngbya* (within the order Pseudanabaenales), which at the top of the cores were reduced with a concurrent increase in taxa within the Nostocales order. Sequences within the Nostocales order in these samples could not be assigned to specific genera, likely due to a lack of available reference sequences in the database used for sequence classification.

Great Slave Lake showed substantial change in cyanobacterial community composition over the last ~100 years based on cores where both top and bottom sections yielded OTUs representative of cyanobacteria (Fig. 5.7). Whereas the bottoms of the cores were composed mainly of Chroococcales, a dominance of Nostocales (>85% of total cyanobacteria being *Dolichospermum*; previously: *Anabaena*) was observed at the top of each core. There was no detection of the *Anabaena* genus in the bottom of the Great Slave Lake cores. The top of the core taken from Yellowknife Bay clustered separately from the tops of the other two cores taken closer to the middle of Great Slave Lake (>75% jackknife support based on weighted UniFrac analysis). The genus *Planktothrix* (part of the Oscillatoriales order) made up the highest proportion of cyanobacteria in the surface sediment sample of Char Lake (37.5%).

5.5 Discussion

Temporal changes in abundance and diversity

Using genes to assess abundance and diversity in a top/bottom analysis of sediment cores is a novel approach. The top/bottom method is limited to producing environmental snapshots of the history of a lake but has been used successfully to illustrate changes related to human development based on classic paleolimnological proxies, such as diatom-inferred phosphorus (Enache *et al.* 2012) and chironomid-inferred oxygen (Quinlan and Smol 2002).

Canada's Arctic has been noted as particularly sensitive to climate warming (Prowse *et al.* 2009). The Hudson Bay Lowlands (sub-Arctic, over the 50° parallel), although not as thoroughly studied, may also be undergoing the effects of climate change (Goldblum and Rigg 2005; Lemelin *et al.* 2010) as is the James Bay area (Bhiry *et al.* 2011). Based on proxy variables measured in sediment cores retrieved from lakes in this region, air temperatures have significantly increased there since the 1990s (Ruhland *et al.* 2014). In northern lakes, where cyanobacteria are typically present as benthic populations, the emergence of bloom-forming strains seen in the top samples is noteworthy. Generally, the abundance of cyanobacterial gene copy numbers in each core of our study was several orders of magnitude higher at the top of the core than at the bottom. The higher numbers of gene copies at the tops of the cores indicate an overall increase in cyanobacteria but also could be due to potential degradation of DNA in sediments representative of earlier times. Sediment with certain characteristics could make a particular site more suitable for preservation of nucleic acids. The oxygen level and organic matter content in sediment, for instance, both affect preservation of DNA (Anderson-Carpenter *et al.* 2011; Kyle *et al.* 2015). The sediment of the Q21 lake (where DNA extracted from the bottom of the core was more concentrated than at the top) may have better DNA preservation conditions than some of the other lakes included in this study. Though DNA concentrations were generally higher at the core tops, the proportional change in gene copy numbers from the bottom to the top of each core was higher than the change in extracted total DNA, indicating that the difference in concentrations was not solely due to preservation differences. This brings up a limitation in using gene copy numbers in a top/bottom approach. Not only could differences be due to preservation of DNA in sediment but the very top sample may be an overestimation of total preserved cyanobacteria due to the presence of overwintering species (Tan *et al.* 2008).

Using DNA sequencing to assess changes in relative diversity revealed more convincing evidence for changes potentially related to climate change. Pyrosequencing results would be less affected by DNA preservation bias between top and bottom core samples. Though there were less cyanobacterial sequences in the bottom samples, the total original sequences amplified were in fact comparable between top and bottom core sections (Table 5.2). Relying solely on copy numbers of the 16s rRNA gene to represent bloom-formers is problematic not only due to the aforementioned preservation differences but also the lack of differentiation between benthic species and potential bloom formers.

Increases in abundance, as depicted by gene copy numbers, were paralleled with shifts to potential bloom-forming taxa seen through pyrosequencing. In the Quebec lake cores there was a temporal shift toward the Nostocales order. Warmer temperatures are thought to be responsible for a higher presence of Nostocales in temperate lakes found in Germany (Mehnert *et al.* 2010) and other areas of the world, (reviewed by Sukenik *et al.* 2012a). However, this is dependent on the genus within Nostocales, which in these cores could not be identified past the taxonomic rank of family. The lack of reference cyanobacterial sequences in public databases based on species of northern regions could be preventing higher taxonomic resolution (Kleinteich *et al.* 2014).

The bottoms of the Great Slave Lake cores, approximately corresponding to the years 1896-1915, were dominated by the order Chroococcales. These sequences were classified as non-*Microcystis* and therefore indicate that toxic blooms were unlikely to have occurred in this lake historically. At the top of all the Great Slave Lake cores, *Anabaena*, a known toxin producer, was found as the dominant genus. The high proportion of *Anabaena* sequences in surface sediment was corroborated by visible surface blooms (J. Chételat, observation, September 2013) and is a remarkable finding for a lake north of the 60th parallel. This clear shift in species-type could be explained by a potentially longer, warmer growing season. Longer thermal stratification, which occurs as a result of a longer growing season, has been shown to favour growth of harmful cyanobacteria (Posch *et al.* 2012). The average air temperature in Yellowknife, Northwest Territories over the growing season (approximately June to September) increased by an average of 17% in the past three years (since 2010) in comparison to the current climate normal set by Environment Canada which is based on temperature averages from 1981 to 2010 (Government of Canada February 27, 2014). At the time of sampling (September 2013) the temperature of the surface water was 13-15°C, depending on the location across the lake. Morphological identification of phytoplankton from various parts of Great Slave Lake in the past 30-50 years reveals conflicting reports of the importance and dominance of cyanobacteria in relation to other phytoplankton (Lund 1962; Fee *et al.* 1985), which could be due to disagreements in taxonomic divisions. This adds further reason for the use of molecular markers to approximate taxonomic classifications through sediment cores.

Latitudinal comparison

A significant decreasing trend of cyanobacterial diversity was seen along the latitudinal gradient of 51° to 74°, represented by the surface sediment samples included in our study. This trend in cyanobacterial diversity is a novel finding in this area though diversity changes in diatom species assemblages have been studied along a similar latitudinal gradient. Large-celled diatoms were found to decrease along a transect of increasing latitude (Fallu *et al.* 2002).

Although Great Slave Lake is further north than the Ontario or Quebec samples, we found a clear dominance of *Anabaena* at the surface of these cores and not in Ontario or Quebec (<1%). The low percent of potentially harmful bloom formers in the Ontario cores corroborated with the low nutrient state of these lakes at the time of coring. Though the diversity of cyanobacteria was lower in Great Slave Lake surface sediment than the cores taken from Ontario or Quebec, the shift toward less diverse, potential toxic bloom-formers was more apparent.

The Char Lake core represents the highest latitude in this study. Previous paleolimnological studies of this high Arctic lake have found it to be oligotrophic and not perturbed by organic pollutants or catchment disturbances, based on diatom assemblages through sediment cores (Michelutti *et al.* 2003). The same analysis did however reveal changes in diatom diversity in connection to warming events over the decade of 1988-1997 that would have decreased the ice-cover period of the lake. Cyanobacterial OTUs were not identified in the bottom sample of our core taken from Char Lake, though they were in the top sample. What was identified in the bottom of the Char Lake core was non-cyanobacterial and dominated by the bacterial phylum *Proteobacteria* (67% of sequences). This may indicate a recent increase in cyanobacteria whereas historical cyanobacterial abundance was below our method detection limits. The cyanobacterial 16S rRNA gene copies were below the qPCR method detection limit for the bottom sample from this core as well. A large portion of the cyanobacterial OTUs at the top of this core were classified within the *Planktothrix* genus. Other genera within the Oscillatoriales order such as *Lyngbya* and *Leptolyngbya* have been detected in polar Arctic mats (Vincent and Quesada 2012). The genus *Planktothrix* includes strains that can form toxic blooms and its presence in this region is surprising though it has been identified north of 60° in Arctic lakes of Norway (Van 2001; Halstvedt *et al.* 2007). However, many sequences that were amplified from the Char Lake sediment were not identifiable, although classified as cyanobacterial. The presence of *Planktothrix* may be explained by certain species within the genus (e.g. *P. rubescens,* which has been observed in European temperate lakes for decades) which can grow in the metalimnion during stratification in low light conditions (Dokulil and Teubner 2012). This result again points to the value in performing more sequencing analyses of cyanobacteria in this region, to add to 16S rRNA databases. In this case, only identifying cyanobacteria down to the species level could determine the presence of potential toxin producers.

5.6 Conclusion

There was a significant trend of decreasing cyanobacterial diversity further north along a latitudinal gradient. Our results indicate an overall increase in cyanobacteria over time coupled with less diversity at certain sites. The low number of classifiable sequences and gene copy numbers found in bottoms samples may confound this result. There is potential value in increasing molecular approaches in environmental DNA in northern regions so that more sequences specific to this area are available in publicly available databases. Studying changes in species diversity in addition to total cyanobacterial abundance yielded a more complete analysis of temporal trends.

Table 5.1: Concentrations and purity (A_{260}/A_{280} ratio) of DNA extracted from surface and bottom samples of lake sediment cores across Northern Canada presented from South to North.

		Used for qPCR		**Used for pyrosequencing**	
Lake		Concentration (ng/μL)	A_{260}/A_{280}	Concentration (ng/μL)	A_{260}/A_{280}
Q9	Top	19.3	1.79	14.0	1.89
	Bottom	12.1	1.53	20.6	1.90
Q21	Top	19.8	1.67	13.0	1.85
	Bottom	21.3	1.67	14.6	1.89
Angling	Top 1	66.9	1.84	70.9	1.88
	Top 2	31.0	1.69	84.5	1.87
Weir	Top 1	102.5	1.86	130.1	1.68
	Top 2	38.4	1.74	85.4	1.86
GSL 1	Top	116.7	1.90	83.1	1.89
	Bottom	18.3	1.60	29.3	1.7
GSL 2	Top	69.2	1.82	87.7	1.89
	Bottom	32.5	1.70	24.5	1.73
GSL 3	Top	79.5	1.83	54.1	1.89
	Bottom	23.0	1.61	20.2	1.76
Char	Top	27.3		4.8	1.47
	Bottom	8.4		1.8	1.9

* GSL represents Great Slave Lake

Table 5.2: Summary of sequence data in sediment cores as determined through pyrosequencing and clustering into Operational Taxonomic Units (OTUs).

	No. of Sequences			**Richness and Diversity**	
	Original total	**Total post-trimming**	**Cyanobacterial only**	**OTUs**	**Shannon Diversity**
Ontario					
Angling top 1	3721	2176	1441	327	6.22
Angling top 2	3287	1969	1335	355	6.70
Weir top 1	3983	2289	1338	287	5.95
Weir top 2	3283	1810	1034	173	5.45
Quebec					
Q9 top	3762	2268	1482	244	4.96
Q9 bottom	4214	2916	14	4	1.81
Q21 top	3296	2158	1095	220	5.93
Q21 bottom	2399	1451	333	76	4.67
Great Slave Lake					
1 top	4163	2024	813	65	2.91
1 bottom	2345	1486	24	7	1.52
2 top	3599	1796	705	73	3.19
2 bottom	2439	1532	11	1	0
3 top	2328	1150	367	41	3.13
3 bottom	2579	1558	0	0	NA
Arctic					
Char top	2759	1700	16	11	2.86
Char bottom	3554	2161	0	0	NA

Figure 5.1: Location of lakes from which sediment core samples were retrieved in northern Canada.

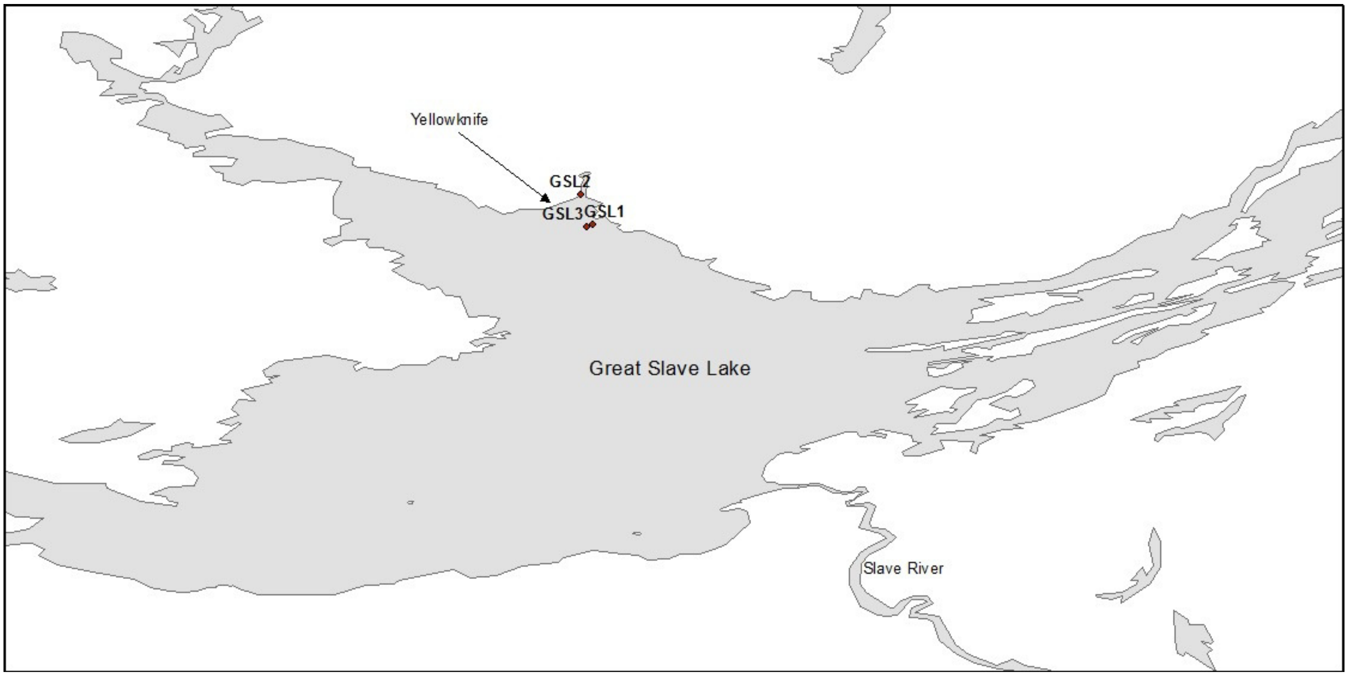

Figure 5.2: Location of the three sampling sites for cores within Great Slave Lake.

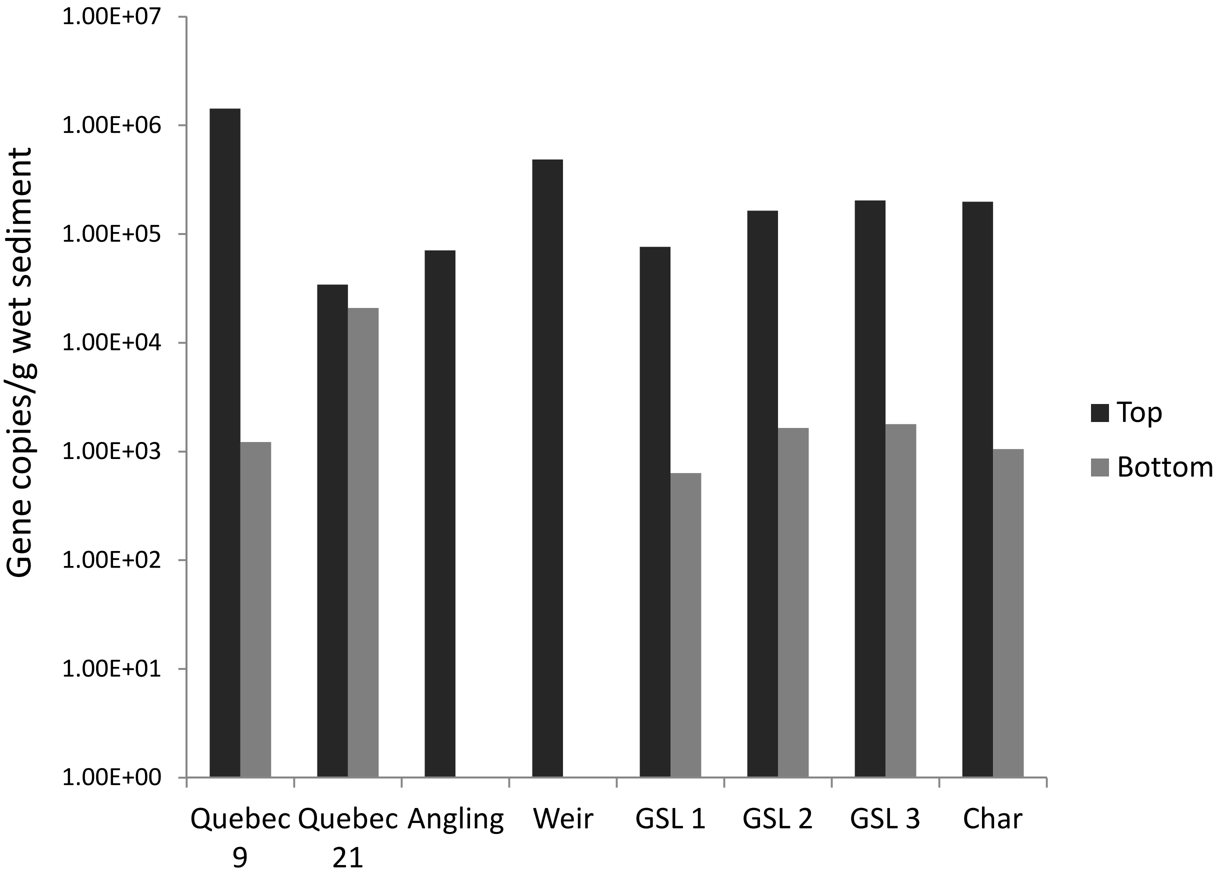

Figure 5.3: Gene copy numbers of cyanobacterial 16S rRNA per gram of wet sediment in top and bottom samples from each sediment core presented on a logarithmic scale. Bottom samples were not analyzed from Angling and Weir Lake cores.

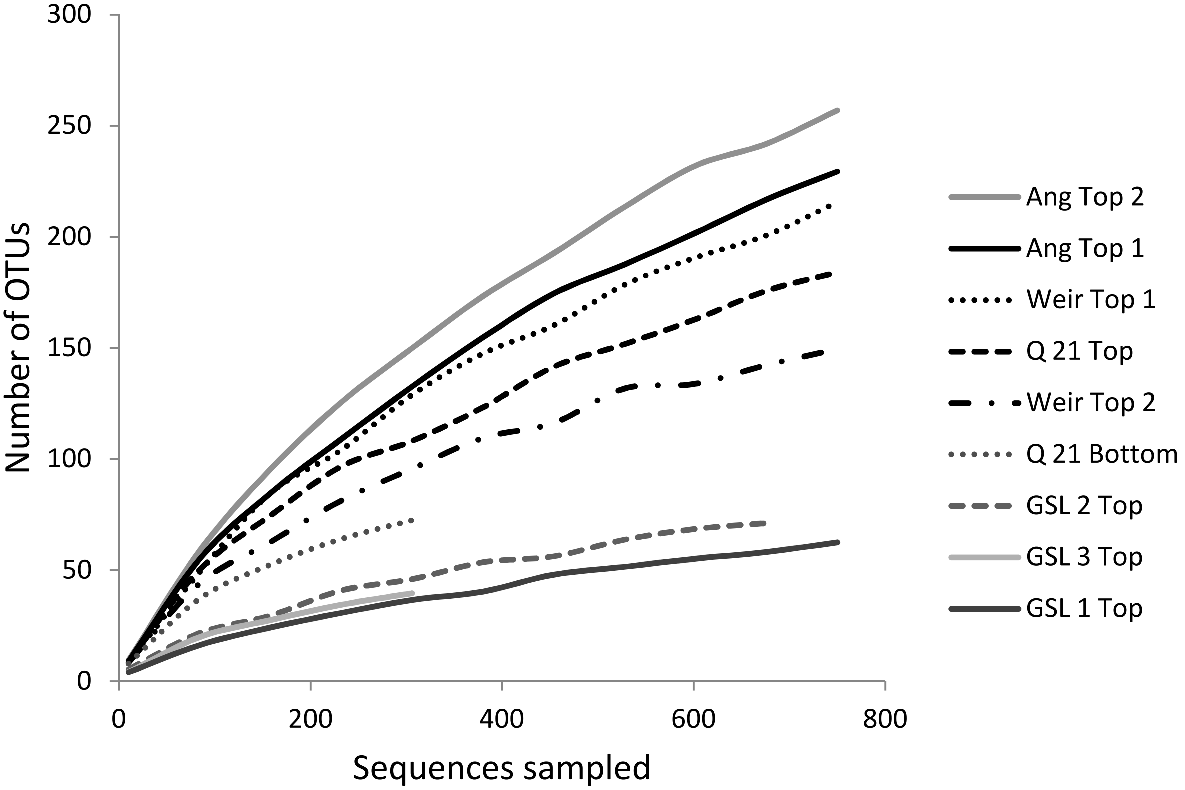

Figure 5.4: Rarefaction curves based on a sequence depth of 333 of cyanobacterial 16S rRNA sequences. Samples with <100 sequences identified as cyanobacterial were not included in this analysis.

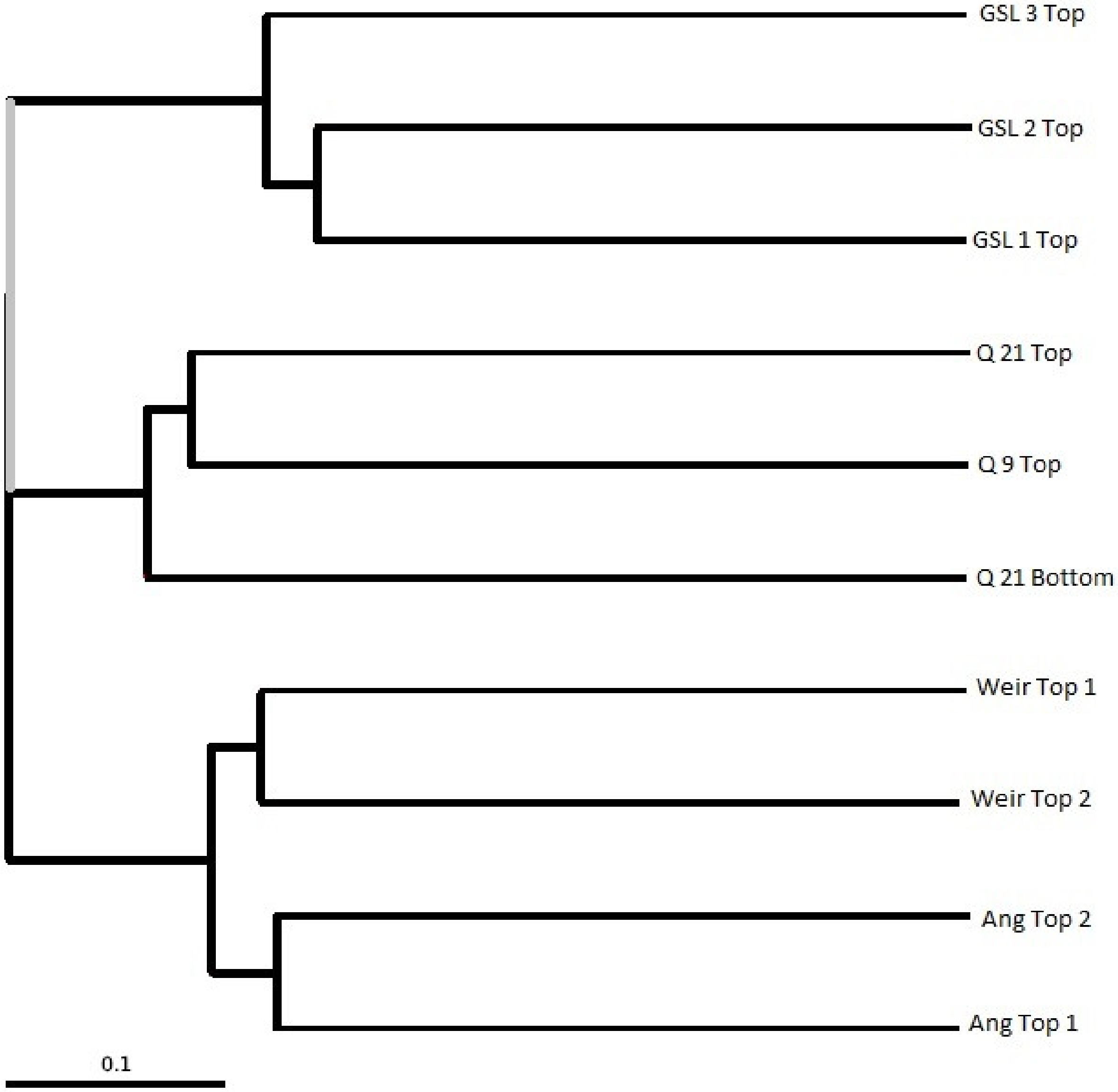

Figure 5.5: Unweighted UniFrac cluster analysis of cyanobacterial 16S rRNA sequences performed using QIIME. Black branches indicate >75% jackknifed diversity support. The gray branch indicates between 50 and 75% jackknife support.

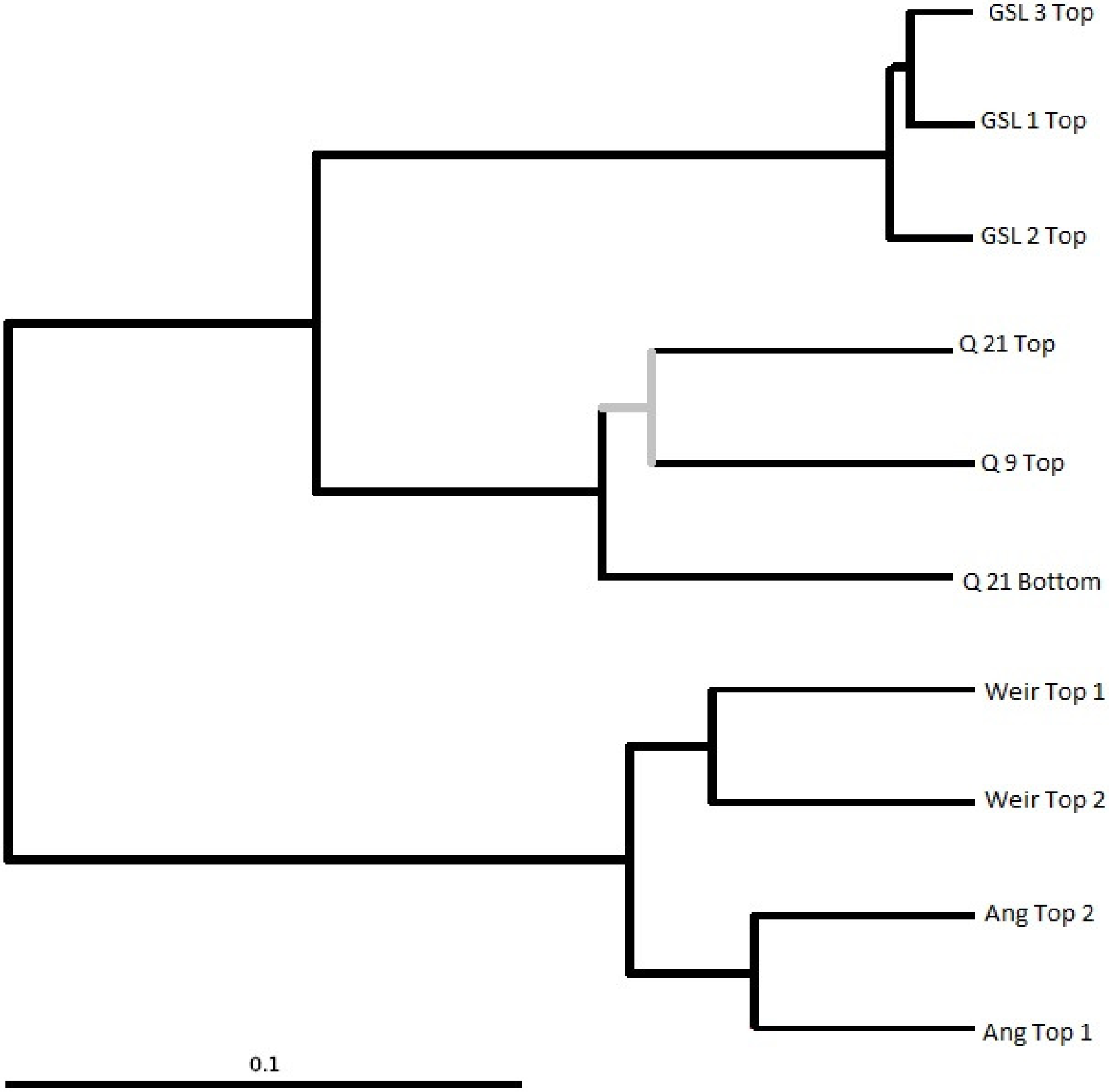

Figure 5.6: Weighted UniFrac cluster analysis of cyanobacterial 16S rRNA sequences performed using QIIME. Black branches indicate >75% jackknifed diversity support. The gray branches indicate between 50 and 75% jackknife support.

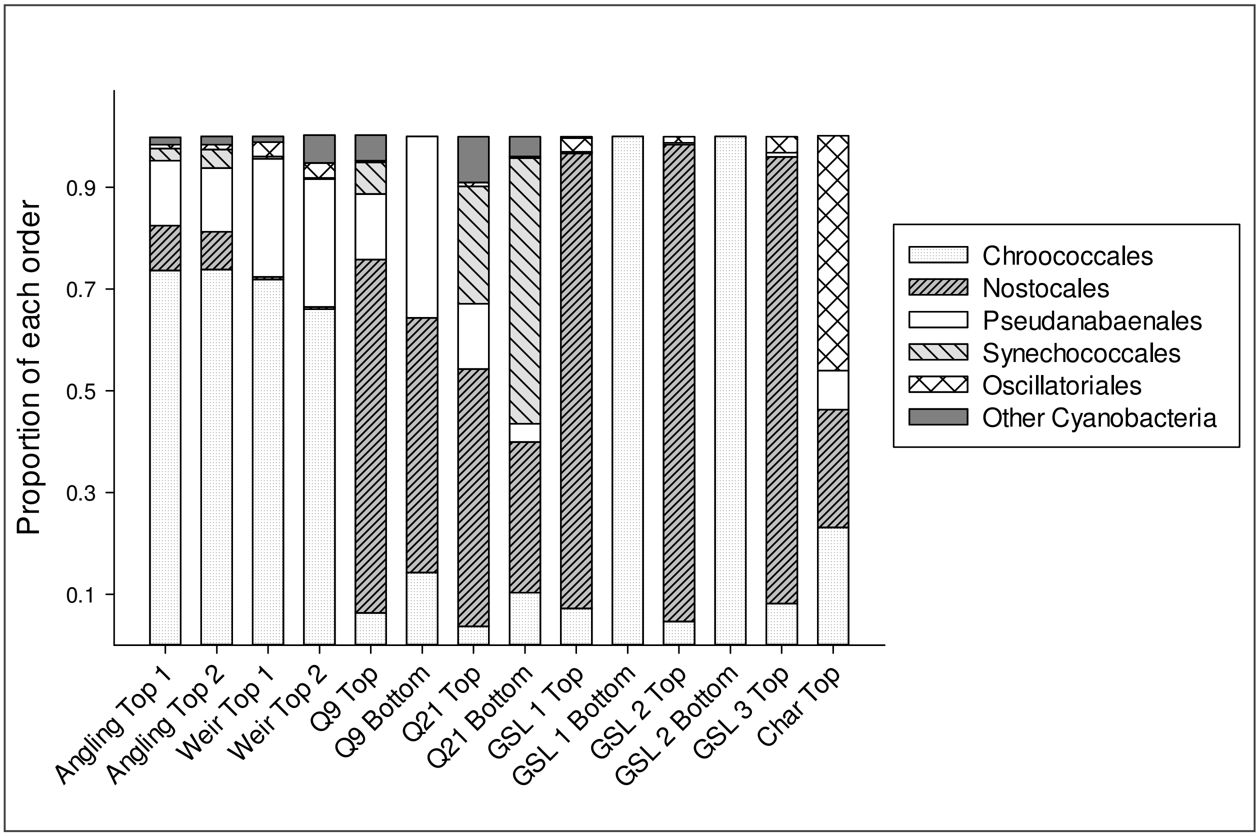

Figure 5.7: Cyanobacterial community composition in top and bottom samples of sediment cores taken along the latitudinal transect of Northern Canada. Composition is depicted at the taxonomic level of order.

CHAPTER 6: GENERAL CONCLUSIONS

6.1 Significance of Findings

There were two overarching goals of this thesis. The first was to establish whether DNA archived in sediment could serve as a proxy for cyanobacterial abundance, toxin synthesis, and community structure. I was able to demonstrate that good quality cyanobacterial DNA can be extracted from sediments and used for downstream molecular applications including quantitative PCR and advanced sequencing methods. The second goal was to determine if changes in climate regime have contributed to shifts in cyanobacterial abundance and community composition in conjunction with the long-established driver of eutrophication. I demonstrated increases in cyanobacterial abundance, including toxin-producers, and changes in diversity in the cyanobacterial community in recent years; even in lakes not strongly impacted by cultural eutrophication. This implies a significant effect of climate warming on lake ecosystems in the regions I studied.

Archived DNA

Good quality DNA was successfully extracted from sediments representative of different organic matter content and other characteristics. Overall, I found concentrations of DNA between about 4 and 120 ng/µL with concentrations typically higher at the surface of cores. I determined (Chapter 2) that DNA can be recovered and amplified with consistency (~20% variation between replicate samples) from both fresh and stored lake sediment. I concluded that storing retrieved sediment at -20°C or -80°C can prevent significant long-term changes in the bacterial community that occur when sediment is stored at 4°C or room temperature.

In Chapter 3, I was able to validate the use of archived DNA by showing positive correlations between cyanobacterial gene copies and pigments through sediment cores representative of the past ~150 years. However, I found that comparatively less DNA was extracted from a lake with a high humic acid content. Copy numbers of genes amplified through the core taken from this lake were also lower than what were found in the other cores, even though this site was the most eutrophic. This suggests that absolute numbers may not be comparable between sediments of different lakes. Interlake comparisons of gene copy abundance should rather be based on percent change, as is recommended for pigment analyses (Leavitt and Hodgson 2001). Quantifying the *glnA* gene, representative of the entire microbial community, in conjunction with genes of interest is useful. This can help ensure that changes seen in copy numbers of the gene of interest are not a result of DNA degradation at lower depths of the core.

In Chapter 4, I successfully tracked the *mcy* gene, which is responsible for microcystin production, through sediment cores. To the best of my knowledge, this was the first time a toxin-synthesizing gene was quantified in sediments representative of more than 100 years before present. I found that the gene copy data could be used as a proxy for microcystins and therefore to determine the site-specific history of toxic blooms. It was clear that in some lakes, toxic cyanobacteria have a long history whereas in others they are a recent phenomenon. Using toxin-synthesizing genes in conjunction with other cyanobacterial genes in temporal analyses can lead to understanding the changes in toxin production within the context of the whole cyanobacterial community.

In Chapter 5, I used archived DNA to determine differences in community composition over time and through space. Sequencing cyanobacterial genes complemented quantitative analyses of gene abundance and explained temporal and spatial variation in cyanobacterial taxa. This was a way of focusing on potentially harmful cyanobacteria, rather than total abundance. Reconstructing community composition by using amplified sequences has the advantage of circumventing researcher bias through microscopy. However, one of the current limitations with sequencing data is that there are often not enough reference genes in publicly available databases to classify all sequences down to a taxonomic level that is ecologically relevant. For example, in Chapter 5, on average, 18% of sequences in each sample were not classifiable past the taxonomic level of order.

One of the other concerns of using extracted DNA as a proxy in paleoecological studies is potential degradation. Cold, anoxic lake or marine sediments have been found most suitable for DNA preservation (Anderson-Carpenter *et al.* 2011). DNA from polar regions has been used to recreate past conditions of over 1000 years. I showed that temperate lakes can also provide good quality DNA from sediments (at least over the past ~150 years). Many functional genes remain to be explored. If temporal patterns of gene expression are of relevance, RNA can be extracted from sediment (Stoeva *et al.* 2014) but due to preservation difficulties of RNA, may be limited to the top few centimetres of the core. Given that genes of interest amplified from archived DNA served as proxies of community composition and abundance in chapters 3 and 4, I was also able to address the primary objective of this thesis: to track temporal patterns in cyanobacterial abundance and composition along with changes in the environment.

Cyanobacteria in the face of environmental change

In Chapter 3, similarly large recent increases of cyanobacterial gene copies were found in cores from lakes surrounded by human development as to those within a protected Park area. Since increases in cyanobacterial abundance did not seem related to human activity, the possibility of warmer temperatures causing or exacerbating the increase seemed highly likely, corroborating previous work in the area (LeBlanc *et al.* 2008). In Chapter 4, temporal dynamics of toxigenic cyanobacteria were compared between a historically eutrophic site (Baptiste Lake) and one of more recent changes to nutrient loading (Lake of the Woods). In Lake of the Woods, I found that microcystin-producing cyanobacterial abundance was indeed increasing over time and independent of total cyanobacteria. In Baptiste Lake, though toxigenic cyanobacteria were historically present (at least since 1825), a shift was seen over time toward a less diverse and potentially more toxic community.

In Chapter 5, quantitative changes in cyanobacterial species diversity and composition were evident in northern temperate lakes of Northern Canada. These lakes represented regions of little direct anthropogenic influence but where effects of climate change are increasingly apparent. In these lakes, significant differences in cyanobacterial diversity were found between sediment samples representative of present day conditions and those representing a time prior to significant warming. A shift toward potential toxic bloom-formers was seen, particularly in Great Slave Lake, Northwest Territories.

6.2 Future Directions

Impending changes in climate regime will ensure that cyanobacterial blooms and toxins are in the forefront of aquatic science research. To develop appropriate mitigation measures for lake management, past lake conditions of nutrient concentrations and species diversity should be considered. Analyzing sediment cores retrieved from lakes can depict cyanobacterial abundance and diversity prior to significant anthropogenic disturbance and help devise objectives for lake management. Just as DNA extracted from water samples has increased in quality, so it has from sediment. The potential benefits of using genes as proxies in sediment cores have even led researchers to devise a piston corer specifically designed to reduce DNA contamination during core retrieval (Feek *et al.* 2011) as well as commercial kits for efficient DNA extraction from soil and sediment (Mahmoudi *et al.* 2011). Further improvements of extraction and amplification of DNA from sediments of a range of chemical and physical properties could improve the use of molecular proxies in paleolimnology.

The Intergovernmental Panel on Climate Change (IPCC) forecasts significant increases in air temperature in future years if mitigation measures are not implemented (IPCC 2013). The effects of climate change are varied and implicate ecosystems and human health through both direct and indirect routes (Mohanty 2009). A mounting body of evidence has prompted various levels of government around the world to develop and implement mitigation efforts to decelerate these changes (Galarraga *et al.* 2011). Climate warming could make recovering from nutrient loading difficult for lake ecosystems (Battarbee *et al.* 2012) particularly since cyanobacteria are predicted to dominate over other phytoplankton as temperatures increase (Elliott 2012; Beaulieu *et al.* 2013). A thorough understanding of the effects of temperature on bloom-forming cyanobacteria and toxin synthesis could aid in developing lake management strategies specific to mitigating temperature increases, as is now being attempted in hypereutrophic lakes such as Lake Taihu, China (Zhang *et al.* 2012).

As evidence of climate change becomes less refutable, understanding the consequences pertaining to lake water and algal blooms could help strengthen policy resolve to reduce activities that contribute to increases in air temperature. This has been seen in the past with the mitigation of phosphorous input (Schindler 1974). Limiting phosphorus loading is now an important component of lake management, leading to restoration in many cases (Schindler 2006). Similarly, appropriate mitigation measures could be put in place to decelerate the effects of anthropogenic-induced warming to freshwater cyanobacteria.

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Appendix A: Example analyses from quantitative PCR

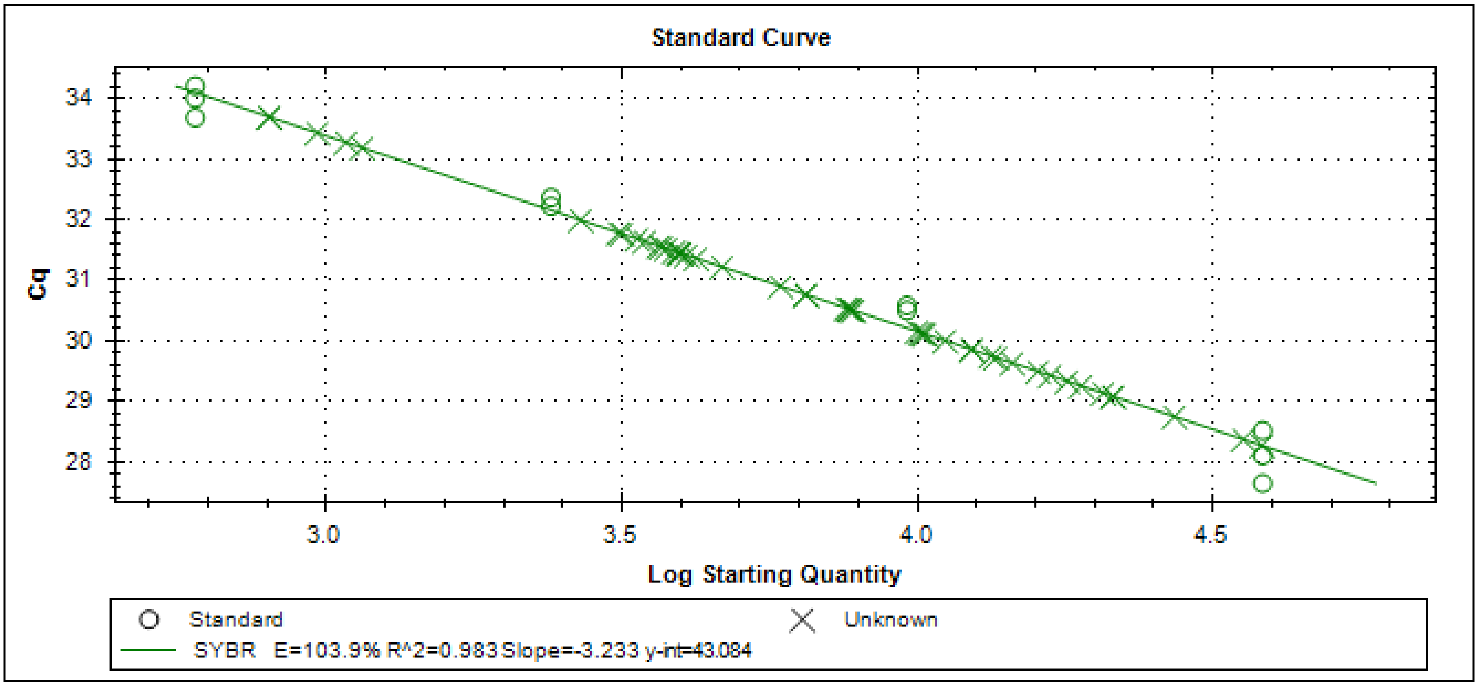

Figure A.1: Example run of quantitative PCR of DNA extracted from sediment using cyanobacterial 16S rRNA primers. Standard curve (open circles) was developed by serially diluting plasmid DNA containing the gene of interest. The samples (crosses) fall within the standard curve.

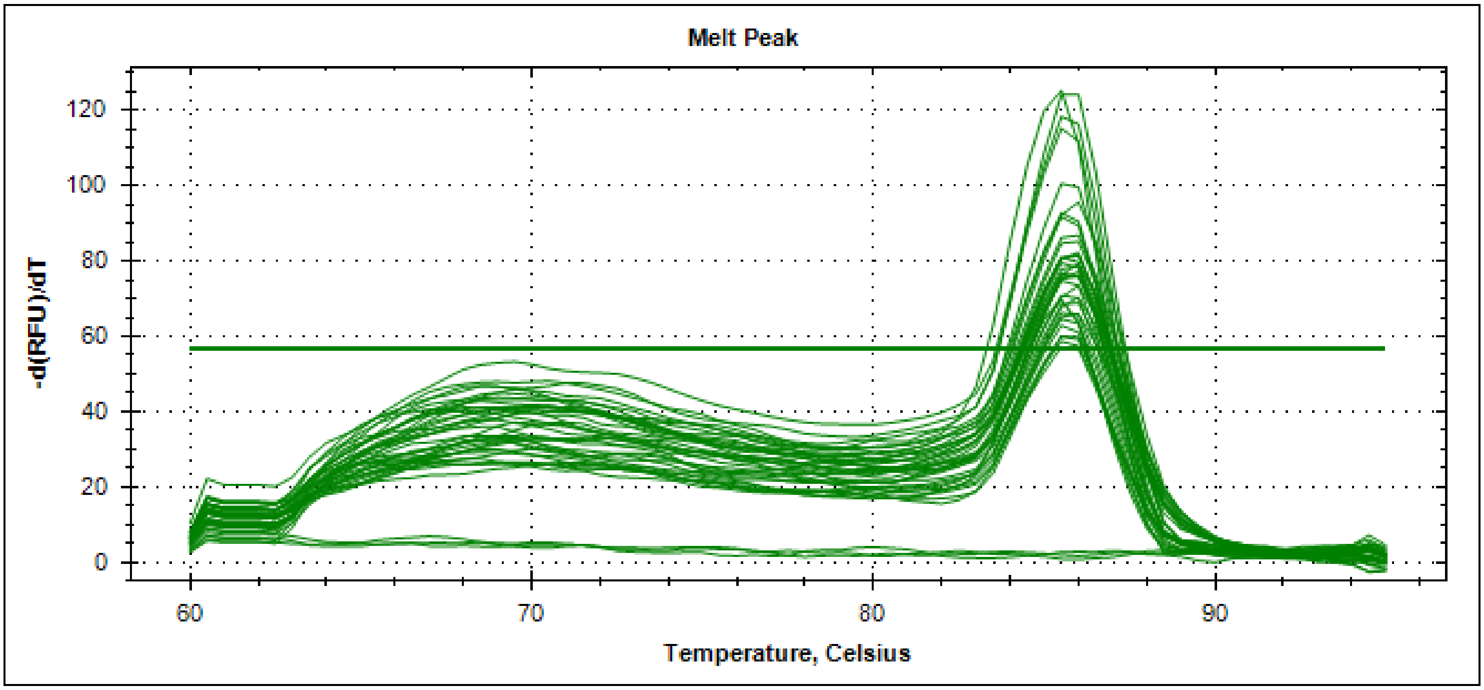

Figure A.2: Melt curve analysis of the cyanobacterial 16S rRNA gene amplified from plasmid DNA and sediment samples from Lake Heney. The flat lines at the bottom of the peak are the negative (no template) controls.

Appendix B: Microcystin concentrations in Lake of the Woods and Baptiste Lake cores

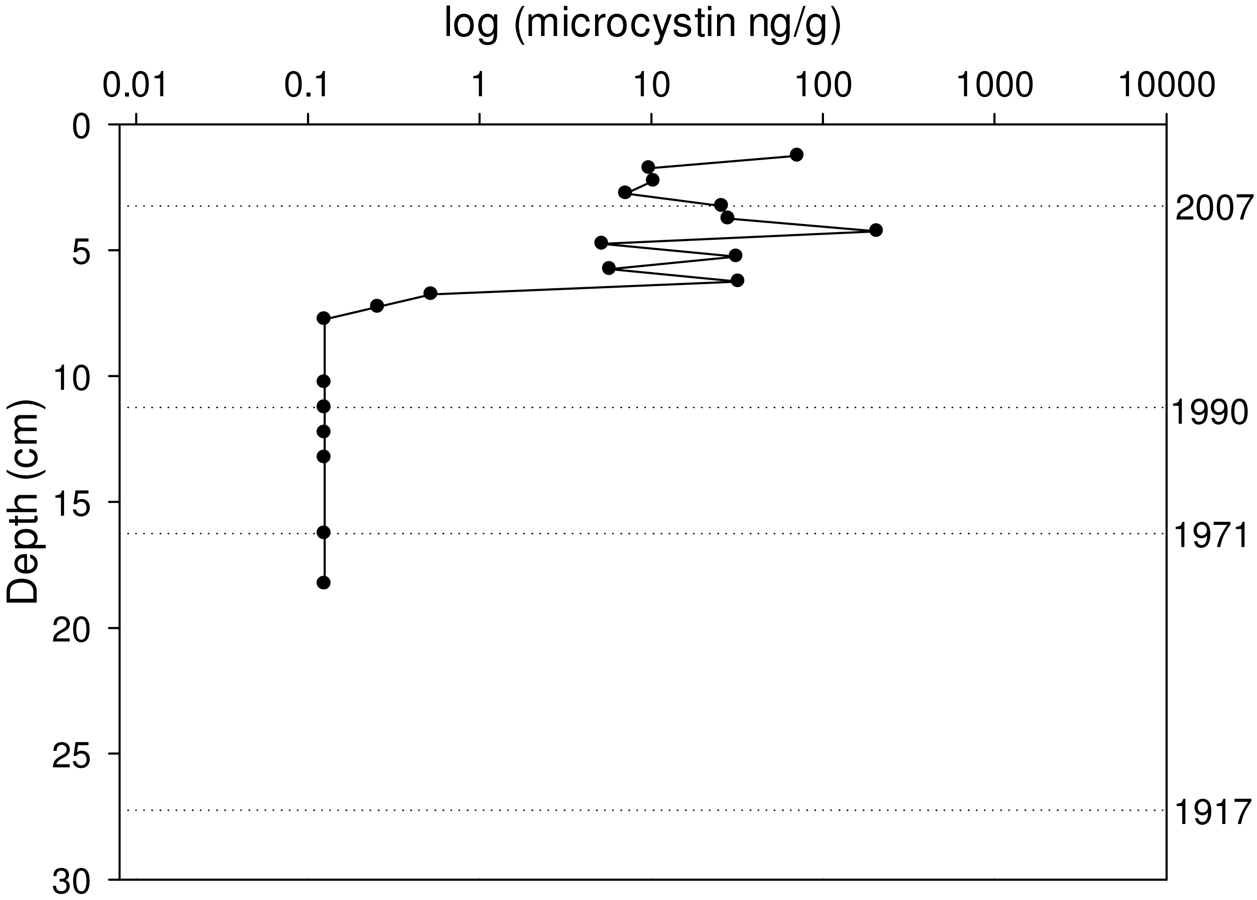

Figure B.1: Microcystin concentrations (ng/g dry sediment) along the depth of the Lake of the Woods core (Zastepa, 2014). Data is presented on a logarithmic scale.

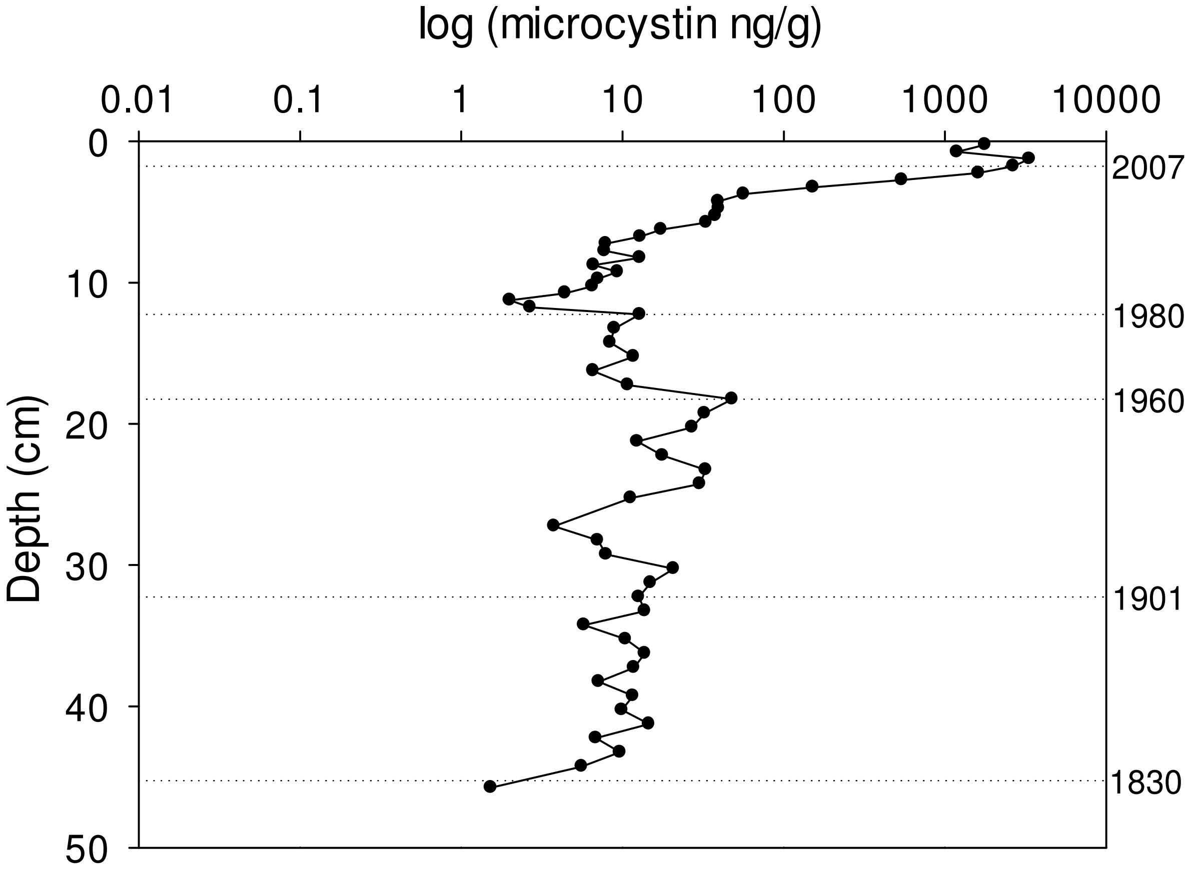

Figure B.2: Microcystin concentrations (ng/g dry sediment) along the depth of the Baptiste Lake core (Zastepa, 2014). Data is presented on a logarithmic scale.