University of Alberta

The Role of Iron in Suppressing Internal Phosphorus Loading and Toxic Cyanobacterial Blooms in Freshwater Lakes

by

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For my father and mother

My parents fled Czechoslovakia in 1968 for a new life in Canada
because they dreamed of giving their children the opportunity
of a university education.

Abstract

Harmful algal blooms occur in nutrient-rich lakes around the world, diminishing the value of these ecosystems for wildlife and humans. Management of algal blooms is an on-going challenge for lake managers and policy makers. The overarching goal of this research was to advance our understanding of the environmental factors leading to blooms of potentially toxic cyanobacteria in shallow eutrophic lakes, in order to guide effective management strategies to reduce their occurrence. First, I evaluated the nutrient conditions that lead to elevated levels of microcystins, a hepatotoxin produced by certain cyanobacteria, by consolidating a national database of nutrient and microcystin concentrations for Canadian lakes. Second, I tested whether nutrients released from sediments stimulate toxic cyanobacterial blooms by culturing the cyanobacterium *Microcystis* in overlying water harvested from incubated lake sediments. Third, I developed and evaluated a conceptual model to explain the biogeochemical pathways leading to toxic cyanobacterial blooms in shallow lakes, based on sediment and culture experiments and lake monitoring in a hypereutophic lake in Alberta, Canada. Fourth, I tested this conceptual model experimentally by manipulating iron loading to in-lake mesocosms and examining changes in sediment chemistry, nutrient cycling, algal biomass and community composition, and microcystin concentrations. The main conclusions of these studies are: (i) microcystins are prevalent in lakes across Canada, but only under high nutrient conditions and at low ratios of nitrogen-to-phosphorus; (ii) lake sediments release bioavailable nutrients that support the growth and toxin production of

microcystin-producing cyanobacteria; (iii) toxic blooms of cyanobacteria in shallow lakes may result from the synergy between iron-deficient sediments and discontinuous polymixis; (iv) iron loading to lakes inhibits internal phosphorus loading, decreases algal biomass, discourages the dominance of cyanobacteria, and reduces microcystin concentrations. This research reaffirms the need for controlling internal phosphorus loading in shallow lakes of the Canadian Prairies, and suggests iron treatment may be an effective remediation strategy to complement external nutrient loading reductions. More broadly, this research emphasizes the important role of iron in influencing the trophic status of lakes, and raises concerns for how sulfur pollution and climate change may be exacerbating the problem of lake eutrophication.

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Finally, the warmth and kindness bestowed on us by the people of Nakamun Lake was truly touching — I hope our research will help to improve the health of this lake and the lives of all those who call Nakamun Lake home.

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Chapter 1. Introduction

Introduction to Eutrophication

In a seminal paper in limnology, A.D. Hasler reported on dramatic changes occurring in lakes across Europe and the United States in the early 1900s in response to nutrient enrichment from domestic sewage (Hasler 1947). Hasler described the case histories of formerly oligotrophic lakes that were overcome with "obnoxious scums of algae" that discolored the water and produced foul odors, and whose decomposition reduced bottom water oxygen to concentrations intolerable by fish. This phenomenon, termed "eutrophication" (derived from the Greek word *eutrophos*, meaning "nutrient-rich") was first coined in 1907 by a wetland ecologist (C.A. Weber, according to Schindler 2006), and was later defined as "the nutrient enrichment of waters, which frequently results in an array of symptomatic changes, among which increased production of algae and other aquatic plants, deterioration of fisheries, deterioration of water quality, and other responses, are found objectionable and impair water use" (A.F. Bartsch, in Schelske and Stoermer 1971). In a letter published in 1970 in *Science*, J.R. Vallentyne warned "if steps are not taken soon to alleviate the problem [of eutrophication], we may find ourselves by the year 2000 in the middle of an algal bowl" (Vallentyne 1970). Unfortunately, Vallentyne's words proved to be prophetic, as eutrophication remains today one of the foremost threats to the health of freshwater ecosystems (Smith et al. 2006, Schindler 2006).

Eutrophication has serious social, ecological, and economic implications that degrade the value of freshwater ecosystems to humans and wildife. Firstly, eutrophication reduces the aesthetic, cultural, and recreational quality of lakes (Carpenter et al. 1998), and potentially expose humans to potent brain and liver toxins (Chorus et al. 2000). Secondly, eutrophication can degrade habitat quality for fish and aquatic birds, lead to fish kills, and cause losses in aquatic biodiversity (Smith and Schindler 2009). Thirdly, eutrophication lowers monetary value of shoreline properties, devalues sport and commercial fisheries, and increases the cost of water treatment. For example, Dodds et al. (2009) estimated that economic losses due to eutrophication of U.S. freshwaters is on the order of $2.2 billion per year.

Cyanobacteria, the photosynthetic prokaryotes that commonly dominate the algal community of eutrophic lakes (Scheffer et al. 1997, Dokulil and Teubner 2000), are responsible for many of the negative impacts of eutrophication (Smith 2003, Paerl and Otten 2013). Record-breaking blooms of cyanobacteria (or 'blue-green algae') are occurring in lakes around the world, from Canada's Lake Erie (Michalak et al. 2013) and Lake Winnipeg (Schindler et al. 2012) to China's Lake Taihu (Paerl et al. 2011), Africa's Lake Victoria (Mugidde et al. 2003) and Israel's Lake Kinneret (Zohary 2004). While the most conspicuous symptoms of cyanobacteria include water turbidity and unsightly scums along shorelines, the production of an array of potent toxins by cyanobacteria is of concern to the health of wildlife, domestic animals, and humans.

Cyanobacteria synthesize hundreds of toxic secondary metabolites (Welker and von Döhren 2006) which are collectively referred to as 'cyanotoxins' (Carmichael 1992). The four main cyanotoxins include microcystin, nodularin, saxitoxin, and cylindrospermopsin; the chemistry, biosynthesis, and toxicology of these cyanotoxins have been extensively reviewed (e.g., Sivonen and Jones 1999, van Apeldoorn et al. 2007, Pearson et al. 2010). Cyanotoxins cause toxic effects in fish and other aquatic organisms (Landsberg 2002, Malbrouck and Kestemont 2006, Martins and Vasconcelos 2009), as well as wildlife, domesticated animals, and humans (Funari and Testai 2008, Bownik 2010, Cheung et al. 2013). Humans are exposed to cyanotoxins through drinking water, consumption of fish and other seafood, contact with recreational waters, and haemodialysis (World Health Organization 2003, Codd et al. 2005, Ibelings and Chorus 2007). Outbreaks of animal and human poisonings from exposure to cyanotoxins have been documented (Chorus et al. 2000, Cheung et al. 2013). Toxic cyanobacteria have been reported in Canada (Kotak and Zurawell 2007) and 45 other countries around the world (Codd et al. 2005). Concerningly, the incidence of toxic cyanobacterial blooms is on the rise globally (Carmichael 2008, Hudnell 2010, Cheung et al. 2013).

Significant advances in our understanding of the causes of eutrophication occurred in the 1960 and 70s, when Canada's Lake Erie was in its "death throes" as fisheries were collapsing and beaches were fouled with decaying algae up to three feet thick (Sperry 1967). Simple models linking the trophic state of lakes to inputs of phosphorus and nitrogen from their watersheds were developed by R.A.

Vollenweider (Vollenweider 1968, 1976), which became the foundation for eutrophication management. However, a heated debate ensued over which nutrient needed to be curtailed in order to control algal blooms (Abelson 1970, Vallentyne 1970). In response to legislative proposals to remove phosphorus from detergents, a so-called "carbon-phosphorus controversy" erupted over the proposal that carbon, rather than phosphorus or nitrogen, limits algal productivity in many aquatic ecosystems (Likens et al. 1971). Experiments on whole lakes at Canada's Experimental Lakes Area generated strong scientific evidence, and stunning photos, that convinced the scientific community and policy makers that phosphorus control was the key to managing algal blooms in nutrient-polluted lakes (Schindler 1974, 1977).

Removal of phosphorus from detergents, coupled to sewage diversion or advanced treatment, led to improvements in water quality in many eutrophic lakes around the world (Cooke et al. 2005). Unfortunately, not all lakes responded to nutrient loading reductions as predicted based on Vollenweider's models (Marsden 1989, Jeppesen et al. 2005). Non-point sources of nutrient pollution (e.g., seepage from septic fields, and runoff from croplands, livestock operations, mining and construction sites, and other urban areas) were recognized as an important, yet challenging to control, part of the eutrophication problem (Carpenter et al. 1998). Further, scientists quickly realized that the recycling of phosphorus from bottom sediments, a phenomenon referred to as "internal phosphorus loading", was responsible for maintaining many lakes in a eutrophic state (Cooke et al. 1977, Søndergaard et al. 1999), even for decades after

substantial reductions in external nutrient loading (Søndergaard et al. 2003, Jeppesen et al. 2007). A number of in-lake remediation techniques were developed in an attempt to control internal phosphorus loading in eutrophic lakes, but these have been met with mixed success (Cooke et al. 2005).

Thesis Goal and Structure

The overall goal of this thesis is to advance our understanding of the contemporary causes of eutrophication of freshwater lakes, with the aim of developing recommendations for the management of this problem. Specifically, my research focuses on the environmental factors contributing to blooms of toxin-producing cyanobacteria in eutrophic lakes. This thesis contains four stand-alone scientific papers/manuscripts.

In chapter one, I assess the prevalence of the cyanobacterial toxin, microcystin, in Canadian lakes by consolidating data available from academics, government agencies, and private consulting companies. In addition, I relate microcystins concentrations in Canadian lakes to phosphorus and nitrogen concentrations and nitrogen-to-phosphorus ratios in order to better understand the conditions associated with episodes of microcystin contamination. I initiated this project because I was intrigued by the inverse relationship between nitrogen-to-phosphorus ratios and microcystin concentrations in three lakes in Alberta, Canada reported by Kotak et al. (2000), which reminded me of the widely known relationship between nitrogen-to-phosphorus and cyanobacterial dominance in the world's lakes published by Smith (1983). This project began with a small data set

for a poster presentation at an annual fisheries meeting (Orihel et al. 2011), but I later expanded the data set to a national scope, and published the findings as a rapid communication in the *Canadian Journal of Fisheries and Aquatic Sciences* (Orihel et al. 2012).

In chapter two, I link nutrient release from sediments of Israel's Lake Kinneret to the production of microcystins by the common cyanobacterium *Microcystis*. I incubated sediments cores under different environmental conditions, then used the overlying water as a medium for growing an toxin-producting strain of *Microcystis* isolated from Lake Kinneret. The impetus behind this project was that environmental factors correlated with microcystin concentrations in lakes are commonly attributed to the direct effect of those factors on cellular toxin production rates. I conducted this study to demonstrate how environmental factors can indirectly influence microcystin concentrations by increasing the pool of nutrients available for the growth of toxin-producing cyanobacterial populations. This project was conducted in collaboration with Israeli scientists during a 3-month internship at the Yigal Allon Kinneret Limnological Laboratory (funded by a Michael Smith Foreign Study Supplement to a Canada Graduate Scholarship). This work is published as a research article in *Annales de Limnologie* (Orihel et al. 2013).

The studies in chapter three and four are the first of a series of papers on a project I initiated and directed called the "University of Alberta's Iron Remediation Study" (funded by the Alberta Water Research Institute). The idea for this project originated in a draft proposal shared with me by David Schindler

entitled “Sulphur Enhancement of Eutrophication in Alberta” written by Dr. Tom Murphy (then, of Environment Canada). The Iron Remediation Study was a multi-year, multi-scale study involving collaborators from academia, government, and community groups, and aimed to investigate the effect of iron on internal phosphorus loading and cyanobacterial blooms in hypereutrophic ecosystems in Alberta. The project manipulated iron loading to lake sediments at incremental scales, first by adding iron to sediment cores (Phase I), then to in-lake mesocosms (Phase II), and finally, to agricultural dugouts (Phase III). Results from Phase I and II are presented in chapters three and four, respectively, in the form of draft manuscripts. The potential impacts of iron treatment on zooplankton and benthic invertebarates in Phase II were assessed in the Master’s thesis by Lindsey Wilson (Wilson 2013). Results from Phase III are not included in my thesis, and will be published with my collaborators in the future.

Chapter three and four are complementary, but employ different approaches. In chapter three, I develop a conceptual model to explain the occurrence of toxic cyanobacterial blooms in shallow nutrient-rich lakes, such as those on the Canadian Prairies. I propose that iron-deficient sediments, coupled to discontinuous polymixis, prime conditions for potentially toxic cyanobacterial blooms. I explore this hypothesis by combining small-scale sediment and culture experiments, a comprehensive one-year sampling program, and a multi-year monitoring data set for hypereutrophic Nakamun Lake, Alberta. This chapter is formatted as a manuscript for possible submission to *Limnology and Oceanography*. In chapter four, I test the conceptual model developed in the

previous chapter by manipulating iron loading to sediments in fifteen mesocosms in Nakamun Lake. I investigate the effect of different doses of iron on internal phosphorus loading, algal biomass and community composition, and microcystin concentrations over the course of this one-year experiment. This chapter is formatted as a manuscript for possible submission to the *Proceedings of the National Academy of Sciences*.

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Chapter 2. High microcystin concentrations occur only at low nitrogen-to-phosphorus ratios in nutrient-rich Canadian lakes[1]

Rapid Communication

The occurrence of cyanobacterial toxins in Canadian freshwaters is a serious environmental and public health concern (Kotak and Zurawell 2007). One common class of cyanobacterial toxins, the microcystins, are potent inhibitors of eukaryotic protein phosphatases. In controlled experiments, microcystins have been shown to cause liver haemorrhage and promote tumour development in mammals. Furthermore, microcystins have been implicated in illnesses and deaths of domestic animals, wildlife, and humans following exposure to these toxins in the natural environment. Concentrations of microcystins are highly dynamic on both temporal and spatial scales, and understanding the factors that drive this variation in and among freshwater systems is key to developing effective water management tools and policies. In the current study, we report the likelihood of microcystin concentrations exceeding water quality guidelines in eutrophic lakes, ponds, and reservoirs in Canada increases as the mass ratio of nitrogen (N) to phosphorus (P) declines.

Smith (1983) first described a strong relationship between the relative amounts of N and P in surface waters and cyanobacterial blooms. In this seminal

[1] A version of this chapter has published. Orihel, D. M., D. F. Bird, M. Brylinsky, H. Chen, D. B. Donald, D. Y. Huang, A. Giani, D. Kinniburgh, H. Kling, B. G. Kotak, P. R. Leavitt, C. C. Nielsen, S. Reedyk, R. C. Rooney, S. B. Watson, R. W. Zurawell, and R. D. Vinebrooke. 2012. High microcystin concentrations occur only at low nitrogen-to-phosphorus ratios in nutrient-rich Canadian lakes. Canadian Journal of Fisheries and Aquatic Sciences 69:1457–1462.

paper, Smith argued that diazotrophic cyanobacteria should be superior competitors under conditions of N-limitation because of their unique capacity for N-fixation. The hypothesis that low N:P ratios favor cyanobacteria has been intensely debated (Lampert 1999) and challenged for its poor performance predicting cyanobacterial dominance (Downing et al. 2001). The dominance of N-fixing cyanobacteria at low N:P ratios has been decisively demonstrated in mesocosm- and ecosystem-scale experiments in prairie and boreal lakes (Schindler et al. 2008, and references therein). Nonetheless, some still question whether these experimental results can be generalized to hypereutrophic lakes with a long history of anthropogenic nutrient loading (e.g., Paerl et al. 2011) .

The main goal of our investigation was to test for a relationship between the mass ratio of N to P and microcystin concentrations in water bodies across Canada. Given that production of microcystins is exclusive to certain members of the Phylum Cyanophyta (Cyanobacteria), the presence of microcystins in lakes should theoretically be higher under low N:P ratios, if cyanobacteria dominate under conditions of relative N deficiency. Noteably, some cyanobacteria that produce microcystins are capable of N-fixation (e.g. *Anabaena*), but many microcystin producers are non-diazotrophic (e.g., *Microcystis*). The dominance of cyanobacteria does not necessarily predicate the occurrence of microcystins because not all cyanobacterial species are capable of synthesizing microcystins, and not all strains of known toxin-producing species are toxic. As such, this hypothesis does not imply a simple linear, negative relationship exists between N:P ratios and microcystin concentrations, but rather, that microcystin

concentrations are potentially elevated at low N:P ratios. A negative relationship between N:P ratio and microcystin concentration has previously been reported in three Albertan lakes (Kotak et al. 2000), four US states (Graham et al. 2004), and Lake Erie (Rinta-Kanto et al. 2009), but has not been tested at the broad geographic extent examined in the current study.

We compiled measurements of microcystin concentrations in freshwaters collected in Canada between 2001 and 2011. A comprehensive program for monitoring cyanobacterial toxins in Canada does not exist, and thus we collated data from academics, government agencies, and private companies. Details of their respective sampling protocols and analytical methods are provided in Table S2-1. Our consolidated data set of microcystin measurements includes 246 water bodies situated in British Columbia, Alberta, Saskatchewan, Manitoba, Ontario, Québec, Newfoundland and Labrador, New Brunswick, Nova Scotia, and Prince Edward Island. In most cases, concentrations of total microcystins were determined by protein phosphatase inhibition assay or enzyme-linked immunosorbent assay. Where microcystins were measured by high performance liquid chromatography or liquid chromatography-(electrospray ionization) tandem mass spectrometry, the sum of individual variants was assumed to approximate total microcystin concentration. In samples below limits of detection (0.005 – 0.22 $\mu g\ L^{-1}$), total microcystin concentrations were assigned a value of half the limit of detection. Total P and total N concentrations were also included in our database if these parameters were measured on the same sample as microcystins (or samples collected on the same day). We considered each water body as one

sampling site, with the exception of three large systems (i.e., Lake Erie: 43 sites; Lake of the Woods: 32 sites; and Lake Winnipeg: 87 sites). In total, our data set includes 405 sites and 3 474 microcystin measurements, of which 982 measurements have associated P and N data. We excluded lakes with only one sample from all statistical analyses.

Microcystins were detected in freshwaters in every province in Canada from coast to coast (Figure 2-1). Concentrations ranged from below minimum detection limits to a maximum of 2 153 µg L^{-1}. Notably, all regions contained lakes where toxin concentrations reached levels of concern. Although the sampling effort was unequal across the country, the highest concentrations tended to occur in the Canadian Prairie provinces, particularly in clusters in central Alberta and southwestern Manitoba. Prairie lakes are typically shallow and warm, with high nutrient concentrations (as a result of high external and internal nutrient loading) –conditions ideal for the proliferation of cyanobacteria. In our national database, 18% of samples and 41% of lakes exceeded the World Health Organization's drinking water quality guideline of 1.0 µg L^{-1}. The Canadian drinking water guideline of 1.5 µg L^{-1} was exceeded in 14% of samples and 35% of lakes, while 1.3% of samples and 9% of lakes surpassed the proposed Canadian guideline for recreational waters of 20 µg L^{-1}.

Our results support the current hypothesis that microcystin concentrations increase with lake trophic status (Kotak and Zurawell 2007). First, we examined the correlation between concentrations of nutrients and microcystins in Canadian lakes (Figure S2-1). Microcystin concentrations were positively correlated with

concentrations of P (Spearman Rank Correlation, $r = 0.36$, $p < 0.01$, $n = 937$) and N (Spearman Rank Correlation, $r = 0.39$, $p < 0.01$, $n = 937$). The strength of these correlations, however, imply that variables other than nutrient concentrations most certainly played a role in determining microcystin concentrations. Next, we calculated minimum thresholds for P and N (independently) as the nutrient concentration above which 95% of values exceeding a specific level of microcystin occurred (Table S2). For example, 95% of the instances where microcystin concentrations exceeded the World Health Organization's drinking water guideline of 1 µg L^{-1} occurred when P concentrations were above 26 µg L^{-1} and N concentrations were above 658 µg L^{-1}. Minimum thresholds for P and N were successively higher for microcystin concentrations of 1, 2, 5, and 10 µg L^{-1} (Table S2). Therefore, correlation analyses and minimum threshold determinations clearly demonstrated that microcystins were primarily a concern in eutrophic and hypereutrophic systems.

As expected based on our hypothesis, our meta-analysis revealed that microcystin concentrations in Canadian freshwaters were high only at low N:P ratios, and conversely, were consistently low at high N:P ratios (Figure 2-2, a-b). These observations were especially apparent for lakes in Alberta (Figure 2-2, a), but also held true for systems in other provinces (Figure 2-2, b). Note that N:P ratio was, as anticipated, weakly correlated with microcystin concentration (Spearman Rank Correlation; $r = -0.09$, $p = 0.004$, $n = 937$). While N:P ratios are not suitable for predicting absolute concentrations of microcystins, we propose N:P ratios are useful for estimating the "risk" of elevated microcystin

concentrations. We performed a simple numerical analysis to quantify the probability of microcystin exceeding specific toxin thresholds at four N:P categories (<20, 20-40, 40-60, and >60)(Table S2). Probabilities were calculated by dividing the number of samples above a specific toxin threshold by the total number of samples in each N:P category. The probability of microcystin concentrations exceeding all toxin thresholds was highest when N:P ratios were less than 20, and decreased at higher N:P ratios (Figure 2-2, c). Importantly, the probability of levels of concern for recreational contact dropped to near zero above an N:P ratio of 40, and similarly, that for drinking water was negligible above an N:P ratio of 60.

Relating microcystin concentration in lakes to nutrient ratios (Figure 2-2, a this study, and similar figures in Kotak et al. 2000 and Graham et al. 2004) ignores the important influence of nutrient concentrations, and thus, a more informative approach is to consider the relationship between microcystin concentrations and both nutrient ratios and concentrations. To begin, we visualized these associations in a scatterplot of N versus P concentration, with microcystin levels displayed categorically with different symbols, and different N:P ratios depicted by diagonal lines (Figure 2-2, d). Shown in this manner, it is evident that microcystin concentrations in Canadian lakes in our study were elevated only in nutrient-rich waters at low N:P ratios. Note that minimum nutrient thresholds for P and N concentrations (Table S2-2) corresponding to microcystin levels of 1, 2, 5, and 10 µg L^{-1} are shown graphically in Figure 2-2, d.

Next, we performed a regression tree analysis to identify the nutrient conditions under which N:P ratios are most strongly associated with high microcystin concentrations. Regression trees explain the variation of a single response variable by repeatedly partitioning the data into homogenous groups using explanatory variables, which is a powerful technique for analyzing complex ecological data with non-linear relationships and higher-order interactions. Using the TREES module in SYSTAT 13, we constructed a regression tree to explain microcystin concentrations in Canadian water bodies based on P concentrations, N concentrations, and N:P ratios (Figure S2-2; total proportional reduction in error = 0.46; n = 937). This analysis indicated that microcystin concentrations remained low (mean ± SD = 0.39 ± 0.99 μg L^{-1}) as long as N concentrations were under 2 600 μg L^{-1}. When N concentrations exceeded this threshold, microcystin concentrations were higher when N:P ratios were below 23 (5.6 ± 8.4 μg L^{-1}), in comparison to when ratios were above 23 (1.5 ± 2.3 μg L^{-1}). In other words, regression tree analysis supported our hypothesis that high microcystin concentrations occur under low N:P ratios, and stipulated this hypothesis is applicable under high nutrient concentrations. In keeping with our findings in Canadian water bodies, high N and P concentrations, coupled with low N:P ratios, were recently determined to favor the growth of toxigenic strains of *Microcystis spp.* in hypereutrophic Lake Taihu, China (Otten et al. 2012).

While the correspondence between high microcystin concentrations and low N:P ratios we observed in Canadian lakes is consistent with our original hypothesis (i.e., conditions of relative nitrogen deficiency favor the dominance of

cyanobacteria, and thus enable the potential for microcystin production), there are several alternative explanations for this observation. First, N:P ratios may physiologically cue cyanobacteria to produce microcystins and thereby increase their intracellular microcystin content (Lee et al. 2000). Second, N:P ratios may be indicative of the nature of nutrient sources to oligotrophic vs. eutrophic ecosystems (Downing and McCauley 1992), and therefore, the association between N:P and microcystin results from the co-variation of N:P ratios and lake trophic status. Third, low N:P ratios may be the consequence, rather than the cause, of cyanobacterial blooms. Many planktonic cyanobacteria, such as *Microcystis,* have a benthic life stage where they engage in "luxury uptake" of P from sediments, and consequently, episodes of cyanobacterial recruitment from sediments can dramatically decrease the N:P ratio in the water column (Xie et al. 2003).

In summary, our meta-analysis of microcystins in Canadian freshwaters has revealed that microcystins are now an issue of national concern as these toxins were detected in every province, and concentrations exceeded water quality guidelines for drinking water, and sometimes for recreational waters, in many eutrophic ecosystems. Consolidating data on microcystin concentrations in water bodies across Canada allowed us to determine nationally-relevant: minimum thresholds for P and N concentrations corresponding to microcystin exceedences, probabilities of microcystin exceedances at different N:P ratios, and a regression tree partitioning variance in microcystin concentrations based on nutrient concentrations and ratios. Once externally validated, these models may become

applicable as screening tools for identifying potentially toxic "hotspots" or "hot times" of unacceptable concentrations of microcystins based on simple chemical parameters that are routinely measured in water quality monitoring programs. Our survey revealed that microcystin concentrations in Canadian freshwaters were elevated only under low mass ratios of N to P, and while this observation is not likely to be disputed, its interpretation and potential ramifications may prove contentious. Is the association between N:P ratios and microcystin concentrations mere coincidence or driven by a biogeochemical mechanism? The present study is a mensurative experiment, and hence we cannot invoke a "cause-and-effect" argument, or identify the causal mechanisms, to explain why high microcystin concentrations coincide with low N:P ratios. We recommend that subsequent experiments should manipulate N:P ratios – at scales relevant to ecosystem management – as the outcome may be germane to the on-going debate regarding the need for a "dual-nutrient management strategy" (Paerl et al. 2011).

Figures

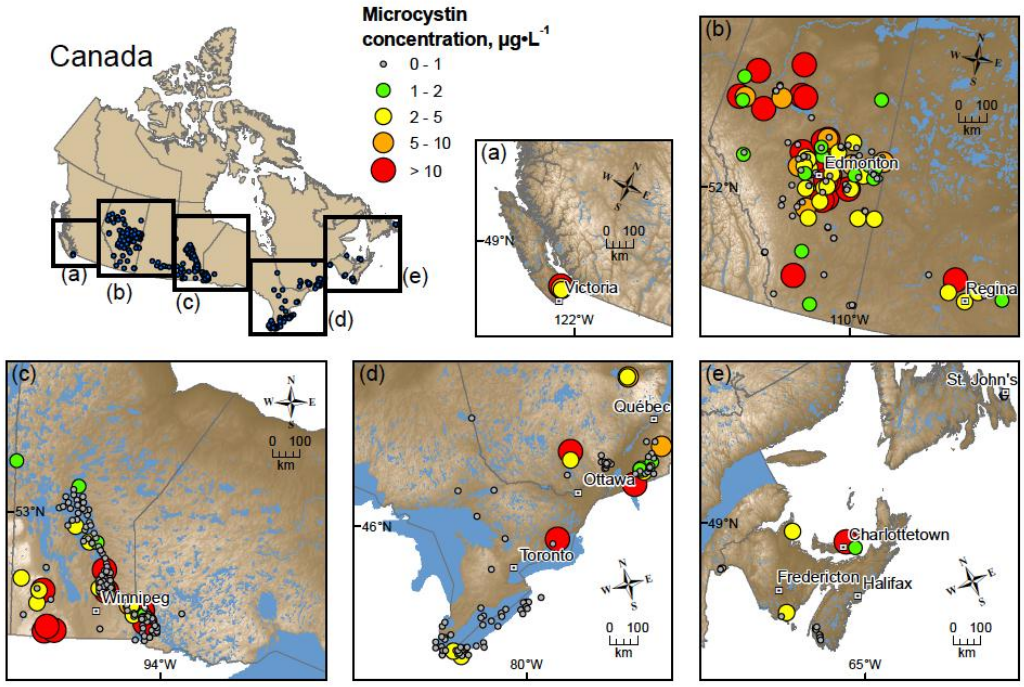

Figure 2-1. Microcystin concentrations in lakes, ponds, and reservoirs in Canada. The map of Canada indicates sampling locations for microcystins. Inset maps (a-e) indicate the maximum concentration of microcystin recorded in each water body (or at each sampling site, in the case of Lake Erie, Lake of the Woods, and Lake Winnipeg).

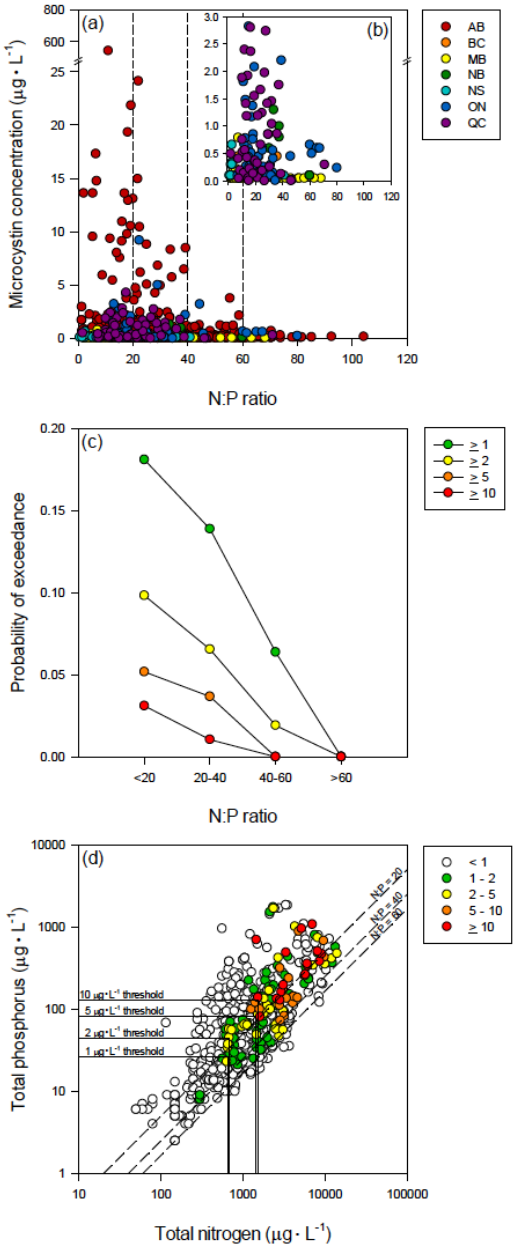

Figure 2-2. Microcystin concentrations in Canadian freshwaters in relation to the mass ratio of total nitrogen (N) to total phosphorus (P). Scatterplot of N:P ratio vs. microcystin concentration, with data for each province denoted by a different symbol (a). Dashed lines indicate N:P ratios of 20, 40, and 60. The inset (b) shows the same plot on a smaller y-axis scale, and with data points for water bodies in Alberta excluded. Probability of exceeding specific microcystin concentrations (1, 2, 5, or 10 µg L^{-1}) at four N:P categories (c). Scatterplot of N concentration vs. P concentration with symbols denoting microcystin concentration classes (µg L^{-1})(d). Dashed lines as in panel (a). Solid lines indicate 95% nutrient thresholds, calculated independently for P and N, above which microcystin concentrations exceed 1, 2, 5, or 10•µg L^{-1}.

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Chapter 3. Internal nutrient loading may increase microcystin concentrations in freshwater lakes by promoting growth of *Microcystis* populations[2]

Introduction

Microcystins are a family of monocyclic heptapeptide toxins which are commonly found in nutrient-polluted freshwaters (Carmichael, 1992). Microcystins are produced by certain species of cyanobacteria, and are well known for their potent toxicity. These compounds strongly inhibit specific protein phosphatases in animal cells, triggering a cascade of events leading to cell necrosis or apoptosis (Campos et al., 2010). Microcystins can promote tumor development in mammals, and are classified as possible human carcinogens (Funari and Testai, 2008). Microcystin toxins have been implicated in poisonings of aquatic invertebrates and fish (Landsberg, 2002), wild and domestic terrestrial animals (Briand et al., 2003), and humans (Chorus et al., 2000). Because of the potential for microcystins to inflict illness or death, the increasing and widespread proliferation of toxin-producing cyanobacteria in freshwaters is serious cause for concern (Carmichael, 2008).

High concentrations of microcystins are frequently associated with blooms of the cyanobacterium *Microcystis* (order Chroococcales) in eutrophic water bodies (Sivonen and Jones, 1999). *Microcystis* is considered a global threat to human health because of its cosmopolitan distribution and ability to thrive in a

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wide range of climates (Pearson et al., 2010). Microcystin production likely evolved in an ancient ancestor of modern cyanobacteria (Rantala et al., 2004), and several extant species of *Microcystis* have retained the genes for microcystin synthesis (Via-Ordorika et al., 2004). Although the biological function of microcystins is still under debate, these compounds may serve as infochemicals (Kaplan et al., 2012), or perhaps as protectants against oxidative stress (Zilliges et al., 2011). Dynamics of microcystins in freshwater lakes have been related to the biomass of *Microcystis* species (Kotak et al., 2000; Ozawa et al., 2005) and seasonal succession of toxic *Microcystis* genotypes (Kardinaal et al., 2007; Davis et al., 2009), confirming the role of *Microcystis* as an important source of these toxins in the natural environment.

Microcystin concentrations in freshwater lakes are often correlated with environmental parameters (Wicks et al., 1990; Kotak et al., 2000; Giani et al., 2005; Rantala et al., 2006; Rinta-Kanto et al., 2009). This observation led to the hypothesis that toxin production by *Microcystis* is controlled, in part, by changes in environmental factors. Indeed, laboratory experiments have demonstrated that physicochemical variables can directly affect microcystin production by *Microcystis* cells. For example, rates of toxin production have been shown to be altered by temperature (Ame et al., 2005), light (Deblois et al., 2010), nitrogen (Dai et al., 2008), phosphorus (Bickel et al., 2000; Oh et al., 2000), inorganic carbon (Jahnichen et al., 2007), iron (Lukac et al., 1993; Sevilla et al., 2008) and sulfur (Long, 2010). However, cellular microcystin quotas of cyanobacteria exposed to different environmental conditions usually do not vary by more than

five-fold (Sivonen and Jones, 1999). Given that microcystin concentrations in surface waters of eutrophic lakes and ponds can vary by several orders-of-magnitude (Kotak and Zurawell, 2007), the direct effects of environmental factors on cellular toxin production cannot alone explain the observed variation in microcystin concentrations in aquatic ecosystems.

While the direct effects of environmental factors on microcystin production have been intensively studied at the cellular level, the complex pathways by which environmental factors indirectly influence microcystin concentrations in aquatic ecosystems are poorly understood. Microcystin concentrations in freshwater lakes are determined by the abundance of cyanobacterial cells, the relative dominance of toxic vs. non-toxic strains, and the microcystin production rate of toxic cells (Zurawell et al., 2005). Environmental factors potentially exert control over concentrations of microcystins in freshwater lakes by triggering natural geochemical processes that stimulate the proliferation of toxigenic cyanobacteria. Specifically, changes in environmental conditions can activate the release of important algal nutrients, such as phosphorus and nitrogen, from bottom sediments. This phenomenon, known as 'internal loading' (Søndergaard et al., 2009) or 'internal eutrophication' (Smolders et al., 2006), can fuel blooms of cyanobacteria in eutrophic lakes (e.g., Burger et al., 2008; Grace et al., 2010). However, the link between episodes of nutrient release from sediments and elevated concentrations of microcystin toxins is not well established.

In the present study, we provide experimental evidence supporting the "indirect effect" hypothesis to explain the relationship between environmental

factors and microcystin concentrations in freshwater lakes. Specifically, we tested the hypothesis that environmental conditions that enhance the release of nutrients from sediments increase microcystin concentrations in lake water by stimulating the growth of *Microcystis* populations. To test this hypothesis, we first exposed natural lake sediments from an important freshwater lake – Israel's Lake Kinneret – to different environmental conditions to mimic seasonal nutrient fluxes from sediments. Next, we cultured a toxigenic strain of *Microcystis* in natural surface waters from Lake Kinneret amended with overlying water harvested from incubated sediments. This latter experiment examined how nutrients released from sediments affect the growth of *Microcystis* populations, and how this change in biomass alters concentrations of microcystins in lake water.

Materials and Methods

Study Lake

We conducted our experiments using sediments collected from Lake Kinneret ('Sea of Galilee'), the largest freshwater lake in Israel (32°42' - 32°55' N; 35°31' - 35°39' E). This subtropical lake covers an area of 170 km^2 and has a maximum depth of 43 m (Serruya, 1978). During the stratification period (April to December), the thermocline depth is typically between 15 and 20 m, and the epilimnion is warm (24-30°C) and well-oxygenated, while the hypolimnion is relatively colder (14-16°C) and anoxic. Lake Kinneret is mesotrophic with annual mean concentrations of total phosphorus and total nitrogen of approximately 20

μg P L^{-1} and 0.6 mg N L^{-1}, respectively (Zohary, 2004). While cyanobacteria were once a minor component of the phytoplankton community of Lake Kinneret, a major regime shift occurred in the mid-1990s towards more frequent and more intense blooms of cyanobacteria (Zohary 2004). Since 1995, toxic populations of *Microcystis* commonly form surface scums in Lake Kinneret (Ostrovsky et al., 2013).

Sediment Core Experiment

On 10 March 2010, sediment cores (n = 12) were retrieved from Lake Kinneret in polycarbonate tubes (inner diameter: 5.5 cm; height: 60 cm) using a Tessenow gravity sampler (Tessenow et al., 1977). Six "profundal" cores were collected from a depth of 32 m, and six "littoral" cores were collected from a depth of 10 m. Care was taken to collect intact sediment columns with an undisturbed sediment-water interface. The height of "overlying water" (i.e., water above the sediment-water interface) was adjusted to exactly 25 cm to ensure each tube contained the same volume of overlying water (0.6 L).

Littoral and profundal sediment cores were randomly assigned to either "summer" or "winter" incubation conditions (n = 3 cores/treatment). The incubation conditions were carefully chosen (based on long-term monitoring data of Lake Kinneret collected by the Yigal Allon Kinneret Limnological Laboratory) to reflect the ambient temperature and oxygen regimes experienced by sediments in Lake Kinneret (see Table 3-1). Littoral cores under "winter" conditions (i.e., cold/oxic) were incubated at 16^{o}C and continuously aerated, whereas littoral cores under "summer" conditions (i.e., warm/oxic) were incubated at 25^{o}C and

continuously aerated. Aeration was achieved by gently and continuously bubbling overlying water with filtered (0.2 µm) atmospheric air using aquarium pumps. Profundal cores under "winter" conditions (i.e., cold/oxic) were incubated at 16^{o}C and aerated, whereas profundal cores under "summer" conditions (i.e., cold/anoxic) were purged with N_2 gas for 10 min, capped and sealed with parafilm, and then incubated at 16^{o}C. All cores were incubated in environmental chambers in darkness.

Overlying water in each sediment core was sampled for nutrients on days 0 and 7 of the incubation period, and filtered (0.45 µm) immediately after collection. Samples were analyzed for soluble reactive phosphorus (SRP), total dissolved phosphorus (TDP), ammonia (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-) using a Flow Injection Auto-Analyzer (QuikChem 8000, Automated Ion Analyzer, Lachat Instruments) following the manufacturer protocol or spectrophotometrically according to Standard Methods (APHA et al., 2005). Total dissolved nitrogen (TDN) was measured using a spectrophotometer (Kontron) following a modified Kjeldahl method (APHA et al., 2005). Dissolved inorganic nitrogen (DIN) is expressed as the sum of NH_4^+, NO_3^-, and NO_2^-. Flux rates of nutrient release from sediments were calculated by dividing the change in the nutrient concentration over the incubation period by the length of the incubation period and the surface area of the sediments.

Microcystis Experiment

We collected and incubated a second set of sediment cores to obtain the growth media for our *Microcystis* experiment, following an approach similar to Cymbola et al. (2008). Four littoral and four profundal sediment cores were collected from Lake Kinneret and incubated in the laboratory under "summer" conditions for 1 wk. As was done in the previous experiment for the summer treatments, littoral cores were subject to "oxic/warm" conditions, whereas profundal cores were subject to "anoxic/cold" conditions. At the end of the 7-day incubation period, overlying water from cores of the same treatment was pooled and filtered through pre-rinsed GF/C filters.

Three types of growth media were prepared in acid-washed glass flasks (see Table 2). The control medium, referred to as the "SW" medium, consisted of only filtered (GF/C) surface water from Lake Kinneret. The "LS" medium consisted of 20% filtered surface water from Lake Kinneret and 80% filtered overlying water from the littoral sediment cores incubated under "summer" conditions. The "PS" medium consisted of 20% filtered surface water from Lake Kinneret and 80% filtered overlying water from the profundal sediment cores incubated under "summer" conditions.

For our study, we used a *Microcystis* strain (MG-K) previously isolated from Lake Kinneret. This strain is green due to a lack of phycoerythrin, does not form colonies in the laboratory, and is known to produce microcystins (Beresovsky et al., 2006). Prior to the experiment, an inoculum culture of *Microcystis* MG-K was prepared in BG (-P) medium, and then transferred to 1 L

of filtered (GF/C) surface water from Lake Kinneret and acclimated in an environmental growth room for 2 d prior to the experiment.

Four replicate experimental cultures were prepared in acid-washed flasks for each of the three treatments (i.e., SW, LS, and PS media). Growth medium (425 mL) was added to each 1-L flask, and then inoculated with 10 mL of the *Microcystis* inoculum culture to achieve a target nominal chlorophyll *a* concentration of 4 μg L^{-1}. This target was about 50% lower than the observed chlorophyll *a* concentration in Lake Kinneret at the time of the experiment. Cultures were incubated for 7 d in an environmental growth room at a temperature of 20^{o}C and under a constant light level of 50 μmol photons m^{-2} s^{-1}. Cultures were gently swirled by hand twice each day.

We assessed nutrient availability in the growth media, as well as the nutrient status, growth, and toxin concentrations of the *Microcystis* cultures. All parameters were measured in each culture on days 0, 1, 4, and 7 (with the exception of toxin levels, as noted below). To examine nutrient availability in the growth media, we measured concentrations of SRP, NH_4^+, NO_3^-, and NO_2^- (following the methods described above for the sediment core experiment). Phosphorus status of phytoplankton was determined by measuring particulate phosphorus (PP) and alkaline phosphatase activity (APA). For PP determination, algal cells were collected on GF/C filters, digested with persulfate, and analyzed using the Flow Injection Auto-Analyzer. For APA determination, algal cells were collected on 2 μm Millipore filters, incubated with 4-methyl-umbelliferyl phosphate at 37^{o}C for 1 h (Hadas et al., 1999), and measured on a Fluoroskan

plate reader (Thermo Scientific Fluoroskan Ascent, USA). To measure growth of phytoplankton, chlorophyll *a* was measured fluorometrically using the method of Holm-Hansen et al. (1965). To assess toxin production, the inoculum culture was sampled on day 0 (50 mL), and the experimental cultures were sampled on day 7 (100 mL) for microcystin determination. Whole water samples for toxin analyses were frozen, freeze-dried, extracted in methanol, and quantitively analyzed by High Performance Liquid Chromatography (Lawton et al., 1994).

Statistical Analyses

All data analyses were performed using SigmaPlot for Windows (Version 11.0). When necessary, data were transformed to satisfy the assumption of normality for parametric statistical methods (assessed by Shapiro-Wilk's Tests). For the sediment core experiment, we performed two-way repeated measures analyses of variance (ANOVA) to compare P and N concentrations at the beginning and end of the incubation period among the four seasonal treatments. We assessed whether fluxes of P and N from sediments were significantly different from zero using t-tests. For the *Microcystis* experiment, we performed two-way repeated measures ANOVA to compare changes in chlorophyll *a* and total microcystin concentrations over time among *Microcystis* cultures with different growth media. Each two-way repeated measures ANOVA was followed by a pairwise multiple comparison procedure (Holm-Sidak Method).

Results

Sediment Core Experiment

At the beginning of the experiment, concentrations of SRP and TDP in overlying water were, as expected, similar among the six replicate cores collected at the same depth in Lake Kinneret (SE < 8% of the mean; Figure 3-1, a-b). In comparison, average phosphorus concentrations in profundal cores from the deep station (21 and 24 μg L^{-1}, for SRP and TDP, respectively) were 3- to 5-fold higher than those of littoral cores from the shallow station (4 and 8 μg L^{-1}, for SRP and TDP, respectively). Over the 7-d incubation period under different environmental conditions, average concentrations of SRP decreased in both winter treatments, from 4 μg L^{-1} to below the limit of detection in the littoral cores, and from 22 to 4 μg L^{-1} in the profundal cores. In contrast, SRP concentrations increased in both summer treatments, from 5 to 31 μg L^{-1} in the littoral cores and from 22 to 134 μg L^{-1} in the profundal cores. The dynamics of TDP in overlying water were similar to those of SRP (c.f. Figure 3-1, a and b). Concentrations of P in overlying water of the sediment cores differed significantly over time and among treatments during the experiment for both SRP (ANOVA; time: $F = 54$, $p < 0.001$; treatment: $F = 50$, $p < 0.001$; Figure 3-1, a) and TDP (ANOVA; time: $F = 213$, $p < 0.001$; treatment: $F = 209$, $p < 0.001$; Figure 3-1, b).

The net flux of SRP and TDP from sediments to overlying water over the incubation period was, on average, negative for the winter treatments, and positive for the summer treatments (Table 3-1). All SRP and TDP fluxes were significantly different from zero, with the exception of the winter treatment of

littoral cores. Phosphorus fluxes were notably higher from profundal than littoral sediments under summer conditions.

Concentrations of DIN and TDN in overlying water were similar among all sediment cores at the start of the experiment collected at the same depth in Lake Kinneret (SE < 2% of the mean), with little difference in the average concentration between littoral (0.38 and 0.75 mg L^{-1}, for DIN and TDN, respectively) and profundal cores (0.45 and 0.85 mg L^{-1}, for DIN and TDN, respectively). At this time, the DIN pool was dominated by NO_3^- (Figure S3-1).

Concentrations of DIN and TDN in the overlying water of the sediment cores increased over the 7-d incubation period in all treatments (Figure 3-1, c-d). The largest changes in DIN were observed for the summer treatments of both the littoral and profundal cores, which increased from 0.38 to 1.55 mg L^{-1}, and from 0.45 to 0.91 mg L^{-1}, respectively. Under summer conditions, the increase in dissolved nitrogen was largely due to an increase in NH_4^+ in the profundal cores, but due to an increase in both NH_4^+ and NO_2^- in the littoral cores (Figure S3-1). An increase in DIN between day 0 and 7 was also observed, but to a lesser extent, under winter conditions, where average concentrations increased modestly from 0.38 to 0.45 mg L^{-1} in the littoral cores, and from 0.45 to 0.69 mg L^{-1} in the profundal cores. Concentrations of N were significantly different over time and among treatments for both DIN (ANOVA; time: $F = 321$, $p < 0.001$; treatment: $F = 25$, $p < 0.001$; Figure 3-1, c) and TDN (ANOVA; time: $F = 91$, $p < 0.001$; treatment: $F = 14$, $p < 0.001$; Figure 3-1, d).

Net fluxes of DIN and TDN from sediments were positive and significantly different from zero under most conditions, with the exception of the littoral winter treatment (Table 3-1). Nitrogen fluxes were greater under summer than winter conditions, with the highest fluxes observed in the littoral sediment cores incubated under warm/oxic conditions (Table 3-1).

Microcystis Experiment

Because sediments acted as sources of nutrients primarily in the summer treatments, we focused on the response of algae to nutrients released from littoral and profundal sediments incubated under summer conditions only. Initial phosphorus and nitrogen concentrations in the three growth media (SW, LS, and PS), prior to inoculation with *Microcystis*, are provided in Table 3-2. Notably, the SRP concentration of the PS medium was an order of magnitude higher than in the LS and SW media, and the DIN concentrations of the LS and PS media were several-fold higher than in the SW media.

Although there was initially more SRP in the PS growth medium (51 $\mu g\ L^{-1}$, on average), concentrations of SRP in all treatments were below detection limits immediately after the addition of *Microcystis*. In contrast to the rapid uptake of SRP, DIN concentrations exhibited little change before and after inoculation of *Microcystis* in all growth media (Figure 3-2, a-c). Over the course of the experiment, DIN slowly declined from an average concentration of 0.15 to 0.09 mg L^{-1} in the SW cultures, and from 0.83 to 0.64 mg L^{-1} in the LS cultures, but a substantial drop in DIN from 0.61 to 0.16 mg L^{-1} was observed in the PS cultures.

Most of the decrease in DIN in growth media was due to losses in the NH_4^+ pool (Figure S3-2).

Steady uptake of phosphorus by *Microcystis* was observed in all cultures, based on the temporal trends in PP levels (Figure 3-2, d-f). The nominal PP concentration in the experimental cultures at time 0 (based on the concentration of the inoculum culture) was 4 µg L^{-1}, but rapidly increased within the first 24 h of the experiment. *Microcystis* cultures in PS media achieved a maximum PP concentration over 100 µg L^{-1}, which was greater than that observed in the LS media (66 µg L^{-1}) and SW media (58 µg L^{-1}).

All cultures had similar APA activity when sampled on day 0 (range: 1065 – 1199 nmol L^{-1} h^{-1}), but differences among treatments in APA activity emerged over the course of the experiment. The chl-normalized APA activities of *Microcystis* grown in SW and LS media remained high during the experiment, whereas the APA activities of cultures in PS media decreased sharply (Figure 3-2, g-i).

The observed chlorophyll *a* concentrations of *Microcystis* cultures at the start of the experiment (range: 3.1 to 4.1 µg L^{-1}, n = 12) were similar and near the target nominal concentration of 4 µg L^{-1}. *Microcystis* cultured only in surface water from Lake Kinneret (SW media) did not grow during the 7-d experiment (Figure 3-2, j). Contrary to these control cultures, *Microcystis* cultured in water previously exposed to sediments increased in biomass (Figure 3-2, k-l). Over 1 wk of incubation, average chlorophyll *a* concentration of *Microcystis* cultures in

LS media doubled (to 6 ± 1 μg L^{-1}), whereas that of cultures in PS media increased by an order of magnitude (to 37 ± 8 μg L^{-1}). Chlorophyll *a* concentrations among the three treatments were statistically different at the end of the experiment, and a significant increase in chlorophyll *a* was observed over time in the LS and PS treatments (ANOVA; time: $F = 25$, $p < 0.001$; treatment: $F = 25$, $p < 0.001$; Figure 3-3, a).

The inoculum *Microcystis* culture contained six microcystin analogues: microcystin–LR, –RR, and –YR, as well as three unidentified microcystin analogues (eluted at 16.47, 17.04, and 18.43 min). The total concentration of microcystins in the inoculum culture was 493 μg L^{-1} on the day inocula were added to the experimental flasks. Based on this measured concentration in the inoculum, the nominal microcystin concentration in the experimental *Microcystis* cultures was 11.6 μg L^{-1} at the beginning of the experiment (Figure 3-3).

Microcystis cultures in SW and LS media produced microcystin–RR and three unidentified microcystin analogues (eluted at 16.47, 17.04, and 18.43 min). *Microcystis* cultures in PS media also produced these microcystin analogues, plus microcystin–LR and –YR. The four replicate cultures of each treatment showed close agreement in terms of both the amount and composition of microcystins (Figure S3-3). The total microcystin concentration of *Microcystis* cultures in SW media did not change during the experiment (11 μg L^{-1}), whereas concentrations of total microcystins of *Microcystis* cultures in LS and PS media increased, on average, to 17 and 45 μg L^{-1} respectively. Total microcystin concentrations

among the three treatments were statistically different at the end of the experiment, and a significant increase in microcystin levels was observed over time in the LS and PS treatments (ANOVA; time: $F = 155$, $p < 0.001$; treatment: $F = 82$, $p < 0.001$; Figure 3-3, b). Average (± SD) microcystin levels of *Microcystis* cultures, normalized to the chlorophyll *a* content of each culture, were 3.62 ± 0.30, 2.89 ± 0.56, and 1.24 ± 0.12 µg toxin per µg chl for SW, LS, and PS media, respectively.

Discussion

In this study, we postulated – and demonstrated experimentally – that nutrient release from lake sediments stimulates growth of the common cyanobacterium *Microcystis*, and this in turn, increases concentrations of microcystin toxins in lake water. Previous studies have been successful in demonstrating that physicochemical factors can directly affect cellular microcystin production by *Microcystis*, but the observed responses have been modest. The effects of environmental factors on the population growth of *Microcystis* and other microcystin-producing cyanobacteria, rather than directly on toxin production, likely explain more of the observed variation in microcystin concentrations in freshwater lakes. Thus, in order to explain the dynamics in microcystin levels observed in natural ecosystems, it is critical to understand the contribution of biogeochemical processes to blooms of toxigenic cyanobacteria. One pathway by which environmental factors indirectly exert control on microcystin concentrations in freshwater ecosystems is through stimulating in-lake biogeochemical processes that supply the nutrients essential to the growth of

toxin-producing cyanobacteria. In this study, we examined nutrient fluxes from intact cores of lake sediments incubated under different environmental conditions carefully chosen to mimic specific seasons. We then assessed the effects of adding nutrients harvested from incubated sediments to natural surface waters collected from Lake Kinneret and inoculated with an endemic, toxigenic strain of *Microcystis*.

Our first main finding was that *Microcystis* cultured in surface water from Lake Kinneret amended with sediment nutrients increased in biomass, whereas *Microcystis* cultured only in surface water experienced no growth. We observed that average chlorophyll *a* concentrations of *Microcystis* cultures increased nearly 2- and 10-fold when exposed to nutrients from littoral and profundal sediments, respectively. This result confirms that the pool of nutrients in natural surface waters is not sufficient to support algal growth even at low cell densities. In agreement with our study, the addition of hypolimnetic water to surface water (5-10%) in laboratory bioassays with a mixed phytoplankton sample from Lake Kinneret resulted in increased algal biomass and carbon fixation (Ostrovsky et al., 1996). Notably, the biomass of *Microcystis* cultures doubled upon exposure to nutrients released from littoral sediments incubated under summer conditions (warm/oxic), but increased by an order of magnitude upon exposure to nutrients released from profundal sediments under summer conditions (cold/anoxic). This finding is consistent with the study by Cymbola et al. (2008), in which growth of a mixed phytoplankton community in laboratory cultures was stimulated to a

greater extent by exposure to overlying water extracted from anaerobic than aerobic sediment cores.

Our observation that the growth of *Microcystis* from Lake Kinneret is enhanced upon exposure to sediment-derived nutrients adds to the existing body of literature that lake sediments provide bioavailable nutrients capable of supporting algal growth. Previous sediment-algal bioassays have demonstrated that a quantitatively small, but biologically important, fraction of nutrients in sediments is available for uptake by algae (Nalewajko et al., 1998; Dzialowski et al., 2008). These bioassays typically involve incubating an algal species in direct contact with sediment in a culture vessel (e.g., Nalewajko et al., 1998), or in indirect contact with sediment in a dual-chambered culture vessel separated by a membrane (e.g., Ekholm et al., 2003). In contrast, we selected the method of Cymbola et al. (2008), in which algae are cultured in lake water harvested from sediment cores, because it allows for incubation of intact sediments under environmentally relevant conditions.

In our laboratory study, *Microcystis* was exposed to nutrients released from littoral or profundal sediments from Lake Kinneret. In aquatic ecosystems, cyanobacteria are exposed to nutrients released from lake sediments through several possible routes. For example, during periods of mixing, water currents entrain bottom waters rich in nutrients released from profundal sediments up to the euphotic zone where they are accessible to cyanobacteria and other algae. The same process brings nutrients from shallow sediments in the epilimnion when lakes are stratified. During periods of stratification, algae are also exposed to

nutrients released from profundal sediments when wind-induced internal seiches cause mixing of epilimnetic and hypolimnetic waters at the lake's periphery – a mechanism that is pronounced in large lakes such as Lake Kinneret (Ostrovsky et al., 1996). Certain species of cyanobacteria can also vertically migrate in the water column, which allows these organisms to translocate sediment-derived nutrients in the hypolimnion up into the epilimnion (Head et al., 1999). Finally, colonies of cyanobacteria, such as *Microcystis*, that over-winter on the sediment surface (Preston et al., 1980) likely accumulate sediment nutrients before migrating up to surface waters (Verspagen et al., 2005).

A second main finding was that microcystin concentrations increased in surface water collected from Lake Kinneret and inoculated with *Microcystis* only when amended with nutrients from sediments. We observed that microcystin concentrations in *Microcystis* cultures exposed to nutrients released from littoral and profundal sediments increased to 17 and 45 μg L^{-1} respectively. These levels of microcystin are well above international drinking water guidelines (1.0 μg L^{-1}) and would be considered a risk for health effects in the case of human recreational exposure (WHO, 2006). In contrast, microcystin concentrations in control cultures incubated in 100% surface water did not change over the course of the experiment.

Microcystin concentrations may have been higher in surface water amended with sediment nutrients simply because *Microcystis* populations achieved greater biomass under these conditions. Theoretically, an increase in the cellular rate of toxin production could have also contributed to the observed change in

microcystin concentrations – because rates of toxin production and cell division are strongly positively correlated (Orr and Jones 1998). However, we observed that microcystin-to-chlorophyll ratios were lower in *Microcystis* cultures amended with sediment nutrients in comparison to those in surface water only. In agreement with our study, Oh et al. (2000) reported that the microcystin content of *Microcystis aeruginosa* was lower at higher growth rates when cultured in media with different amounts of phosphorus.

In aquatic ecosystems, microcystin concentrations are determined in part by competition between toxic and non-toxic strains of cyanobacteria (Davis et al. 2009). One limitation of our experimental design was that we isolated one toxigenic strain of *Microcystis* to examine its response to nutrients released from lake sediments. An interesting avenue of future research would be to compare the growth of toxic and nontoxic strains of *Microcystis* in response to sediment-derived nutrients, and to assess how competition for sediment nutrients among toxic and non-toxic strains affects microcystin concentrations in freshwaters.

In conclusion, our study provides experimental evidence of a linkage between nutrient release from sediments and concentrations of microcystins, known human hepatotoxins, in lake water. Because high rates of nutrient regeneration from sediments are common in eutrophic lakes (Welch and Cooke, 1995; Jepessen *et al*., 2005), our hypothesized pathway of effects connecting internal nutrient loading and microcystin concentrations could potentially be operating in a broad range of freshwater systems. Furthermore, our study serves as an example of how environmental factors can be responsible for indirectly

influencing microcystin concentrations in freshwater lakes by triggering the release of nutrients from sediments and stimulating the growth of *Microcystis*. We hope our study encourages future research on the impacts of anthropogenic activities on internal nutrient loading, and potentially microcystin concentrations, in freshwater lakes. Climate warming (Malmaeus et al., 2006) and sulfur pollution (Caraco, 1993) are suspected of enhancing internal nutrient loading in freshwaters. If sediment nutrient release and microcystin production are indeed intimately linked, such global environmental problems may be having the unforeseen effect of increasing levels of harmful toxins in eutrophic freshwaters.

Tables

Table 3-1. Phosphorus and nitrogen fluxes (mean ± SD, n = 3) from Lake Kinneret sediments

Code	Treatment	Flux (mg m^{-2} d^{-1})			
		SRP	TDP	DIN	TDN
LW	Littoral Winter (16^oC/oxic)	-0.1 ± 0.1	-0.2 ± 0.1	2.5 ± 3.3	2.1 ± 2.2
LS	Littoral Summer (25^oC/oxic)	1.0 ± 0.2*	1.5 ± 0.2*	44 ± 10.1*	44 ± 9.6*
PW	Profundal Winter (16^oC/oxic)	-0.7 ± 0.2*	-0.5 ± 0.1*	8.8 ± 1.8*	12.0 ± 11.5
PS	Profundal Summer (16^oC/anoxic)	4.4 ± 0.6*	5.1 ± 0.9*	17.6 ± 3.5*	24.3 ± 3.7*

SRP = soluble reactive phosphorus; TDP = total dissolved phosphorus; DIN = dissolved inorganic nitrogen; TDN = total dissolved nitrogen; * mean flux is significantly different from zero (t-test; $P < 0.05$).

Table 3-2. Initial composition and nutrient concentrations (mean ± SD, n = 4) of growth media for the *Microcystis* experiment

Code	SRP ($\mu g\ L^{-1}$)	NH_4^+ ($mg\ L^{-1}$)	NO_3^- ($mg\ L^{-1}$)	NO_2^- ($mg\ L^{-1}$)	DIN ($mg\ L^{-1}$)
SW	n.d.	n.d.	0.14 ± 0.01	0.02 ± 0.00	0.16 ± 0.01
LS	n.d.	0.42 ± 0.02	0.21 ± 0.01	0.12 ± 0.01	0.75 ± 0.03
PS	50.7 ± 2.6	0.61 ± 0.02	n.d.	0.01 ± 0.00	0.61 ± 0.02

SW = 100% surface water; LS = 80% littoral core water and 20% surface water; PS = 80% profundal core water and 20% surface water; SRP = soluble reactive phosphorus; DIN = dissolved inorganic nitrogen; n.d. = non-detectable.

Figures

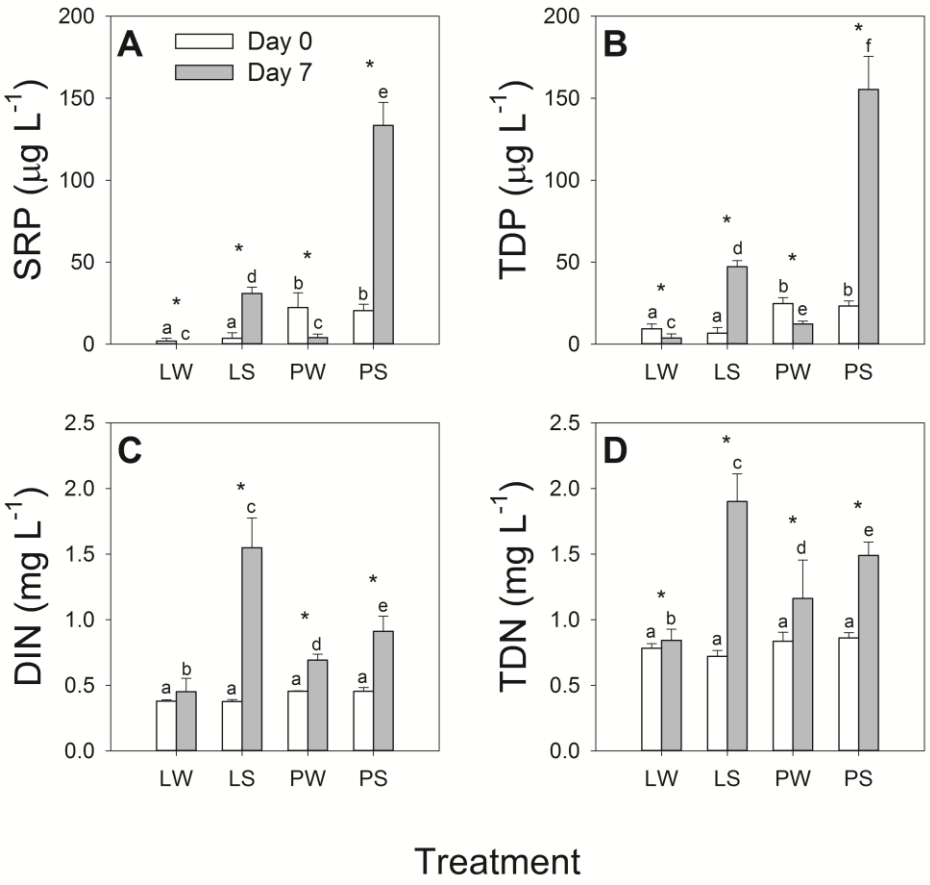

Figure 3-1. Concentrations of soluble reactive phosphorus (A), total dissolved phosphorus (B), dissolved inorganic nitrogen (C), and total dissolved nitrogen (D) in overlying water of sediment cores on days 0 and 7 of the incubation experiment. Average (± SD) calculated from 3 cores per treatment. Treatments are defined in Table 3-1. Lower case letters denote significantly different values among treatments, and astericks denote significantly different values between time periods ($p < 0.05$).

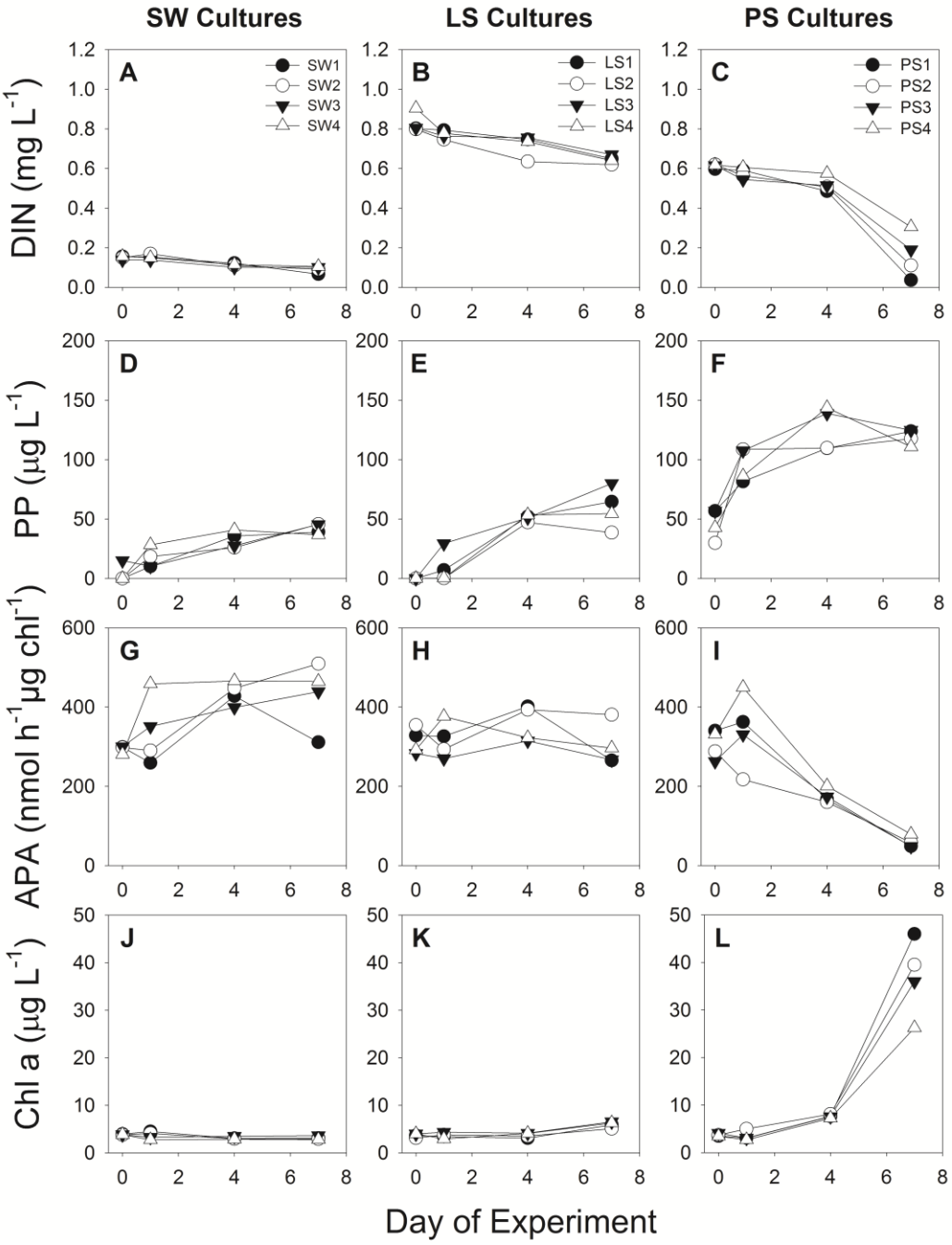

Figure 3-2. Temporal changes in concentrations of dissolved inorganic nitrogen (A-C), particulate phosphorus (D-F), alkaline phosphatase activity (G-I), and chlorophyll *a* (J-L) of *Microcystis* cultures over the 7-day experiment. Results for cultures in SW media are shown in panels A, D, G, J; for LS media in panels B, E, H, K; and for PS media in panels C, F, I, and L. The four cultures of each treatment are indicated by different symbols. Growth media are defined in Table 3-2.

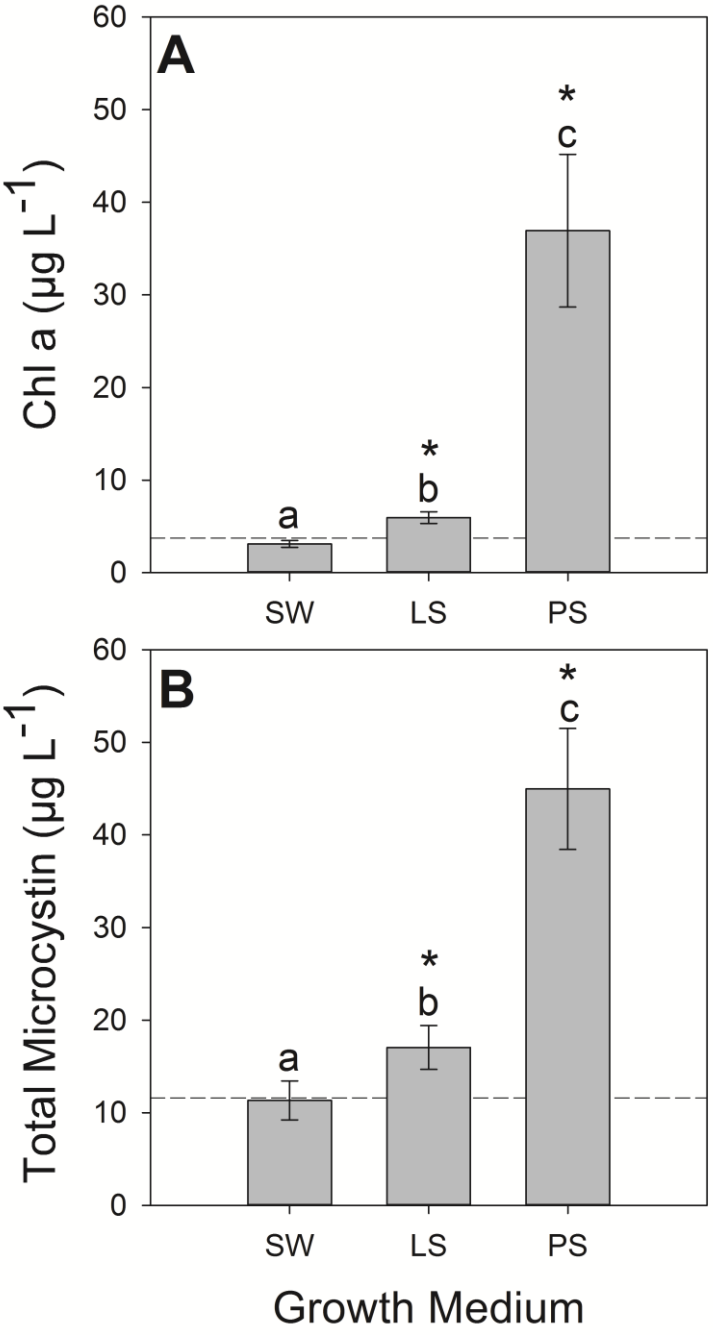

Figure 3-3. Average concentrations of chlorophyll *a* (A) and total microcystin (B) in *Microcystis* cultures at the end of the experiment (day 7). Nominal concentrations of chlorophyll *a* and total microcystins on day 0 are indicated by horizontal dashed lines. Average (± SD) calculated from 4 cultures per treatment. Growth media are defined in Table 3-2. Lower case letters denote significantly different values among treatments, and astericks denote significantly different values between time periods ($p < 0.05$).

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Chapter 4. Iron deficiency and discontinuous polymixis: a recipe for cyanobacterial blooms in shallow eutrophic lakes

Introduction

Blooms of cyanobacteria are a growing problem in freshwater lakes worldwide, including those on the Canadian Prairies. The problems associated with these blooms include poor water clarity, taste and odor problems, and production of cyanotoxins. One group of cyanotoxins, the microcystins, are potent mammalian liver toxins produced by species that commonly bloom in nutrient-rich ponds, lakes, and reservoirs. A correspondence between high concentrations of microcystins and low ratios of nitrogen-to-phosphorus (N:P) in eutrophic lakes occurs across Canada (Orihel et al. 2012) and in other regions of the world, such as the midwestern United States (Graham et al. 2004) and the Yangtze River region of China (Wu et al. 2006). Although a number of hypotheses exist for why microcystin concentrations are elevated only at low N:P ratios (see Orihel et al. 2012), there is no authoritative explanation for this phenomenon. Considering the health risks to humans, domestic animals, and wildlife posed by microcystins (van Apeldoorn et al. 2007, Funari and Testai 2008), it is prudent to understand the biogeochemical mechanisms responsible for the interrelated dynamics of N:P ratios and microcystin concentrations in freshwater lakes.

Lake sediments can harbour large stores of phosphorus (P) from many years of nutrient loadings from discharges of sewage effluent, fertilizer leached from

agricultural fields and livestock operations, and runoff from urban centers. In many lakes, P accumulated in sediments is efficiently recycled into surface waters (Cooke et al. 2005). Because P is an essential, and often limiting, nutrient for algal growth, releases of P from sediments can fuel algal blooms, particularly in shallow lakes (Søndergaard et al. 2003). Iron (Fe) has long been known to play a key role in P cycling across the sediment-water interface. According to the 'classic model', P cycling is controlled by the adsorption of P to solid Fe(III) oxyhydroxides in the presence of oxygen, and the reductive dissolution of Fe(III) and release of P in the absence of oxygen (Mortimer 1941). However, the paradigm that oxygen controls P release from sediments is misleading (Hupfer and Lewandowski 2008), as oxygenation of bottom waters is often ineffective at inhibiting P release (Gächter and Wehrli 1998), and anoxia does not necessarily induce P release (Schindler et al. 1987, Caraco et al. 1991).

An alternative model suggests the interplay of Fe and sulfur during diagenesis controls P release from sediments. The long-term sequestration of P in sediments is postulated to be controlled by the burial of Fe(III) oxyhydroxides, and the subsequent formation of Fe(II) phosphate minerals [namely, vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$)](Gächter and Müller 2003, Katsev et al. 2006). However, P sequestration in sediments is impeded by the presence of sulfide (H_2S) – a product of dissimilatory sulfate reduction by anaerobic bacteria. Sulfide directly mobilizes P from sediments by scavenging Fe from Fe(III)-phosphate complexes and Fe(II) phosphate minerals (Murphy et al. 2001). Further, H_2S depletes the pool of dissolved Fe in porewaters by precipitating insoluble iron sulfides (e.g., pyrite,

FeS_2) (Smolders and Roelofs 1993), and thereby, precluding the formation of Fe(II) phosphate minerals (Nriagu 1972).

A deficiency of available Fe in lake sediments may play a critical role in determining microcystin concentrations in freshwater lakes. Here, we put forward a new hypothesis to explain why shallow lakes tend to be hotspots for microcystin contamination, as well as why low N:P ratios seemingly increase the probability of encountering potentially harmful levels of microcystins. During the ice-free season, shallow lakes in the temperate zone commonly experience complete water column mixing punctuated by short, intermittent stratification events ("discontinuous cold polymictic", as defined by Lewis 1983). We propose that Fe deficiency in sediments, coupled to the discontinuous polymictic nature of many shallow lakes, are synergistically responsible for creating the ideal conditions for cyanobacterial blooms, which in turn, may result in outbreak of microcystin contamination (Figure 4-1). More specifically, Fe deficiency results in a disproportionately greater mobilization of P than N from sediments; therefore, discontinuous polymixis acts as a 'P pump', driving down N:P ratios in surface waters and delivering bioavailable P from sediments to algae in the euphotic zone. These P-rich conditions, coupled to low N:P ratios, stimulate blooms of cyanobacteria, and thus, enable the potential for microcystin production.

We assessed our model through a multifaceted investigation in a shallow, hypereutrophic lake on the Canadian Prairies (Figure 4-2, a). First, we conducted a detailed suite of chemical and mineralogical analyses to determine the availability of Fe in sediments and to probe for P-bearing minerals. Second, we

performed a series of sediment incubation experiments and algal bioassays to test whether Fe-deficient sediments: (i) exhibit high P flux which lowers the N:P ratio in water above the sediment interface, and (ii) release bioavailable nutrients that stimulate the growth of natural phytoplankton communities. Third, we monitored the thermal structure, water chemistry, phytoplankton community, and microcystin dynamics in our study lake over a one-year period to determine if discontinuous polymixis creates high P/low N:P conditions in the euphotic zone, and whether these conditions, in turn, stimulate blooms of microcystin-producing cyanobacteria. Fourth, we examined a multi-year data set of surface water quality to determine if seasonal minima in N:P ratios coincide with seasonal maxima in algal biomass and microcystin concentrations.

Methods

Study Site

Nakamun Lake (53°52'58.42"N, 114°12'14.16"W) is located 65 km northwest of Edmonton, Alberta, Canada (Figure 4-2, b-d). The characteristics of Nakamun Lake have previously been summarized (Mitchell and Prepas 1990). Briefly, the lake's drainage basin (48.6 km^2) is nearly 14 times larger than its surface area (3.54 km^2). Small streams flow into the lake from the east and south intermittently, but the outlet stream in the northwest corner of the lake flows only during wet periods, resulting in a long residence time (21 years). The maximum and mean depths are 8.0 m and 4.5 m, respectively. Much of the natural forest cover surrounding the lake has been converted to pasture for cattle operations and

to cropland for cereal production. Over 300 residences are situated in four subdivisions on the southern shoreline, and a large youth camp is established on the north shore. Large extents of shoreline have been cleared of natural vegetation, and in some cases, hardened with rock, concrete, or wood. Where natural riparian zones remain intact, the dominant vegetation includes awned sedges (*Carex atherodes*) and common cattails (*Typha latifolia*) near shore, and aspen (*Populus tremuloides*) and balsam poplar (*Populus balsamifera*) at the outer edges (Alberta Riparian Habitat Management Society 2009). Sampling stations (stn.) were positioned at the maximum (Stn. A) and mean (Stn. B) depth of the lake.

Sediment and Porewater Characterization

Samples to characterize the chemistry and mineralogy of sediments were collected in March 2010 from Stn. B. Intact cores (n = 3) were retrieved with a Glew gravity-driven sediment corer. Duplicate high-resolution profiles of dissolved oxygen, pH, and hydrogen sulfide gas (H_2S) across the surface-water interface were measured using microelectrodes with 50-µm tips (OX50, H2S50, and PH50; Unisense A/S, Denmark), calibrated following procedures outlined in their respective Unisense A/S manuals. To account for drift in the H2S50 microelectrode signal, H_2S values were re-zeroed for each profile using the average zero signal from the oxic part of the sediment profile. Values of H_2S were later calculated as ΣH_2S ($H_2S + HS^- + S^{2-}$) based on temperature and pH equilibria (as per the procedure outlined in the Unisense A/S manual). Sediment cores were then extruded at 2.5 cm intervals to a depth of 20 cm, after which samples were

centrifuged (at 4000 rpm) to separate pore waters from sediments. Pore waters were extracted using Luer-lock syringes and filtered through Target GL Microfiber syringe filters (0.7 μm). A subset of porewater samples were analyzed for a suite of elements by inductively coupled plasma mass spectrometry (ICP-MS) on a Perkin Elmer Elan6000 quadrupole ICP-MS (Perkin Elmer, USA) at the Radiogenic Isotope Facility at the University of Alberta. Other chemical parameters were analyzed as described below under 'Standard water analyses'. Samples for ICP-MS were acidified with analytical grade HNO_3, and those for N analyses were acidified with 1 M H_2SO_4.

Surface sediments were analyzed in duplicate for: loss-on-ignition at 550 and 950°C (Heiri et al. 2001), total N and total carbon (C) by the Dumas Combustion Method on a Costech 4010 Elemental Analyzer System (Costech Analytical Technologies Inc, USA), a suite of elements by ICP-MS (after HF-HNO3 digestion), and mineral content on a Rigaku Powder X-Ray Diffractometer (Rigaku, USA). Sediments were extracted sequentially to determine P speciation based on the method of Ruttenberg (1992), but modified to incorporate an extra alkaline step as proposed by Baldwin (1996). Extractions were done in duplicate at room temperature, and solutions were agitated continuously during extraction (200 rpm). Suspensions were centrifuged and supernatant was decanted using syringe needles. Orthophosphate was measured on a UV-VIS (Thermo Evolution 260) using the molybdate blue method (Murphy and Riley 1962). The reactive orthophosphate in the citrate-bicarbonate-dithionite extracts were analysed by the isobutanol method (Watanabe and Olsen 1961), after reacting the solution with

1% v/v $FeCl_3$ to minimize the interference of citrate with reduction of the molybdate complex (Ruttenberg 1992).

Microcosm Experiments

In Experiment (Exp.) 1, Fe was added to sediment cores to examine its effect on P concentrations and N:P ratios in water above the sediment-water interface (hereafter, 'overlying water') under simulated spring (cold and oxic) and summer (warm and anoxic) conditions. In August 2008, 12 sediment cores were retrieved from Stn. A. Cores were adjusted to ensure a similar volume of overlying water (i.e., 0.5 L). In a cold (4^oC) room, cores were fitted with aquarium bubblers to gently aerate the water column. Following a regression-based design, cores were randomly assigned to one of six treatments (2 replicates/treatment): 0, 0.5, 1.0, 1.5, 2.0, or 2.5 mg Fe per core. Doses of liquid Fe (standard grade ferric chloride, 13.8% Fe; Kemira Water Solutions, Inc., Canada) were added to overlying water. To simulate the "spring" phase, cores were incubated for 3 days under cold, oxic conditions in the laboratory. To subsequently simulate the "summer phase", cores were bubbled with N_2 gas to strip the water column of oxygen, sealed closed with rubber bungs, and incubated for 9 days at the bottom of Nakamun Lake (Stn. A) in a 'Sediment Core Lander'(Orihel and Rooney 2012). During the *in situ* phase, cores were exposed to water temperatures ranging from 14.4 to 16.3°C, with an average of 15.2°C. Overlying water from the mid-point of the core water column was sampled on days 0, 3, and 12 of the experiment.

In Exp. 2, we quantified the effect of Fe on the P fluxes from sediments under ambient summer conditions, and to improve our mechanistic understanding, we also examined sediment porewater chemistry. Cores (n = 12) were collected in July 2009 from Stn. B, and adjusted to achieve a similar volume of overlying water (0.5 L), and treated with Fe the same day in the field. Following an ANOVA-based design, cores were randomly assigned to one of four treatments (3 replicates/treatment): 0, 4.4, 23.6, or 82.8 mg Fe per core. To compare P release explicitly among treatments, overlying water was replaced with lake water of a known dissolved P concentration (0.02 mg L^{-1}) after the added Fe precipitated from the water column. Cores were incubated in a Sediment Core Lander at Stn. B to measure P flux from sediments. Overlying water and porewater (0-5 cm) of each core were sampled at the end of the 2-week incubation.

In Exp. 3 and 4, overlying water from incubated sediments (from Exp. 2) was used as a culture medium for growing natural, mixed phytoplankton communities. Exp 3. assessed algal growth in overlying water from untreated sediments in comparison to surface water from Nakamun Lake. Surface water was collected from Stn. A, and overlying water was harvested from control cores after a 2-week incubation. Surface water ('SW') and overlying water from control sediments ('OW-0') were passed through Whatman GF/C filters for the culture media. Aliquots (100 mL) of media was placed in autoclaved glass flasks (6 flasks/medium). For the phytoplankton stock, surface water from Stn. A was passed through a 150 µm mesh to remove large zooplankton. Cultures were inoculated with 50 mL of phytoplankton stock, and incubated in a growth

chamber for 20 days under constant light at 17°C. Exp. 4 followed a similar procedure as Exp. 3, but also included media from incubated Fe-treated sediments. Cultures for this bioassay included (3 flasks/medium): surface water (SW), overlying water from control cores (OW-0), and overlying water from cores treated with 2 g Fe m^{-2} (OW-2), 12 g Fe m^{-2} (OW-12), or 42 g Fe m^{-2} (OW-42). Each flask contained 180 mL of medium and 25 mL of phytoplankton stock. Inoculated cultures were incubated in a growth chamber for 30 days under constant light at 17°C (note: temperature was unexpectedly turned down to 10°C). Cultures were sampled at the end of each bioassay.

Samples from Exp. 1 and 2 were analyzed for P, N, and Fe, and from Exp. 3 and 4 for chlorophyll *a*, according to methods described under 'Standard water analyses'.

Lake Sampling

Our sampling program conducted in Nakamun Lake from June to October 2009 focused on lake stratification, water chemistry, particle sedimentation, phytoplankton biomass and species composition, and microcystin concentrations. Water chemistry sampling was also conducted under ice from January to March 2010.

Profiles of water temperature, dissolved oxygen, pH, conductivity, and oxidation-reduction potential were performed monthly using a multi-parameter water quality sonde (Hydrolab Series 5 Datasonde Multiprobe). Data loggers (Hobo Pendant UA-002-64) were installed every 0.5 m to measure hourly water

temperatures. Supporting data on air temperature, wind speed, and precipitation at Busby, Alberta (53°55'51" N, 113°55'18"W) were obtained from Environment Canada's National Climate Data and Information Archive.

Integrated samples for water chemistry, phytoplankton analyses, and microcystin determination were collected every 2 weeks by sampling the top 0-4 m of the water column using a polyvinyl chloride tube fitted with a foot valve. On alternate weeks, discrete samples (0, 4, and 7.5 m) were collected using a van Dorn water sampler. Integrated samples (for water chemistry only) were also collected monthly from January to March. Samples were kept cool and dark during transport back to the laboratory. Between sampling events, equipment was soaked overnight in 10% hydrochloric acid and rinsed thoroughly with Milli-Q water.

Between biweekly water sampling events, three sets of paired sedimentation traps were deployed at a depth of 3 m to measure sedimentation rates. Traps were constructed from acrylic tubes (7 cm diameter, 68 cm height) with 1-L bottles screwed into the bottom. Each set of traps had one bottle poisoned with 3% formalin, and one non-poisoned bottle. Collection bottles were changed every 2 weeks, and between deployments, traps were rigorously scrubbed and filled with lake water. Samples were homogenized in a blender, subsampled, and analyzed in duplicate for total suspended solids, dissolved P, and particulate P (as described below).

Standard Water Analyses

Chemical analyses were conducted in the Biogeochemical Analytical Service Laboratory at the University of Alberta. All porewater and overlying water samples, and any sample analyzed for soluble reactive P, dissolved inorganic N species, or dissolved inorganic carbon (DIC), were filtered by syringe (Target GL Microfiber, 0.7 μm). Other samples were filtered under vacuum (Whatman GF/F, 47 mm, 0.7 μm) in a filtration tower, and the filtrate and filter were collected for 'dissolved' and 'particulate' analyses. Soluble reactive P and chlorophyll *a* samples were frozen (-20^{o}C), dissolved inorganic N species and dissolved Fe samples were acidified with sulfuric and nitric acids, respectively, and all other samples were stored at 4^{o}C until analysis. Phosphorus was determined following method 4500-P F (American Water Works Association 2011) using a Lachat QuikChem 8500 FIA Automated Ion Analyzer (Lachat Instruments, USA). Samples for total P (unfiltered water), dissolved P, or particulate P were digested with potassium persulfate in an autoclave prior to analysis, whereas samples for soluble reactive P were not digested. Ammonia (NH_3) and nitrate + nitrite (NO_3+NO_2) were determined by methods 4500-NH3 F and 4500-NO3 I (American Water Works Association 2011) on the Lachat QuikChem 8500 FIA Automated Ion Analyzer. The sum of NH_4 and NO_3+NO_2 is referred to herein as 'dissolved inorganic N'. Samples for total N (unfiltered water) and dissolved N were analyzed using the same method as NO_3+NO_2 after digestion with potassium persulfate. Dissolved organic carbon (DOC) was determined following EPA methods 415.1 (EPA 1983) on a Shimadzu 5000A

TOC analyzer (Shimadzu, Japan); DIC was also analyzed on this instrument following protocols supplied by the manufacturer. Particulate carbon was measured by CE440 Elemental Analyzer (Exeter Analytical Inc, USA). Sodium, potassium, calcium, magnesium, and iron were measured by Inductively Coupled Plasma-Optical Emission Spectrometer ICAP6300 (Thermo Scientific, USA). Sulfate (SO_4) and chloride (Cl) were determined by EPA method 300.1 (EPA 1983) using Dionex DX600 Ion Chromatography (Dionex, USA). Other analyses included: total Alkalinity (Standard Method 2320 Alkalinity, American Water Works Association 2011), pH (Standard Method 4500-H^+ pH value, American Water Works Association 2011), Total Dissolved Solids (Standard Method 2540 B, American Water Works Association 2011), and Total Suspended Solids (Standard method 2540C, American Water Works Association 2011). Chlorophyll *a* was measured fluorometrically after extraction in ethanol on a Shimadzu RF-1501 Spectrofluorophotometer (Shimadzu, Japan).

Phytoplankton and Microcystin Analyses

Samples for pigment analyses were filtered under low light on Whatman GF/F filters and kept frozen (-80°C) until analysis. Algal pigments were estimated using standard reversed-phase high performance liquid chromatography (Vinebrooke et al. 2002). Pigments were extracted by soaking freeze-dried filters in a mixture of acetone:methanol (80:20, by volume) for 24 h in darkness at -80°C. Extracts were filtered through a 0.2 µm membrane filter (Millipore Millex-FG Hydrophobic PTFE), then dried under a stream of N_2 gas, shielded from direct light. Dried extracts were dissolved into a precise volume of injection solvent (70% acetone:

25% ion-pairing reagent: 5% methanol). Ion-pairing reagent consisted of 0.75 g tetra-butyl ammonium acetate and 7.7 g ammonium acetate in 100 mL water. Pigment concentrations were quantified on a Hewlett-Packard (Hewlett-Packard Canada Ltd., Mississauga, Ontario, Canada) model 1100 system equipped with a Varian Microsorb-100Å C-18 column (10-cm length, 5-µm particle size), and detected with an in-line Hewlett-Packard model 1046A fluorescence detector (435-nm excitation wavelength, 667-nm detection wavelength) and a model 1100 scanning photodiode array spectrophotometer (435-nm detection wavelength). Pigments isolated from samples were identified by comparision to those from unialgal cultures and authentic standards.

Samples for algal cell counts were fixed with Lugol's solution and formaldehyde-acetic acid alcohol. Samples collected on four dates (19 June, 30 July, 27 August, and 24 September) were enumerated. Aliquots of preserved samples were gravity settled for 24 h. Counts were performed on an inverted microscope at magnifications of 125X, 400X, and 1200X with phase contrast illumination. Cell counts were performed using the Ütermohl technique as modified by Nauwerck (1963) and Findlay and Kling (1998). Cell counts were converted to wet weight biomass by approximating cell volume. Estimates of cell volume for each species were obtained by measurements of up to 50 cells of an individual species and applying the geometric formula best fitted to the shape of the cell (Vollenweider 1968, Rott 1981). A specific gravity of 1 was assumed for cellular mass, and biomass estimates are expressed as mg m^{-3}.

Samples for microcystin determination were stored frozen (-20°C) and in darkness until analysis. Microcystin concentrations (expressed in microcystin–LR toxicity equivalents) were determined by protein phosphatase inhibition assays based on the work of An and Carmichael (1994) on a Bio-Tek ELx800TM Absorbance Microplate Reader (Fisher Scientific, Canada). In each sample analyzed for microcystins, cyanobacteria species known to produce microcystins were also enumerated using an inverted microscope. After aliquots of fixed samples were settled in an Ütermohl chamber, cyanobacteria were identified to species level and counted in 16 fields of view under 125X magnification. Counts were converted to biomass using average species-specific cell volumes as measured on a subset of samples.

Multi-Year Water Quality Data Set

Surface water quality in Nakamun Lake was monitored by a provincial government agency (now, Alberta Environment & Sustainable Resource Development) every other year between 2004 and 2012. Composite samples of the euphotic zone were collected every 3-4 weeks from May to October according to protocols in Alberta Environment (2006). Samples were analyzed for total P, total dissolved P, NH_4, NO_3+NO_2, chlorophyll *a*, and microcystin-LR using the same methods as described above. Total Kjeldahl N (TKN) was determined by standard method 4500-C.Total N was calculated as the sum of TKN and NO_3+NO_2. Soluble reactive P was not measured.

Statistical Analyses

All graphs and statistical tests were performed with SigmaPlot for Windows (Version 12.3). Normality and equal variance of data was assessed prior to performing parametric tests, and data were log-transformed as required. For data that did not meet the assumptions of parametric tests, the non-parametric alternative was carried out. Linear regression (Exp. 1) or one-way ANOVA (Exp. 2) was used to model relationship between Fe dose and P, N, N:P, and Fe concentrations. T-test (Exp. 3) or one-way ANOVA (Exp. 4) were used to test for differences in chlorophyll *a* among treatments. The Holm-Sidak method was employed to determine significant differences between controls and treatments following ANOVA (Exp. 2 and 4).

Results

Sediment Characterization

Sediments from Nakamun Lake consisted of a dark brown gyttja with no discernible annual laminations. Surface sediments were rich in organic matter (loss-on-ignition at 550°C = 45.9%), poor in carbonates (loss-on-ignition at 950°C = 3.1%), and contained pyrite, quartz, carbonates (calcite; dolomite), feldspars (albite; orthoclase), and clays (montmorillonite; kaolinite; illite). Element concentrations in surface sediments are provided in Table 4-1. Notably, concentrations of P and Fe were 0.2 and 1.7% (by weight), respectively, corresponding to a molar Fe:P ratio of 4.5. Sequential P extractions established

that the majority of P in sediments was organic or associated with humic substances, and furthermore, Fe-bound P was not detectable (Figure 4-3, a).

Microelectrode analysis of the sediment water-interface revealed that oxygen disappeared within 1-2 mm of the interface, pH decreased by more than one unit to 7.5, and ΣH_2S concentrations reached 2 mmol L^{-1} in the top 3 cm of the sediments (Figure 4-3, b-d). Chemical analyses of discrete porewater samples from a 20-cm core confirmed the rapid switch to anaerobic respiration and the accumulation of high concentrations of nutrients and major cations (Figure 4-3, f-q). Nitrate and SO_4 were quickly consumed near the interface, and peaks in Mn and Fe occurred within the top 5 cm. Dissolved P and NH_4^+ reached concentrations in pore water in excess of 0.5 and 5 mg L^{-1} respectively, which is an order of magnitude greater than concentrations in water above the sediments. Notably, Fe concentrations in pore water did not exceed 0.5 mg L^{-1}, and the molar Fe:P ratio was less than 1 in all samples.

Experiment 1

Low doses of Fe (from 0.25 to 1.25 g Fe m^{-2}) precipitated P from overlying water of sediment cores under cold, oxic conditions in the laboratory, and modestly slowed P release from sediments under warm, anoxic conditions in Nakamun Lake (Figure S4-1, a-b). At the end of the experiment, dissolved P in overlying water of sediment cores was negatively related to the amount of Fe applied (Figure 4-4, a; $R^2 = 0.57$, $F_{1,10} = 13.4$, $p = 0.004$). Similarly, soluble reactive P significantly decreased with increasing Fe dose (Figure S4-1, c; $R^2 = 0.49$, $F_{1,10} =$

9.5, $p = 0.012$). Dissolved inorganic N was mainly in the form of NH_4^+, and in contrast to P, concentrations were not significantly related to Fe dose (Figure S4-1, d; $R^2 = 0.14$, $F_{1,10} = 1.6$, $p = 0.24$). Consequently, molar ratios of dissolved N:P at the end of the experiment varied from 18 to 57, and were positively related to Fe dose (Figure 4-4, b; $R^2 = 0.84$, $F_{1,10} = 51.7$, $p < 0.001$). Fluxes of Fe from control sediments were very low under anoxic conditions, and little of the precipitated Fe in treated cores under oxic conditions was recycled back to overlying water under anoxic conditions (Figure S4-1,e-f). The Fe precipitate was initially reddish-brown, but turned black under anoxia, suggestive of pyrite formation.

Experiment 2

This experiment determined the effect of a broader range of Fe doses (0, 2, 12, or 42 g Fe m^{-2}) on the chemistry of overlying water and sediment porewater, as well as explicitly on P fluxs from sediments. In sediment cores with the same starting concentration of P in overlying water, dissolved P in overlying water of sediments treated with 0 and 2 g Fe m^{-2} increased 30-fold over the 2-week incubation to over 0.50 mg L^{-1}, whereas average concentrations in the 12 and 42 g Fe m^{-2} treatments only reached 0.12 and 0.03 mg L^{-1}, respectively (Figure 4-4, c; Figure S4-2, a). Soluble reactive P comprised most (> 80%) of the dissolved P in the 0 and 2 g Fe m^{-2} treatments, but was undetectable at higher Fe doses (Figure S4-2, a). Iron affected dissolved P in overlying water ($F_{3,8} = 128$, $p < 0.001$) and porewater ($F_{3,8} = 53$, $p < 0.001$), with the means of the 12 and 42 g Fe m^{-2} treatments significantly lower than of the control (Figure 4-4, c). Iron additions reduced fluxes of

dissolved P from 6.8 mg m^{-2} d^{-1} in control cores to 0.2 mg m^{-2} d^{-1} in cores treated with 42 g Fe m^{-2} (Table 4-2).

As observed in Exp. 1, Fe had no impact on dissolved inorganic N (Figure S4-2, b). Dissolved inorganic N (> 99% NH_4^+) ranged from 2 to 4 mg L^{-1} in overlying water and 9 to 16 mg L^{-1} in porewater, and was not significantly different among treatments in overlying water ($F_{3,8}$ = 1.3, p = 0.35) or porewater (H = 4.179, p = 0.24, df = 3). Because Fe did not affect N, but had a strong effect on P, this translated into substantial changes in N:P (e.g., molar ratios of N:P were below 15 in all control samples, but exceeded 137 in overlying water, and 660 in porewater, in cores treated with 42 mg Fe m^{-2}). The observed effect of Fe on N:P ratios was significant in overlying water ($F_{3,8}$ = 767, p < 0.001) and pore water ($F_{3,8}$ = 36, p < 0.001), with the means of 12 and 42 g Fe m^{-2} treatments significantly different from the control (Figure 4-4, d).

Dissolved Fe differed among treatments in overlying water ($F_{1,10}$ = 481, p < 0.001) and porewater ($F_{3,8}$ = 77, p < 0.001), with all Fe treatments significantly different from the control (Figure S4-2, c). Dissolved Fe in controls was consistently below 0.5 mg L^{-1}, whereas Fe in treated cores reached 4.5 and 21.6 mg L^{-1} in overlying water and pore water, respectively. In all treated sediments, the Fe precipitate turned from red to black during incubation. Notably, the addition of 2 g Fe m^{-2} resulted in a 5-fold increase in porewater Fe, but had a negligible effect on porewater P concentrations (Figure 4-4, c) or the flux of dissolved P (Table 4-2). Only when the molar ratio of Fe to P in porewater exceeded 1 did treatment affect P concentrations (Figure S4-2, d).

Experiments 3 and 4

Bioassays with natural, mixed phytoplankton communities from Nakamun Lake demonstrated the influence of sediment nutrients on algal growth. In Exp. 3, algae cultured in overlying water harvested from incubated control sediments had chlorophyll *a* concentrations approximately an order of magnitude greater than cultures grown in surface water (Figure 4-4, e; $t = -24$, $p < 0.001$, $df = 10$). In Exp. 4, chlorophyll *a* of algal cultures grown in surface water, overlying water from control sediments, or overlying water from sediments treated with 2, 12, or 42 g Fe m^{-2} were significantly different (Figure 4-4, f; $F_{4,10} = 22$, $p < 0.001$). Chlorophyll *a* concentrations of cultures grown in overlying water harvested from control sediments were significantly higher (7-fold) than those of cultures grown in surface water. Moreover, chlorophyll *a* of cultures grown in overlying water from sediments treated with 12 and 42 g Fe m^{-2} (but not 2 g Fe m^{-2}) were significantly lower than those grown in overlying water from control sediments (Figure 4-4, f).

Weather and Stratification

In June 2009, during a period of increasing air temperatures and low wind speeds (Figure S4-3), a thermal stratification event occurred in Nakamun Lake in which oxygen was depleted in the hypolimnion, but redox potential remained high (Figure 4-5, a-e; 19 June). A major storm took place in early July, but stratification set up again in late July and persisted for more than 2 weeks (Figure 4-5, f). By 6 August, oxygen was absent below 5 m, which coincided with a sharp drop in redox potential and an increase in conductivity (Figure 4-5, a-e). High

wind speeds in mid-August destratified the lake and replenished oxygen in bottom waters (Figure 4-5, b; 20 August). A few calm days at the end of August with air temperatures around 30°C resulted in another brief stratification event which led to bottom water anoxia but little change in redox potential or conductivity (Figure 4-5, a-e; 3 September). Soon after, temperatures cooled and the lake was well-mixed for the remainder of the fall.

Water Chemistry

Nakamun Lake is a moderately hardwater lake, with nutrient concentrations and turbidity values typical of a eutrophic ecosystem (Table 4-3). In 2009, concentration of total P increased 4-fold over the summer, reaching a maximum of 100 μg L^{-1} (Figure 4-5, g). This increase in P occurred in August, during a time of low water renewal, and corresponded to the two-week stratification event in which the hypolimnion was anoxic and the redox potential dropped (Figure 4-5, a-f). During this event, the total mass of P in the lake more than doubled to over 800 kg (Figure 4-5, g), and dissolved P in bottom waters increased by an order of magnitude to over 300 μg L^{-1} (Figure 4-5, h-i). After the lake mixed in mid-August, dissolved P in bottom water decreased, but then increased again during the next stratification event in early September (Figure 4-5, j-k). The majority of dissolved P in bottom waters was in the form of soluble reactive P (66-78%), in contrast to surface waters, where soluble reactive P accounted for less than 15% of dissolved P.

Whereas total P during the open water season ranged from 24 to 100 μg L^{-1}, of which less than half (18-54%) was usually in the dissolved phase (Figure 4-6, a), total N ranged from 1.5 to 2.5 mg L^{-1}, and was primarily (46-92%) in the dissolved phase (Figure 4-6, b). During this period, soluble reactive P remained below 5 μg L^{-1}, while dissolved inorganic N ranged from 21 to107 μg L^{-1} (66-100% as NH_4^+)(Figure 4-6, a-b). An imbalance in the increases in N and P over the summer caused the molar ratios of total N to total P to drop from 148 to 56, and of dissolved inorganic N to soluble reactive P to drop from 46 to 13 (Figure 4-6, c).

Under ice, total P increased modestly from 33 to 44 μg L^{-1}, as a result of an increase in dissolved P, while total N was fairly stable around 2 mg L^{-1} and was almost entirely (>96%) dissolved (Figure 4-6, a-b). In March, dissolved oxygen concentrations dropped to 1-2 mg L^{-1} in water above the sediments, and concentrations of soluble reactive P (17 μg L^{-1}) and dissolved inorganic N (454 μg L^{-1}) were at their maximum values, and in contrast to the summer, most (>94%) of the dissolved inorganic N was as NO_3+NO_2. Ratios of N:P were high under ice in comparison to mid-summer (Figure 4-6, c).

Suspended Particles and Sedimentation

Concentrations of suspended solids, particulate P, and particulate N in Nakamun Lake in 2009 peaked in August, and then again in late fall (Figure S4-4; Figure 4-6, d-e). Relative to the ideal 'Redfield ratio' (106 C:16 N:1 P;), the molar ratio of particulate C:N:P on 4 June (162:18:1) and 30 July (141:17:1) was C-rich, on

27 August (221:42:1) and 21 October (92:9:1) was P-poor and P-rich, respectively. Particulate N concentrations roughly tracked counts of cyanobacterial heterocysts (Figure 4-6, e). Fluxes in poisoned sedimentation traps ranged from 2.5 to 21 g m^{-2} d^{-1} for total particles, and 11 to 57 mg m^{-2} d^{-1} for particulate P, with the highest fluxes occurring in late fall (Figure S4-4; Figure 4-6, d). Sedimentation in poisoned traps were usually higher than in non-poisoned traps, particularly in summer.

Phytoplankton

Results from HPLC and microscopy showed a shift in the phytoplankton community of Nakamun Lake in 2009 from eukaryotic algae in the spring to cyanobacteria in late summer and fall (Figure 4-7). Chlorophyll *a*, an essential pigment produced by all photosynthetic algae, peaked in late summer, and again, to a lesser extent, in late fall (Figure 4-7, a). Note that chlorophyll *a* measured by fluorometry and HPLC were strongly correlated ($r = 0.95$, $p < 0.001$, $n = 10$), but fluorometry tended to overestimate concentrations (by 55%, on average). The pigments canthaxanthin, produced by filamentous cyanobacteria, and myxoxanthophyll, produced by colonial cyanobacteria, increased through the summer and were at maximum values in August (Figure 4-7, b-c). A second, smaller peak in myxoxanthophyll occurred in early October. In contrast, fucoxanthin and diadinoxanthin, pigments produced by chrysophytes, diatoms, and dinoflagellates, and alloxanthin, a pigment produced by cryptophytes, were highest in June and decreased through the summer, and were low or undetectable

in the fall (Figure 4-7, d-e). Chlorophyll *b*, produced by chlorophytes, peaked in late July/early August, and again in late September (Figure 4-7, f).

Shifts in the phytoplankton community deduced through changes in pigments were confirmed by biomass estimates of algal species (Figure 4-7; bars). On 19 June, when algal biomass was low, the community consisted predominately of the chrysophyte *Chrysochromulina parva*, the dinoflagellate *Ceratium hirundenella*, and the cryptophyte *Rhodomonas minuta*. Algal biomass increased 5-fold by Jul 30, largely as a result of the dinoflagellate *Ceratium hirundenella* and the cyanobacterium *Anabaena flos-aquae*. On 27 August, algal biomass was very high, and the community was almost exclusively dominated (98%, in biomass) by cyanobacteria. At this time, N-fixing cyanobacteria (namely, *A. flos-aquae, Aphanizomenon klebahnii,* and *Anabaena crassa*) were the most abundant algae (Figure 4-7, b), and heterocysts exceeded 10 million cells L^{-1} (Figure 4-6, e). Colonies of non-N-fixing cyanobacteria, mainly *Microcystis ichthyoblabe*, were also present in the August bloom. By 24 September, algal biomass had decreased, but the community remained dominated by cyanobacteria, largely by *A. klebahnii,* and to a lesser extent, by the colonial cyanobacterium *Woronichinia naegelianum*.

Microcystins

In 2009, concentrations of microcystins in Nakamun Lake were low (< 0.5 µg L^{-1}) in June and July, climbed to 2.25 µg L^{-1} by the end of August, then dropped and peaked a second time in September The first peak in microcystin corresponded to

the seasonal low in N:P ratio and the seasonal high in chlorophyll *a* concentration (Figure 4-6, c). The main species of cyanobacteria present that are known to produce microcystins were the filamentous, N-fixing species *A. crassa* and *A. flos-aquae,* and the colonial, non-N fixing species *M. ichthyoblabe* and *W. naegelianum*. Temporal changes in total biomass of known microcystin-producing species roughly corresponded to the increase in microcystin concentrations in late summer, but could not account for the second peak in late fall (Figure 4-6, f). During the first peak in microcystin in August, there were dense populations of *A. crassa* and *A. flos-aquae,* and some large colonies of *M. ichthyoblabe,* and during the second peak in the fall, *A. crassa* and *A. flos-aquae* were rare, but colonies of *M. ichthyoblabe* and *W. naegelianum* still persisted.

Multi-Year Water Quality Data Set

To examine whether the temporal trends in nutrients, chlorophyll *a*, and microcystin concentrations observed during our sampling program in 2009 are typical of Nakamun Lake, we examined surface water quality data collected by Alberta Environment (Figure 4-8). For the last three sampling years (2008, 2010, and 2012), total P concentrations in the euphotic zone of Nakamun Lake were below 50 µg L^{-1} in May and June, and then peaked in August to 134, 87, and 101 µg L^{-1} in 2008, 2010, and 2012, respectively. Total N concentrations also increased and peaked (> 2.3 mg L^{-1}) in August of these 3 years. Consistent with 2009, the increase in total P concentration to its maximum value in August of each year was disproportionately greater than the increase in total N concentration, resulting in a decline in the ratio of total N to total P from spring to

summer. In 2008, 2010, and 2012, large blooms of algae occurred each year during the summer low in N:P ratio, as was the case in 2009. Furthermore, in agreement with observations in 2009, peaks in microcystin concentrations in 2008, 2010, and 2012 consistently occurred during the seasonal low in the N:P ratio, and at the same time, or slightly after, the chlorophyll *a* maxima. In contrast to the other years, nutrient concentrations in 2004 and 2006 were already very high in the spring, and N:P ratio showed little seasonal variation.

Discussion

We propose that Fe deficiency and discontinuous polymixis in shallow lakes interact to create the ideal set of conditions required to stimulate toxic cyanobacterial blooms. Here, we discuss how the results of our multifaceted investigation in a hypereutrophic, polymictic lake on the Canadian Prairies, along with research from other lakes around the world, support the numbered arrows in our model outlined in Figure 4-1. While earlier works have focused on specific aspects contained within our working model, our study is unique in that we have taken an ecosystem perspective to the causes of cyanobacteria blooms and potential outbreaks of microcystin contamination, and have provided a theoretical framework to hopefully guide future research in this field.

Arrow (1)

The ratio of Fe:P in surface sediments or sediment porewaters is commonly used as an indicator for the P-binding capacity of sediments. Based on a large suite of Danish lakes, Jensen et al. (1992) concluded that Fe exerts control on P flux from

sediments only when the Fe:P ratio in surface sediments exceeds 8. Experimental and survey studies indicate that P mobilization from sediments is greatly enhanced when the Fe:P ratio in porewater is below about 1 (Smolders et al. 2001, Geurts et al. 2010). In our study, the ratio of Fe:P in Nakamun Lake was 4.5 in surface sediments (Table 4-1), and was consistently below 1 in sediment porewaters (Figure 4-3), and thus, our study lake can be considered 'Fe-deficient'. Furthermore, porewater Fe concentrations in Nakamun Lake (< 0.5 mg L^{-1}) are among the lowest measured in sediments of Canadian lakes (Table 4-4). The availability of Fe in Nakamun Lake is limited by high H_2S production in sediments (Figure 4-3) and subsequent formation of iron sulfides.

The effects of low Fe on the speciation of P in sediments were readily apparent from the results of sequential P extractions of Nakamun sediments, where Fe-bound P was not detectable, and most P was associated with humic and organic substances (Figure 4-3, a). Metal ions, such as Fe^{+3}, are well-known to bind to humic substances (Benedetti and Riemsdijk 1995), and phosphate can interact with humic substances to form stable complexes via metal bridges (Guardado et al. 2008). Organically bound P is typically a major component of the total P pool in eutrophic lake sediments (e.g., Andersen and Ring 1999, Kisand 2005). Although much of this organic P pool can be recalcitrant (Bai et al. 2009), it also contains labile forms that play an important role in P cycling (Ahlgren et al. 2011). For example, many microorganisms, including cyanobacteria, can store excess P in their cells in the form of polyphosphate, which can be released from decaying algae and bacteria by enzymatic hydrolysis (i.e., alkaline phosphatases)

(reviewed by Hupfer et al. 2007). Polyphosphate has been shown to be rapidly transformed during early sediment diagenesis (Hupfer et al. 2004). In other words, in Fe-deficient sediments, P exists in labile forms that are susceptible to recycling, rather than in insoluble Fe minerals that are a more stable sedimentary sink.

Fluxes of P from anoxic sediments in our study were high (6.8 ± 0.6 mg m^{-2} d^{-1}), but comparable to previous measurements in Nakamun Lake (Riley and Prepas 1984) and other eutrophic lakes and reservoirs (Andersen and Ring 1999, Penn et al. 2000, Carter and Dzialowski 2012). The addition of Fe to sediments greatly reduced P flux during warm, anoxic incubations (Table 4-2). This suggests that Fe sequesters P in Nakamun sediments by a mechanism other than the classical model described by Mortimer (1941) (i.e., adsorption to redox-sensitive Fe(III) oxyhydroxides). Although we did not examine the mineralogy of Fe-treated sediments, evidence for the formation of Fe(II) phosphate minerals can be found in other studies. For example, vivianite, the most stable Fe(II) phosphate mineral, has been detected in Narrow Lake, an unusual Fe-rich mesotrophic lake in Alberta fed by groundwater (Manning et al. 1991), as well as Lake Baikal, an oligotrophic lake in Russia with high porewater P and Fe (Fagel et al. 2005).

Arrow (2)

Shallow lakes often respond differently to nutrient pollution than their deeper counterparts because of the predominance of internal P loading in the former (Cooke et al. 1993, Søndergaard et al. 2005, Taranu et al. 2010). Several features unique to discontinuous polymictic lakes facilitate high rates of P release from

sediments. First, warm temperatures at the bottom of shallow lakes in summer stimulate the microbial processes in sediments that drive internal P loading, in contrast to dimictic lakes where the hypolimnion in summer remains near spring temperatures. Second, stratification periods in discontinuous polymictic lakes lower the redox potential at the sediment-water interface enabling redox-sensitive processes that mobilize P at the sediment surface, in contrast to continuous polymictic lakes where bottom waters are constantly reoxygenated by mixing. Third, destratification in discontinuous polymictic lakes allows for P accumulated in bottom waters during stratification to be efficiently transported to the euphotic zone in summer when warm temperatures promote cyanobacterial blooms. This is in contrast to dimictic lakes, where P is largely retained beneath the thermocline in summer, and thus, largely inaccessible to phytoplankton. In Nakamun Lake, we observed that discontinuous polymixis acted like a 'P pump', translocating bioavailable P from sediments to phytoplankton in the euphotic zone: a large accumulation of dissolved P in bottom waters during a prolonged stratification event led to a doubling of whole lake P mass, and upon destratification, a doubling of total P concentrations in surface waters (Figure 4-5). The transient build-up of P in the hypolimnion and its subsequent transport to the euphotic zone has been reported in other polymictic lakes, such as Lake Müggelsee, Germany (Wilhelm and Adrian 2007) and Severson Lake, Minnesota (Schindler and Comita 1972).

Arrow (3)

The importance of internal P loading in lakes became widely recognized in the 1970s and 1980s after reductions in external P loading, through sewage treatment or diversion, resulted in unexpectedly little change in P concentrations in some lakes (reviewed by Marsden 1989). Based on long-term case histories of a large suite of European and North American lakes, internal P loading has been estimated to delay the response of shallow lakes to nutrient loading reductions by 10-15 years (Jeppesen et al. 2005, 2007). In agreement, P release from sediments is expected to take 19-26 years to reach a new steady state in Onondaga Lake, USA, after nutrient loading reductions based on a dynamic mechanistic model (Lewis et al. 2007). Simple mass balance P budgets have long indicated that internal loading from sediments can exceed external P loading on an annual basis in numerous lakes around the world (Cooke et al. 1977, Garber and Hartman 1981, Kozerski et al. 1999) – a conclusion which has been confirmed through more sophisticated process-based dynamic models (Schauser et al. 2004, Burger et al. 2008). Notably, a well-constrained mass balance (Schindler et al. 1987) and a radioisotope P tracking study (Levine et al. 1986) indicated internal P loading is negligible in Lake 227, Experimental Lakes Area, an experimentally fertilized lake with some of the highest porewater Fe concentrations recorded in Canada (Table 4-4). Nonetheless, authors of a comprehensive book on lake management and restoration have concluded that lakes which experience significant internal loading of P are "the rule rather than the exception" (Cooke et al. 2005).

Although internal P loading is relatively under-studied in the Canadian Prairies, the available evidence from pore water analyses (Shaw and Prepas 1990, Ballard 2011), incubation experiments (Riley and Prepas 1984, Burley et al. 2001, Loh et al. 2013), and lake nutrient budgets (Riley and Prepas 1984, Mitchell 1984) indicates bottom sediments are an extremely important source of P to eutrophic prairie lakes. This conclusion is strengthened by our study of Nakamun Lake, as sediments exhibited high P release under warm, anoxic conditions (Table 4-2), the total mass of P in the lake doubled in the summer of our study year (Figure 4-5, g), and episodes of internal P loading were commonly observed in the multi-year water quality data set (Figure 4-8).

Arrow (4)

Ratios of N:P in surface waters are controlled by the balance of inputs and losses of N and P to lakes, as well as the internal processes that consume or regenerate these elements. In general, N:P is inversely related to lake trophic status, which has been attributed to the different sources of nutrients to oligotrophic lakes and eutrophic lakes (e.g., precipitation and runoff from forested catchments is N-rich, whereas sewage discharges and feedlot runoff are P-rich) (Downing and McCauley 1992). While external inputs from watersheds and airsheds may set the total pool of nutrients in lakes, *in situ* processes – such as uptake and excretion by organisms, particle sedimentation, recycling from sediments, and nitrogen fixation by cyanobacteria – are responsible for short-term dynamics in nutrient concentrations.

Low N:P can be caused by internal P loading from sediments in Fe-poor shallow lakes, as demonstrated by our investigation in Nakamun Lake. First, porewater and overlying water from sediments had dissolved N:P of 8 and 14, respectively (Figure 4-4, d). In contrast, N:P in porewater and overlying water of Fe-treated sediments were much higher (up to 457 and 141, respectively). Second, a decrease in N:P from spring to summer was observed in our sampling campaign (Figure 4-6), and in the last 3 years of the 5-year data set (Figure 4-8), in tandem with an increase in P concentration. Similar seasonal dynamics in N:P have been observed, for example, in Lake Limmaren, Sweden (Vrede et al. 2009), Ford Lake, USA (Lehman 2010), and shallow Danish lakes (Søndergaard et al. 2005). Although we focused our study on the release of dissolved P from sediments, recruitment of resting stages of cyanobacteria from sediments to the water column also contributes to internal P loading (Barbiero and Welch 1992, Hellweger et al. 2008) and may cause sudden decreases in N:P in surface waters of eutrophic lakes (Xie et al. 2003).

Some cyanobacteria fix atmospheric N for their physiological needs in specialized cells (i.e., heterocysts) when N is in short supply in the environment. Evidence for the importance of this process in lakes comes from models based on N fixation measurements (Hendzel et al. 1994, Mugidde et al. 2003), isotopic N signatures of particulate organic matter (Patoine et al. 2006, Jankowski et al. 2012), and whole-lake budgets (Schindler et al. 2008). Nitrogen fixation has been shown to modify N:P in experimental lakes and mesocosms with different nutrient supply ratios (Levine and Schindler 1992, Vrede et al. 2009). In Nakamun Lake,

particulate N (and total N) increased during a bloom dominated by N-fixing cyanobacteria (Figure 4-6, b; Figure 4-7, b). Although we did not directly quantify N fixation, heterocysts counts are a reliable indicators of N fixation rates (Findlay et al. 1994, de Tezanos Pinto and Litchman 2010). The lack of a corresponding decrease in dissolved N (Figure 4-6, b) and the extremely high number of heterocysts (Figure 4-6, e) suggests N fixation likely contributed to the increase in N, and subsequent increase in N:P (Figure 4-6, c).

Arrow (5)

Phosphorus is considered the ultimate limiting nutrient (sensu Vitousek et al. 2010) of algal productivity in freshwater lakes, based on findings from whole-ecosystem manipulation experiments and case histories of lake recovery following reductions in nutrient loading (reviewed by Schindler 2012). There is broad agreement that cyanobacteria tend to dominate the phytoplankton community of nutrient-rich lakes, and furthermore, that nutrient pollution increases the frequency and intensity of cyanobacterial blooms (Smith and Schindler 2009). For example, in a study of nearly 100 north temperate zone lakes, cyanobacterial biomass accounted for an increasing fraction of total algal biomass as a function of total P concentration (up to ca. 100 µg L^{-1}) (Downing et al. 2001). Because P is released from sediments in a form highly bioavailable for uptake by algae, internal P loading can stimulate algal blooms during times of the year when algae are nutrient-starved. The stimulation of algal growth by sediment-derived nutrients was demonstrated by our algal assays performed with overlying water

from incubated Nakamun Lake sediments (Figure 4-4, e-f). Similar findings were reported earlier by Cymbola et al. (2008) and Orihel et al. (2013).

Arrow (6)

The theory that low N:P favors the dominance of cyanobacteria was advanced by Smith (1983) based on the premise that habitats deficient in inorganic N confer a competitive advantage to N-fixing cyanobacteria. Although not supported by some studies (e.g., Dolman et al. 2012), this theory has been corroborated by experiments manipulating N:P loading to mesocosms and whole lakes (Schindler et al. 2008, Vrede et al. 2009), and monitoring of eutrophic lakes after reductions in N loading, such as Lakes Peipsi and Võrtsjärv, Estonia (Noges et al. 2008) and Lake Kinneret, Israel (Zohary 2004). Nonetheless, cyanobacteria may not always dominate the phytoplankton community at low N:P because this relationship is modulated by other environmental factors, such as temperature and light (Levine and Schindler 1999, de Tezanos Pinto and Litchman 2010). Low N:P may also promote cyanobacterial blooms by other mechanisms, including inducing recruitment of resting stages of cyanobacteria (Stahl-Delbanco 2003), providing a competitive advantage to migrating cyanobacteria that can monopolize benthic N sources (Ferber et al. 2004), or triggering pulses of "new" N (via N fixation) that support the growth of nonheterocystous cyanobacteria (Beversdorf et al. 2013).

In Nakamun Lake, we observed a substantial decrease in both dissolved inorganic N:P and total N:P from spring to summer (Figure 4-6, c). Consistent with our expectations, a corresponding shift in the phytoplankton community

from a mixed assemblage composed of chrysophytes, dinoflagellates, and cryptophytes to one dominated by cyanobacteria (Figure 4-7). This cyanobacterial bloom was mainly comprised of *Anabaena* and *Aphanizomenon* spp. with high numbers of heterocysts, but also included the nonheterocystous species *Microcystis icthyoblabe* and *Woronochinia naegelianum*. This result is consistent with the hypothesis that low N:P stimulates N fixation by heterocystous cyanobacteria, which in turn, sustains populations of nonheterocystous cyanobacteria.

Arrow (7)

Cyanobacterial blooms are a prerequisite for microcystin outbreaks, yet not all cyanobacterial blooms lead to microcystin outbreaks. Genes for microcystin synthesis evolved in an ancient ancestor of modern cyanobacteria (Rantala et al. 2004), and many extant species of cyanobacteria can synthesize microcystins (Sivonen and Jones 1999, Codd et al. 2005). Predicting microcystin dynamics in natural ecosystems is complicated by the fact that non-toxic and toxic stains of species known to produce microcystins often co-exist (Welker et al. 2003, Davis et al. 2009, Rinta-Kanto et al. 2009), and that microcystin production of toxic cells can vary under different environmental conditions (reviewed by Neilan et al. 2013). Concentrations of microcystins in freshwater lakes, therefore, are not only controlled by the biomass of microcystin-producing species, but also by the relative proportion of toxic strains in populations of known toxin producers, and by their respective toxin production rates (Kotak and Zurawell 2007). This is consistent with the findings in Nakamun Lake, where seasonal changes in

microcystin concentrations corresponded poorly to total algal biomass (Figure 4-6, c, Figure 4-8) and the total biomass of known toxin producers (Figure 4-6, f).

Arrow (8)

Iron is an essential micronutrient for all algae, but cyanobacteria have a particularly high requirement for this element as a result of their evolutionary history (reviewed by Kranzler et al. 2013). In addition to the numerous well-documented physiological responses of Fe starvation in algal cells, Fe deficiency may also stimulate microcystin synthesis by cyanobacteria. Early studies generated conflicting results as to the effect of Fe on the cellular yield of microcystin in laboratory cultures of *Microcystis aeruginosa* (Lukac and Aegerter 1993, Utkilen and Gjolme 1995, Yan et al. 2004), but recent studies have demonstrated that Fe starvation triggers the transcription of microcystin synthetase genes (Sevilla et al. 2008) and microcystin production (Alexova et al. 2011). The stimulation of microcystin synthesis by Fe starvation may be linked to the physiological function of microcystins. Utkilen and Gjolme (1995) proposed that microcystins function as intracellular Fe chelators to increase the efficiency of Fe uptake into the cell. Alternatively, microcystins may function as radical scavengers to protect cells from oxidative stress (Dziallas and Grossart 2011) – a role which would be required under Fe-deficient conditions because Fe starvation causes the accumulation of reactive oxygen species in cells (Latifi et al. 2005). In addition to stimulating microcystin synthesis, Fe deficiency may influence the partitioning of this toxin in freshwater lakes. Half-lives for microcystins in lake water are on the order of days, but are substantially shorter in sediments (Chen et

al. 2008). Microcystins form moderately stable complexes with Fe(III) (Klein et al. 2013), thus it may be that dissolved microcystins persist longer in the water column of Fe-poor than Fe-rich lakes because microcystins would be precipitated from the water column in the latter. We did not investigate the direct effects of low Fe on the production or complexation of microcystin in our study. Given the paucity of studies that have explicitly examined this question, particularly in the field, this topic would be an exciting avenue of future research.

Arrow (9)

Although several field studies have documented an inverse relationship between N:P ratios and microcystin concentrations, there is scant evidence to support the hypothesis that N:P ratios directly influence microcystin synthesis by cyanobacteria. In the study of Lee et al. (2000), microcystin concentrations generated by *Microcystis* grown in media with different N:P ratios (1-100) exhibited little variation among N:P in treatments with a fixed N concentration, and was somewhat lower at N:P ratios of 1 and 5 in treatments with a fixed P concentration. Vézie et al. (2002) concluded that the intracellular microcystin content of two *Microcystis* strains was related to N:P ratios of growth media, but the overall variation in toxin content was small and the range in N:P ratios examined was not environmentally relevant (3 to 845). Downing et al. (2005) observed the highest microcystin concentrations in cells grown in media with N:P ratios between 18 and 51, but found no significant correlation between N:P ratio and cellular microcystin content in either of two strains of *M. aeruginosa*. Although N:P seems to have little direct effect on microcystin production, the

authors of a recent study suggested low N:P may select for toxin-producing strains, based on the observation that toxic strains of *Microcystis* spp. in Lake Taihu, China were more common than non-toxin producing strains in regions of the lake with low N:P (17-23) than in regions with high N:P (50-58) (Otten et al. 2012).

In summary, field studies from lakes around the world have previously reported that outbreaks of microcystin contamination in eutrophic lakes only occur below a certain N:P threshold value (Graham et al. 2004, Wu et al. 2006, Orihel et al. 2012). These natural surveys documented an intriguing relationship that warranted further investigation, considering its potential implications on nutrient reduction strategies. This study proposed and tested a model to explain the apparent relationship between low N:P and microcystin concentrations. We conclude this relationship may not necessarily be causal, but rather, that low N:P may simply be a symptom of high rates of internal P loading in shallow lakes. Further, this study advanced the hypothesis that a synergy between Fe deficiency in sediments and discontinuous polymixis in shallow lakes is responsible for the occurrence of cyanobacterial blooms, which in turn, may lead to elevated levels of microcystins.

Tables

Table 4-1. Element concentrations of surface sediments in Nakamun Lake.

Parameter	Concentration[a] (mg g^{-1} dw)
C	242.0
Al	37.5
N	30.5
Ca	25.4
Fe	17.1
K	9.26
Mg	6.94
P	2.12
Ti	1.78
Mn	0.81
Sr	0.16
Zn	0.073
V	0.055
Cu	0.037
Cr	0.032
Pb	0.021
Ni	0.019
Y	0.011
Co	0.0075
As	0.0046
U	0.0018
Mo	0.0013
Ag	0.0002

[a] average of duplicate surface samples (0-2.5 cm) collected at Stn. B in March 2010.

Table 4-2. Phosphorus flux from Nakamun Lake sediments treated with different amounts of iron.

Fe Dose g m^{-2}	Dissolved P Flux[a] mg m^{-2} d^{-1}
0	6.8 ± 0.6
2	6.7 ± 0.8
12	1.2 ± 0.3
42	0.2 ± 0.2

[a] Mean ± SD (n = 3) from sediment cores in Experiment 2.

Table 4-3. Water chemistry of Nakamun Lake in 2009-2010.

Date	Units	Open Water[a]		Under Ice[b]	
		Average	n	Average	n
Total Phosphorus	µg L^{-1}	48	11	35	4
Particulate Phosphorus	µg L^{-1}	29	11	14	1
Dissolved Phosphorus	µg L^{-1}	17	11	25	4
Soluble Reactive Phosphorus	µg L^{-1}	2	11	17	1
Total Nitrogen	mg L^{-1}	1.8	11	2.1	4
Particulate Nitrogen	mg L^{-1}	0.33	4	0.12	1
Dissolved Nitrogen	mg L^{-1}	1.4	11	2.1	4
Ammonium	µg L^{-1}	33	4	27	1
Nitrate+Nitrite	µg L^{-1}	10	4	427	1
Particulate Carbon	mg L^{-1}	2.0	4	0.9	1
Dissolved Organic Carbon	mg L^{-1}	21	4	22	1
Dissolved Inorganic Carbon	mg L^{-1}	39	4	45	1
Sodium	mg L^{-1}	37	4	38	1
Potassium	mg L^{-1}	15	4	17	1
Calcium	mg L^{-1}	23	4	25	1
Magnesium	mg L^{-1}	12	4	13	1
Chloride	mg L^{-1}	6	4	7	4
Sulfate	mg L^{-1}	13	4	13	4
Total Alkalinity	mg L^{-1} $CaCO_3$	177	4	196	1
Bicarbonate	mg L^{-1}	204	4	239	1
Carbonate	mg L^{-1}	12	4	0	1
pH	-	8.6	4	7.8	4
Total Dissolved Solids	mg L^{-1}	239	4	312	1
Total Suspended Solids	mg L^{-1}	4	11	2	4
Chlorophyll *a*	µg L^{-1}	18	11	2	1
Secchi Depth	m	1.75	13	-	-

[a] Integrated samples (0-4 m) collected between June and October 2009.
[b] Integrated samples (0-4 m) collected between January and March 2010.

Table 4-4. Range in porewater iron concentrations in Nakamun Lake in relation to other Canadian lakes.

Lake	Province	Fe (mg L^{-1})	Reference
Nakamun Lake	Alberta	0 - 0.5	This study
		0.03 - 0.1	Ballard (2011)
Tucker Lake	Alberta	0.2[a]	Shaw and Prepas (1990)
Island Lake	Alberta	0.2[a]	Shaw and Prepas (1990)
Narrow Lake	Alberta	0.2[a]	Shaw and Prepas (1990)
Baptiste Lake	Alberta	0.3[a]	Shaw and Prepas (1990)
Jenkins Lake	Alberta	0.4[a]	Shaw and Prepas (1990)
Lake Holland	Quebec	0 - 0.4	Chappaz et al. (2008), Couture et al. (2010)
S-9 Lake	Alberta	0.7[a]	Shaw and Prepas (1990)
Long Lake	Alberta	0.9[a]	Shaw and Prepas (1990)
Minnie Lake	Alberta	1[a]	Shaw and Prepas (1990)
Figure Eight Lake	Alberta	1[a]	Shaw and Prepas (1990)
McFarlane Lake	Ontario	1 - 3	Chen et al. (2003)
Moira Lake	Ontario	0.02 - 4	Azcue et al. (1994)
Lake Winnipeg	Manitoba	0 - 5	Brunskill and Graham (1979)
Lake Du Syndicat	Quebec	0.5 - 5	Couture et al. (2010)
Lake Tantare	Quebec	0.08 - 7	Alfaro-De la Torre and Tessier (2002), Laforte et al. (2005), Chappaz et al. (2008)
Balmer Lake[b]	Ontario	0.3 – 7	Martin and Pedersen (2002)
Chevreuil Lake	Quebec	0.08 - 9	Huerta-Diaz et al. (1998)
Anderson Lake[b]	Manitoba	0.1 - 9	Pedersen et al. (1993)
Sanctuary Pond	Ontario	2 - 10	Mayer et al. (1999)
Lake Desperiers	Quebec	6 - 11	Chappaz et al. (2008)
Lake Vose	Quebec	4 - 12	Laforte et al 2005, Chappaz et al. (2008)
Lake Erie	Ontario	3 - 13	Azcue et al. (1996)
Great Slave Lake[b]	Northwest Territories	1 – 17	Andrade et al. (2010)
Clearwater Lake	Ontario	0 - 24	Huerta-Diaz et al. (1998), Chen et al. (2003), Belzile et al. (2000)
Jacks Lake	Ontario	1 - 28	Carignan and Lean (1991)
Lakes (n=7)	Ontario	0.6 – 40[c]	Bendell-Young et al. (2002)
Lake Bedard	Quebec	28 - 55	Couture et al. (2010)
ELA Lake 227	Ontario	13 - 58	Cook (1984)
ELA Lakes (n=16)	Ontario	0.06 – 111	Brunskill et al. (1971)

[a] reported as a mean for each lake; [b] lake impacted by mining; [c] reported as a maximum for each lake.

Figures

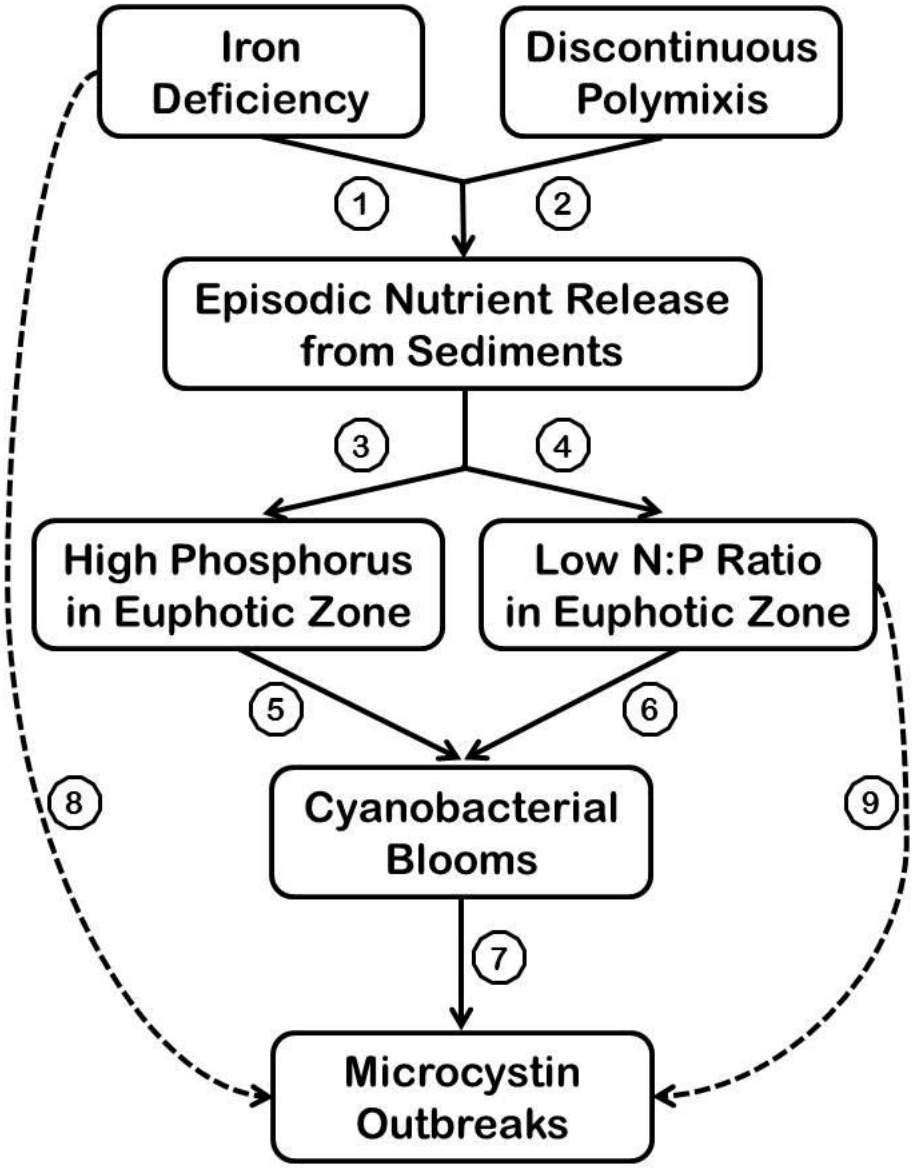

Figure 4-1. Conceptual model for how iron deficiency and discontinuous polymixis synergistically create the ideal conditions for toxic algal blooms in shallow lakes. Numbered arrows are discussed in the paper.

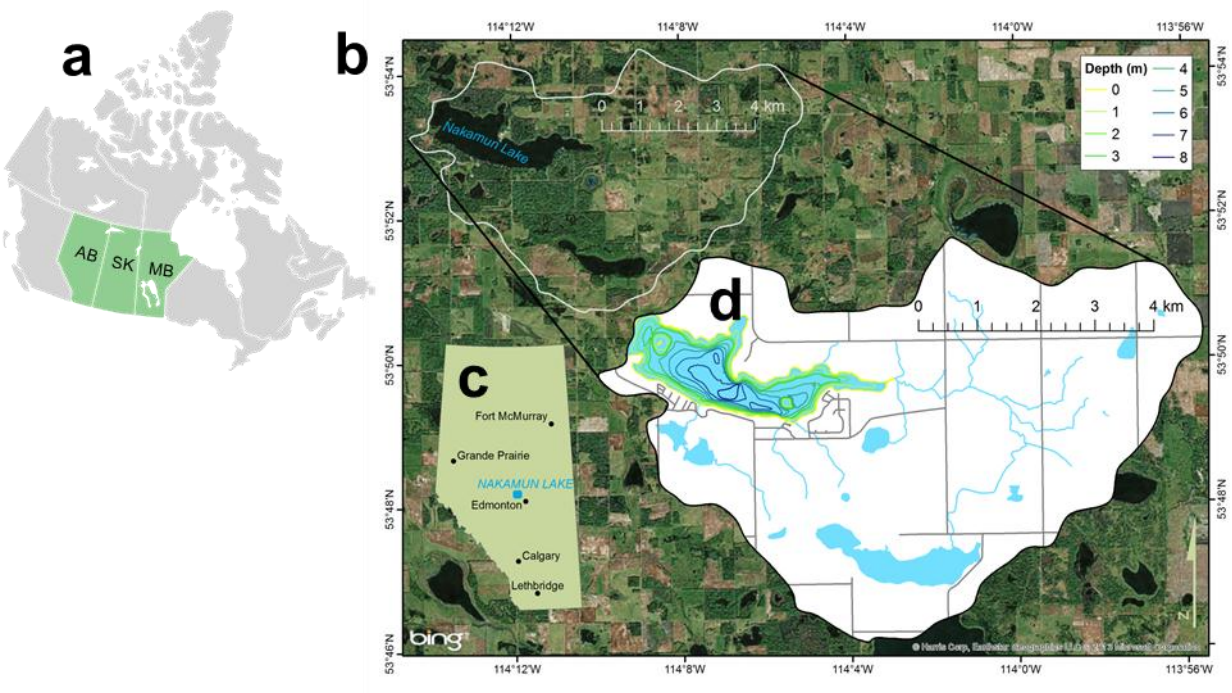

Figure 4-2. Map of Canada, with the Prairie Provinces of Alberta (AB), Saskatchewan (SK), and Manitoba (MB) shown in green (a). Map of Nakamun Lake and its surrounding landscape, with the watershed border outlined in white (b). Inset (c) shows the location of Nakamun Lake in Alberta. Inset (d) depicts the watershed hydrology and lake bathymetry.

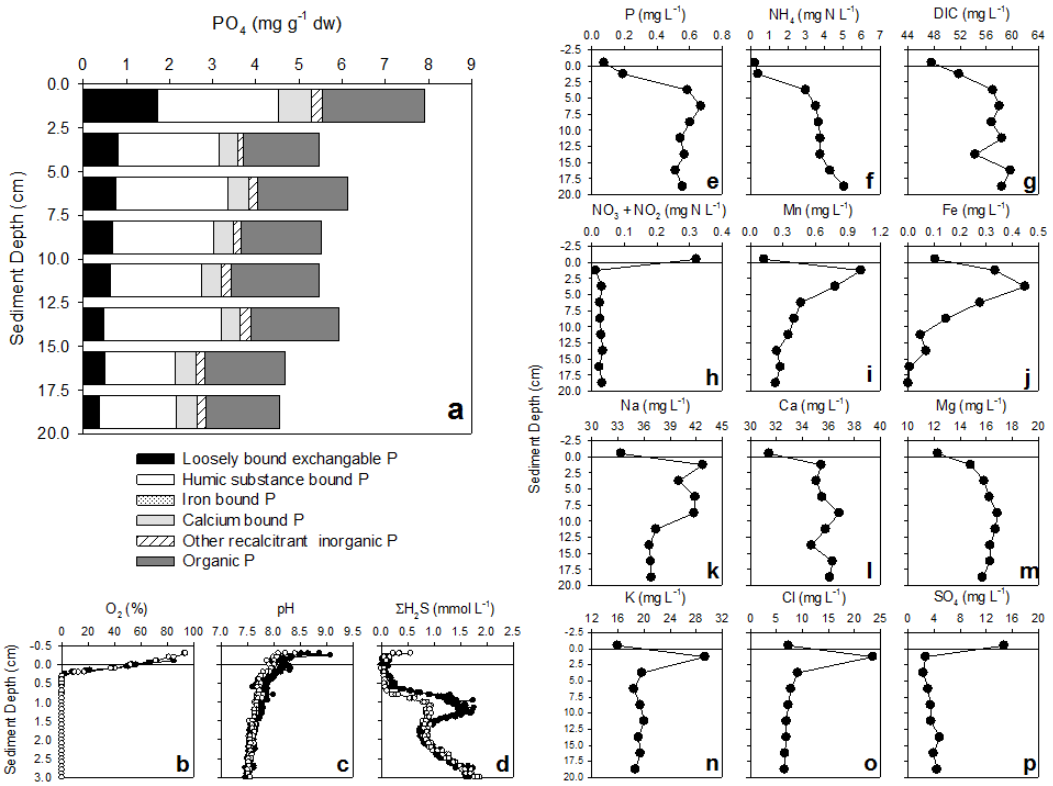

Figure 4-3. Characteristics of Nakamun Lake sediments. Phosphate speciation at various sediment depths (a), profiles of oxygen, pH, and total sulfide at the sediment-water interface (b-d), and porewater profiles of phosphorus, nitrogen, metals, and major ions (e-o).

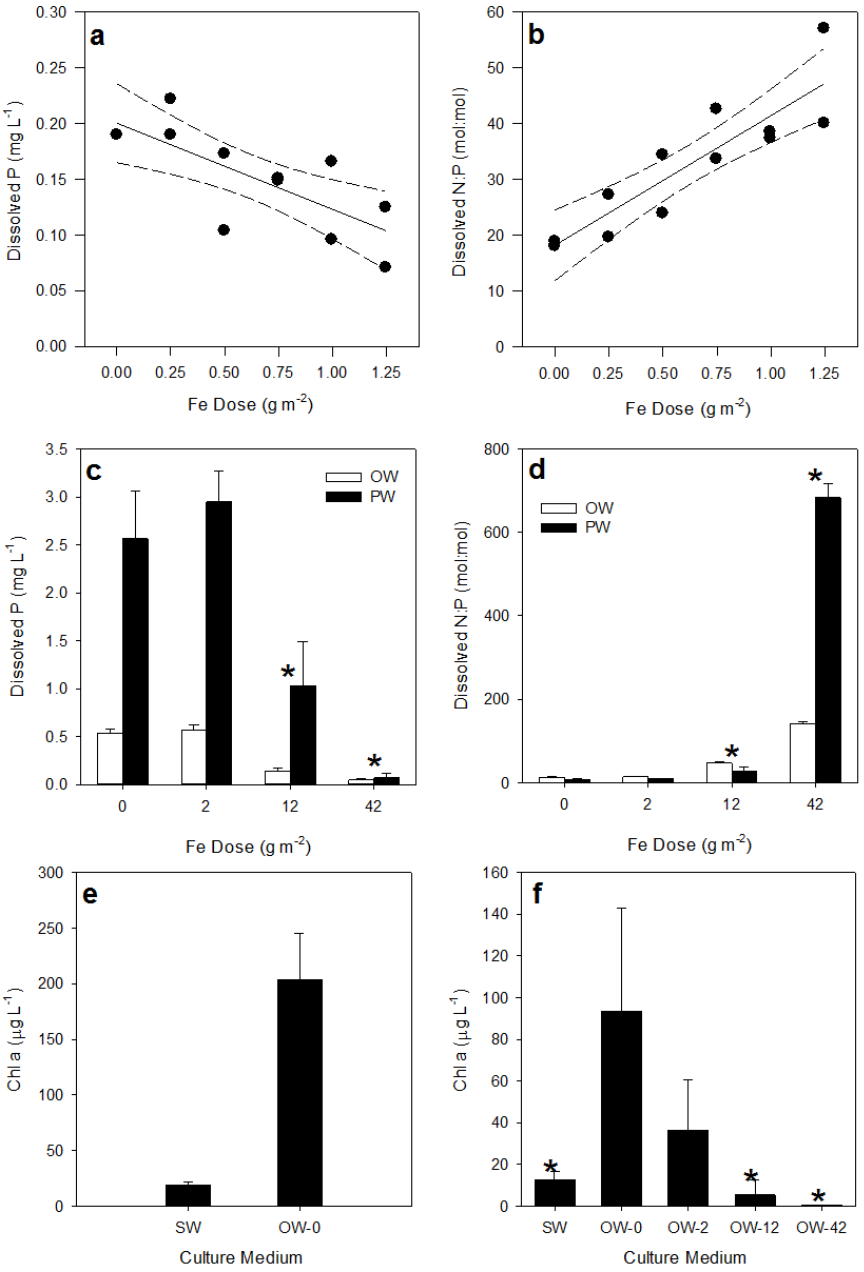

Figure 4-4. Results from Experiments 1-4. Relationships between iron dose and dissolved phosphorus concentration (a) and nitrogen-to-phosphorus ratio (b) in overlying water of cores (Exp. 1). Mean (± SD) of dissolved phosphorus concentrations (c) and nitrogen-to-phosphorus ratios (mean ± SD) (d) in overlying water (OW) and pore water (PW) of cores treated with different doses of iron (Exp. 2). Chlorophyll *a* (mean ± SD) of phytoplankton grown in surface water (SW) or overlying water harvested from sediments treated with 0 g Fe m^{-2} (OW-0), 2 g Fe m^{-2} (OW-2), 12 g Fe m^{-2} (OW-12), or 42 g Fe m^{-2} (OW-42) in Exp. 3 (e) and 4 (f). Asterisks denote treatments that differ significantly from control cores in (c-d) or the OW-0 treatment in (f).

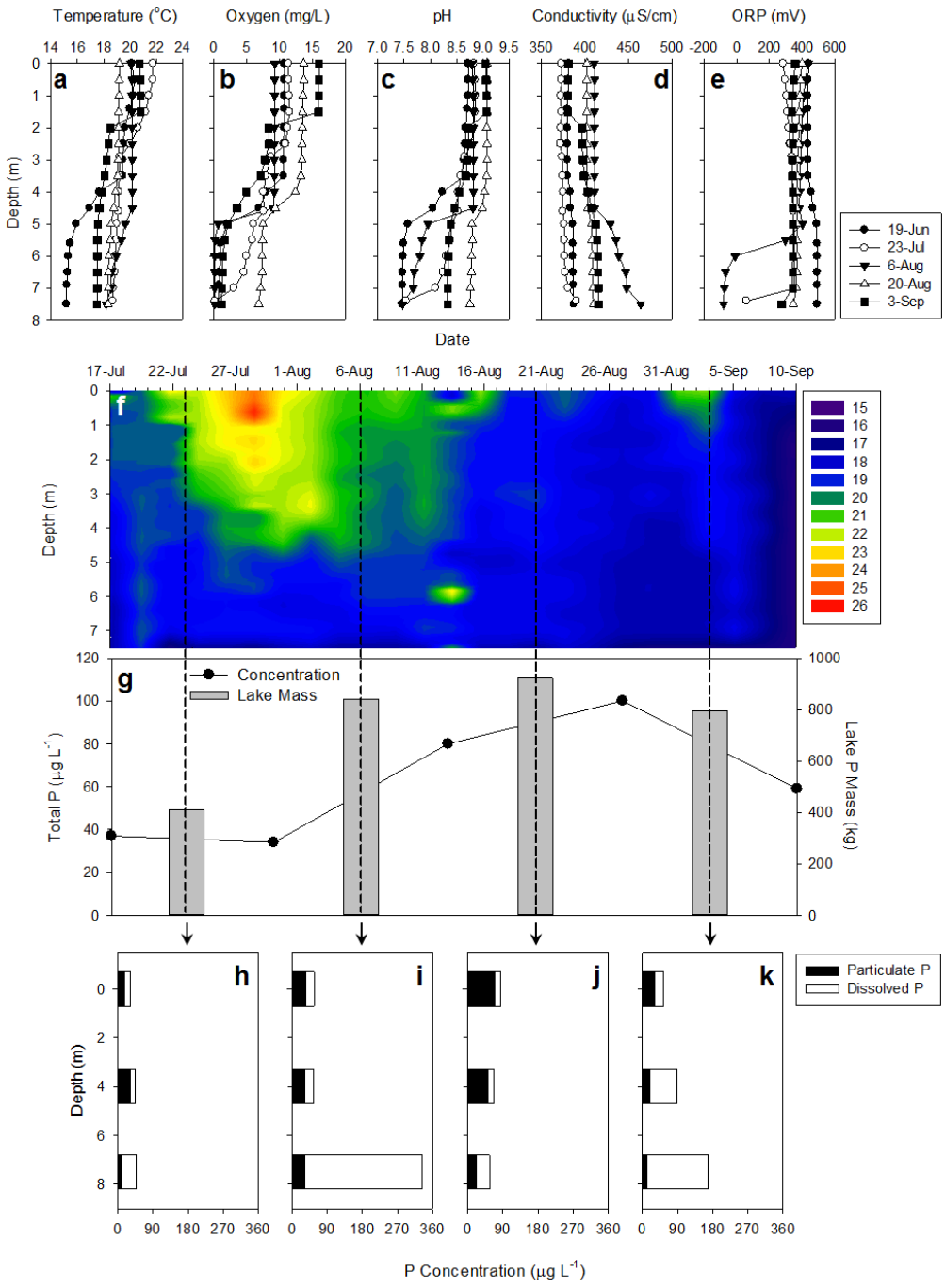

Figure 4-5. Discontinuous polymixis in Nakamun Lake in 2009. Vertical profiles of temperature, dissolved oxygen, pH, conductivity, and oxidation-reduction potential (a-e). High-resolution profiles of water temperature, in degrees C (f), shown with changes in total phosphorus concentration in the upper water column (0-4 m) and whole-lake phosphorus mass (g). Dissolved and particulate phosphorus at discrete depths on 23 July, 6 August, 20 August, and 3 September (h-k).

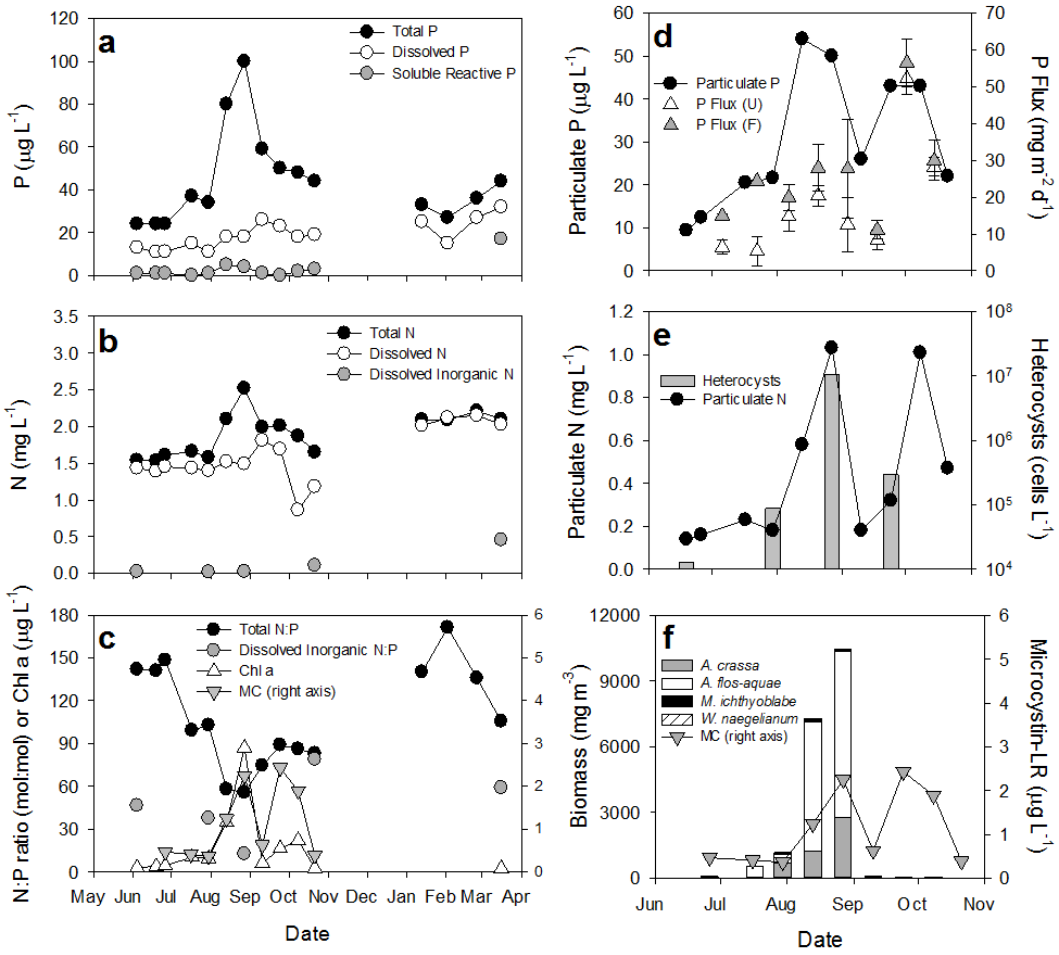

Figure 4-6. Seasonal dynamics in Nakamun Lake in 2009 and under ice in 2010. Phosphorus concentrations (a) nitrogen concentrations (b), and nitrogen-to-phosphorus ratios, chlorophyll *a* concentrations, and microcystin concentrations (c). Particulate phosphorus concentrations and sedimentation rates (mean ± SD) measured by unpoisoned (U) and poisoned (F) traps (d). Particulate nitrogen concentrations and heterocyst densities (enumerated on four dates) (e). Total biomass of potential microcystin producers, shown with microcystin concentrations(f).

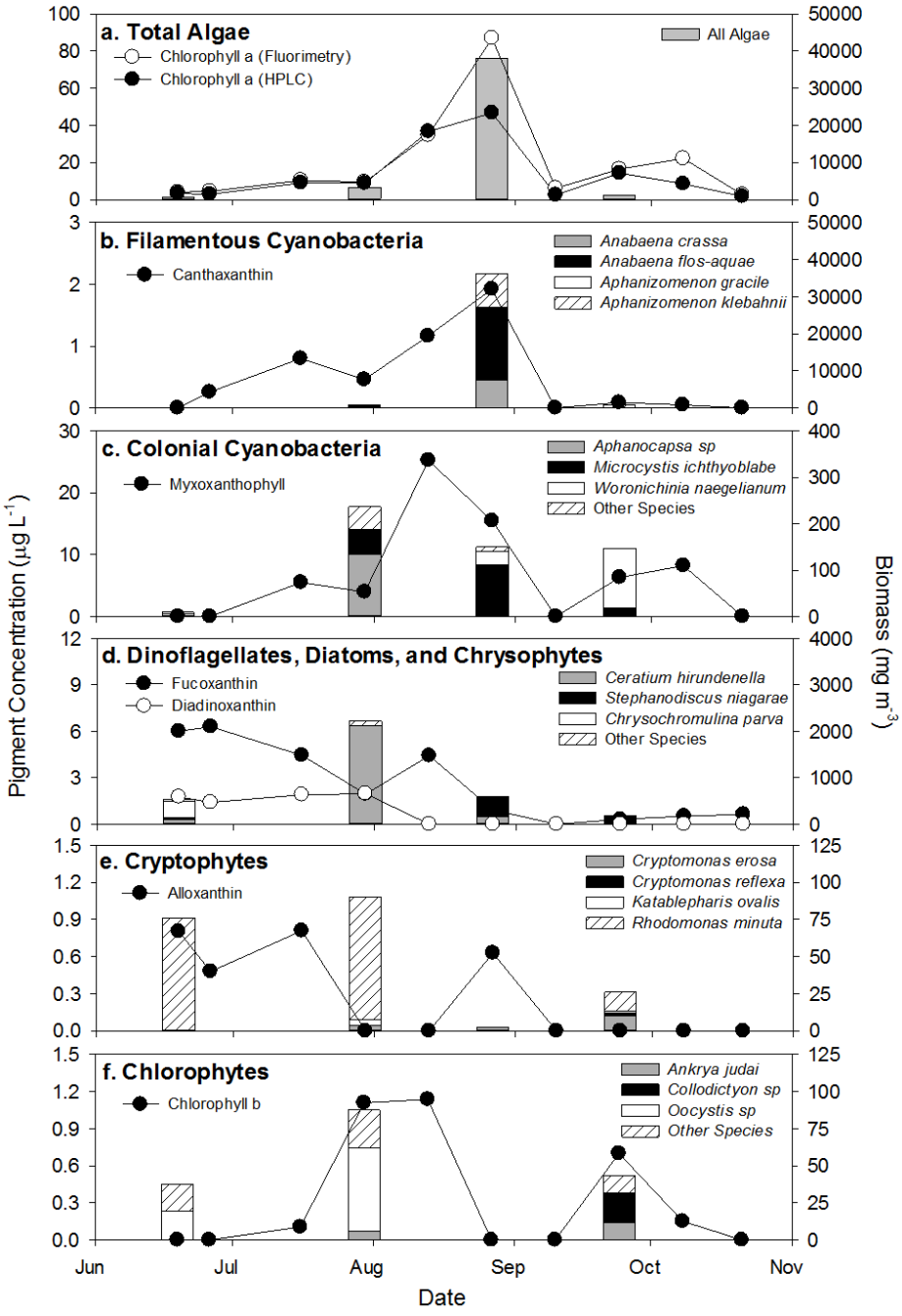

Figure 4-7. Phytoplankton biomass and community composition in Nakamun Lake in 2009. Seasonal trends in pigment concentrations representative of various algal groups (circles; left axes), shown with biomass estimates of individual algal species on four dates (bars; right axes).

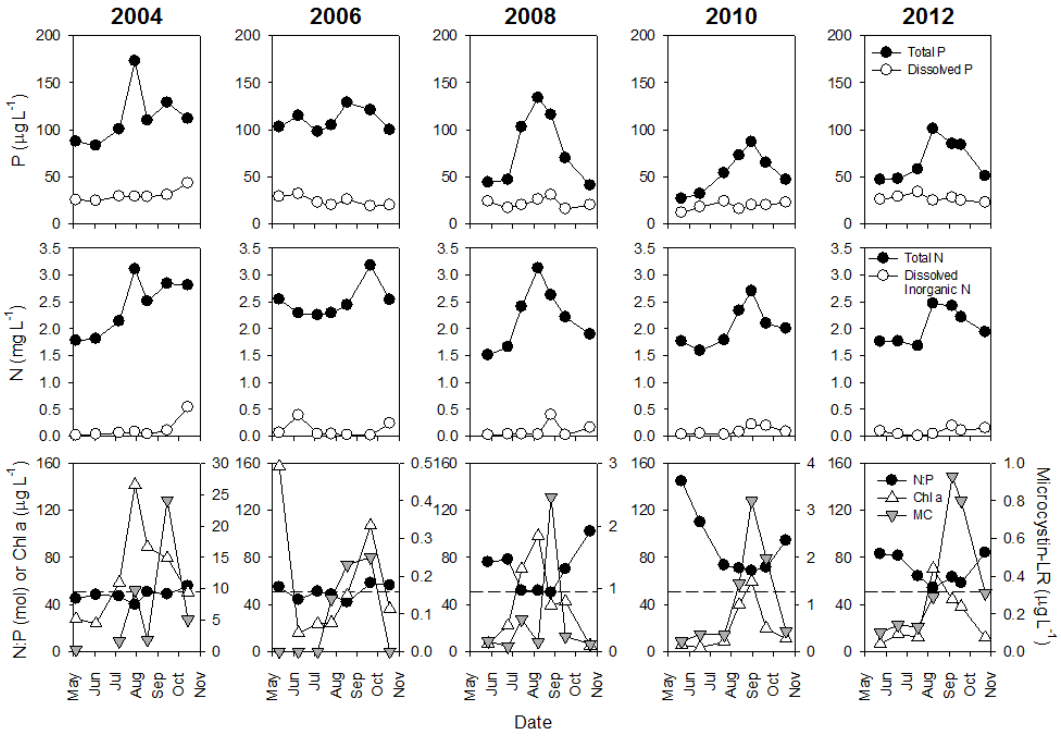

Figure 4-8. Seasonal dynamics in nutrients, algae, and microcystins in the euphotic zone of Nakamun Lake (2004-2012). Total and dissolved phosphorus concentrations (top row), total and dissolved inorganic nitrogen concentrations (middle row), total nitrogen-to-phosphorus ratios, chlorophyll *a* concentrations, and microcystin-LR concentrations (bottom row). Dashed line indicates the N:P threshold determined by Orihel et al. (2012) below which microcystin-LR concentrations are likely to be elevated.

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Chapter 5. Iron suppression of internal phosphorus recycling and toxic cyanobacterial blooms

Introduction

Eutrophication has emerged as one of the key drivers of global change damaging freshwater and marine ecosystems over the last century (Millenium Ecosystem Assessment 2005). Nutrient loading to freshwater ecosystems stimulates growth of nuisance algae, particularly cyanobacteria (Figure 5-1, a-b). The negative consequences of excessive algal growth include reduced water clarity, oxygen depletion, mortality of fish, and losses in aquatic biodiversity, among others, which lead to the impairment of ecosystem functions and services to human society (Carpenter et al. 1998, Smith 2003). Ecological changes associated with nutrient enrichment can amplify infection and disease in wildlife and humans (Johnson et al. 2010), and many cyanobacteria that are favored by eutrophication can produce neurologically and liver-damaging toxins (van Apeldoorn et al. 2007, Funari and Testai 2008). Eutrophication also comes at an enormous economic price due to its depreciation of fisheries, tourism, and property values, as well as increased costs of water treatment and health care. For example, in the United States alone, estimated economic losses from eutrophication of freshwaters are estimated at 2.2 billion dollars per year (Dodds et al. 2009).

Excess phosphorus is considered to be the main cause of algal blooms in lakes (Hecky and Kilham 1988). Several lines of evidence support that phosphorus is the ultimate nutrient limiting algal productivity in most freshwaters

ecosystems. First, variation in the biomass of phytoplankton, as inferred through concentrations of chlorophyll *a*, among lakes can be explained, in part, by the supply of phosphorus to lakes (Vollenweider 1976). Second, experimental addition of phosphorus transforms previously oligotrophic lakes to eutrophic lakes (Schindler 1977). Third, case histories of reductions in phosphorus loading to nutrient-polluted lakes, typically achieved through advanced wastewater treatment, have successfully decreased algal biomass in many lakes (Cooke et al. 2005). In addition to the role of phosphorus in controlling total algal biomass, the dominance of cyanobacteria has also been shown to increase as a function of total phosphorus concentrations (Downing et al. 2001)

Internal phosphorus loading from sediments is a common phenomenon in shallow eutrophic lakes that perpetuates high concentrations of phosphorus in surface waters (Riley and Prepas 1984, Søndergaard et al. 1999, Burger et al. 2007). Differences among lakes in internal phosphorus loading appears to be largely driven by variation in sediment chemistry that allows for the sequestration and removal of P from the bioavailable pool (Boström 1984). Although the exact mechanisms responsible for internal phosphorus loading in lakes are still under debate, a compelling hypothesis was proposed by Gächter and Müller (2003), in which phosphorus sequestration in sediments is the result of authigenic formation of ferrous phosphate minerals. The most stable ferrous phosphate mineral is vivianite [$Fe_3(PO_4)_2 \cdot 8H_2O$], a colorless or greyish mineral that precipitates in anaerobic sediments when porewaters are supersaturated with phosphate and iron (Nriagu 1972, Postma 1981). Importantly, vivianite will not form in sulfide-rich

environments, as sulfide directly causes the dissolution of vivianite, and indirectly prevents its formation by precipitating dissolved iron in sulfidic minerals (e.g., pyrite, FeS_2).

Industrial sulfur pollution is hypothesized to have enhanced internal phosphorus loading by decreasing the pool of dissolved iron available to form stable minerals that sequester phosphorus in sediments (Caraco et al. 1989, Smolders and Roelofs 1993). However, the implications of sulfate-mediated iron deficiency on the trophic state of lakes are currently unclear, as iron plays a complex – and potentially antagonistic – role in controlling primary productivity of aquatic ecosystems. On the one hand, iron forms minerals in lake sediments that may inhibit internal phosphorus loading, and thereby, limit algal productivity. On the other hand, iron is an essential micronutrient required by all plants. Therefore, iron may stimulate algal productivity in freshwater lakes (Vrede and Tranvik 2006, North et al. 2007), as it does in large parts of the ocean (Boyd and Ellwood 2010). The influence of iron on cyanobacterial dominance of phytoplankton communities is also unclear. Cyanobacteria have a particularly high requirement for iron as a product of their evolutionary history (Kranzler et al. 2013), and therefore, limited iron availability may allow eukaryotic algae to out-compete cyanobacteria. However, high levels of sedimentary iron may not necessarily increase the dominance of cyanobacteria because iron sequestration of phosphorus can increase nitrogen-to-phosphorus in surface waters (Chapter 3). Because low nitrogen-to-phosphorus ratios can stimulate the growth of nitrogen-

fixing cyanobacteria (Schindler et al. 2008), high iron conditions may possibly reduce the competitive advantage of nitrogen-fixing cyanobacterial species.

Here, we investigate these competing hypotheses for the effect of iron on the trophic state of lakes and cyanobacterial dominance. Our study was performed in a hypereutrophic lake located in the prairie parkland of Alberta, Canada (Figure 5-1). Like many shallow, nutrient-rich lakes, Nakamun Lake experiences seasonal releases of phosphorus from sediments that fuel intense blooms of cyanobacteria and potentially their production of microcystins. We manipulated iron concentrations across 15 in-lake mesocosms and monitored the changes in sediment chemistry, nutrient cycling, algal biomass and community composition, and microcystin concentrations.

Results and Discussion

We hypothesized that experimentally increasing porewater iron in a sulfate-rich, low-iron lake would lower concentrations of dissolved phosphorus in sediment porewaters by reducing the accumulation of sulfide in porewater and promoting the formation of ferrous phosphate minerals. Nakamun Lake is modestly rich in sulfate for a freshwater lake (ca. 10 mg L^{-1}), but has low concentrations of iron in surface sediments (16 mg g^{-1} d.w) and porewaters (< 0.5 mg L^{-1}). Consequently, sulfide concentrations upwards of 2 mM accumulate in sediment porewaters, and phosphorus exists in sediments in organic forms that are susceptible to recycling (Chapter 3). The addition of different doses of iron (2 to 225 g Fe m^{-2} sediment) to mesocosms in Nakamun Lake generated a continuum of iron concentrations from

background levels up to 94 mg g^{-1} d.w in the solid phase and 6 mg L^{-1} in porewaters (Figure 5-2, a-b). Phosphorus concentrations in solid sediments were similar among treatments (Figure 5-2, c), but as expected, iron treatment significantly decreased concentrations of dissolved phosphorus in sediment porewaters from 1-2 mg L^{-1} to less than 0.5 mg L^{-1} at the highest doses (Figure 5-2, d). Iron and phosphorus concentrations in porewaters were also impacted in deeper sediments, down to depth of 15 to 20 cm (Figure 5-2, e-f). Iron suppressed the build-up of dissolved sulfide in porewaters in a dose-dependent manner (Figure 5-2, g), as a result of the precipitation of iron sulfides. A black solid was visible on the surface of sediments in iron-treated mesocosms, and the examination of sediment minerals using x-ray diffraction confirmed the formation of pyrite (Orihel, unpublished data).

The importance of internal loading in Nakamun Lake is manifested in seasonal patterns of nutrient concentrations, in which phosphorus concentrations in surface waters typically double from spring to fall, and to a lesser extent, under ice in winter (Chapter 3). Based on the a priori hypothesis that iron would increase the binding capacity of sediments, we predicted the addition of iron would depress episodes of internal phosphorus loading in Nakamun Lake. Indeed, iron diminished internal phosphorus loading (Figure 5-3, a-h), with maximum total phosphorus concentrations in summer reaching > 100 μg L^{-1} in Nakamun Lake and mesocosms receiving low Fe doses to a low of 33 g L^{-1} in the mesocom receiving the highest Fe dose. This effect was strongly dependent on the dose of iron applied during the summer open water season (Figure 5-4, a-b) and during

winter under ice (Figure S5-1, a-b). Soluble reactive phosphorus accumulated only in control mesocosms and those receiving low iron doses (Figure S5-2). Somewhat unexpectedly, iron also decreased nitrogen concentrations (Figure 5-3, i-p), but this effect was significant in the open water season (Figure 5-4, c-d) and not in the winter (Figure S5-1, c-d). More dissolved inorganic nitrogen tended to accumulate in the water column at higher iron doses than lower ones, whereas the opposite was true for particulate nitrogen (Figure S5-3).

The ratio of nitrogen and phosphorus in freshwater lakes is an important metric as the ratio of these elements has been shown to influence phytoplankton community composition. Specifically, a low ratio of nitrogen-to-phosphorus favors species of cyanobacteria that fix atmospheric nitrogen, as this capacity gives them a competitive advantage over other algae that rely on the pool of dissolved inorganic nitrogen in lakes (Schindler et al. 2008 and references therein). Based on our earlier work in Nakamun Lake (Chapter 4), we expected the nutrient flux from iron-enriched sediments to be characterized by higher nitrogen-to-phosphorus ratios than iron-deficient sediments. Consistent with this prediction, the addition of iron to mesocosms in this study had a disproportional effect on phosphorus and nitrogen, which translated into notable changes in nutrient ratios. Iron significantly increased the ratio of total nitrogen to total phosphorus, and of dissolved nitrogen to dissolved phosphorus, in the open water season (Figure 5-4, e-f) and under ice (Figure S5-1, f). Further, the ratio of dissolved inorganic nitrogen to soluble reactive phosphorus was elevated in mesocosms receiving the higher iron doses, particularly under ice (Figure S5-4).

One of the most conspicuous symptoms of eutrophication is the growth of planktonic cyanobacteria that form unsightly floating masses on the lake surface and foul beaches with thick green odorous scums (Figure 5-1, b-c). This study supports that successively higher amounts of iron increasingly suppress the total biomass of phytoplankton, as indicated by water column concentrations of the pigments chlorophyll *a* (Figure 5-5, a-d), as well as another pigment produced by all algae, beta carotene (Figure 5-5, e-h). Average concentrations of chlorophyll *a* over the open water season in the iron-treated mesocosms ranged from 5 to 31 μg L^{-1}, as measured by high performance liquid chromatography (or 6 to 44 μg L^{-1} as measured by standard fluorometry), and were significantly negatively related to the dose of iron applied (Figure 5-6, a). Total algal biomass estimated from algal counts and biovolumes showed similar patterns as measurements of chlorophyll *a* by two different methods (Figure S5-5). Interestingly, chlorophyll *a* concentrations in mesocosms receiving the three lowest iron doses (which, incidentally, had little effect on internal phosphorus loading) were higher than the mean of the control mesocosms (Figure 5-6, a). Therefore, the hypothesis that iron stimulates productivity cannot confidently be ruled out. Note that some of the variability in phytoplankton biomass may be related to the growth of periphyton on the walls of the mesocosms (Figure S5-8), which accumulated phosphorus and increased in biomass as a function of iron dose (Figure S5-9).

The addition of iron to Nakamun Lake not only reduced the biomass of the phytoplankton community, but also considerably changed the species composition. Temporal trends in the pigments zeaxanthin (produced by all

cyanobacteria), canthaxanthin (produced by filamentous cyanobacteria), and myxoxanthophyll (produced by colonial cyanobacteria) showed a high degree of variability (Figure 5-5, i-t), but on average, concentrations of all three cyanobacterial pigments were significantly decreased by iron treatment (Figure 5-6, c-e). Concentrations of the pigment echinenone, which is commonly used to indicate cyanobacteria, was absent in most samples (data not shown). In contrast to cyanobacteria, pigments representative of chlorophytes (chlorophyll *b*), cryptophytes (alloxanthin), and chrysophytes/diatoms/dinoflagelletes (fucoxanthin and diadinoxanthin) were variable among mesocosms (Figure S5-6, a-p) and not significantly related to iron dose (Figure S5-7, a-d). Concentrations of bacterial chlorophyll were notably elevated in most iron-treated mesocosms relative to controls (Figure S5-6) and decreased with increasing iron dose (Figure S5-7, e). Changes in community composition were clearly evident from enumeration of algal species (Figure 5-7). Low-iron mesocosm predominately contained cyanobacteria in the summer and diatoms and chlorophytes in the fall, whereas high-iron mesocosms contained mixed assemblages of chrysophytes, euglenophytes, and cryptophytes.

Blooms of cyanobacteria in freshwater lakes and drinking water reservoirs are a concern to human health because exposure to potent toxins produced by cyanobacteria can cause illness, and in some cases, death (Chorus et al. 2000). Microcystins are a family of hepatotoxins synthesized by several common species of cyanobacteria and have been found in lakes worldwide (Sivonen and Jones 1999, Dittmann and Wiegand 2006, Orihel et al. 2012). One of the most

remarkable finding of this study was that iron appeared to have an effect on microcystins above and beyond its control on cyanobacterial biomass. We hypothesized that greater sediment iron would reduce concentrations of microcystin by reducing the phosphorus supply to, and thus the growth of, toxin-producing cyanobacteria. To our surprise, even low doses of iron kept microcystin concentrations low, irrespective of the high cyanobacterial biomass. Microcystin concentrations in all iron-treated mesocosms, with one exception, largely remained below the 1 $\mu g\ L^{-1}$ drinking water guideline set by the World Health Organization, whereas microcystin concentrations in the lake and in the control sites were above this guideline for much of the late summer and fall (Figure 5-5, u-x). Seasonal average concentrations of microcystins in the control mesocosms ranged from 0.6 – 2.4 $\mu g\ L^{-1}$. In comparison, average microcystin concentrations in the iron-treated mesocosms ranged from 0.2 – 0.6 $\mu g\ L^{-1}$ (excluding the 42 g m^{-2} mesocosm) and were unrelated to iron dose (Figure 5-6, f). This is a particularly compelling finding in light of a recent discovery that iron starvation triggers the transcription of microcystin synthetase genes and the production of this toxin in the cyanobacterium *Microcystis* (Sevilla et al. 2008, Alexova et al. 2011).

In summary, substantial changes in nutrient cycling, algal biomass and species composition, and microcystin concentrations in Nakamun Lake occurred in response to rather modest changes in sediment iron. The porewater iron concentrations achieved even at the highest iron dose are still lower than other lakes in the region connected to groundwater iron sources (Ballard 2011) and an

order of magnitude lower than some Canadian Shield lakes (Chapter 4). By sequestering phosphorus in sediments, iron decreased phosphorus concentrations and increased nitrogen-to-phosphorus ratios in the water column. This in turn, reduced the biomass of phytoplankton and periphyton, as well as shifted the composition of the algal community from one largely dominated by cyanobacteria to a more mixed community. Iron also reduced microcystin concentrations, not only by reducing cyanobacterial biomass, but by some other mechanism, possibly related to the inhibition of microcystin synthetase gene transcription.

These findings reinforce the important role that iron plays in controlling the trophic state of sulfate-rich lakes, and the sensitivity of these lakes to changes in iron supply. In this context, sulfur pollution may be a potential threat to the health of sensitive freshwater lakes. It is well-established that anthropogenic sulfur emissions began increasing on a global scale in the late 1800s and rose to over 70 million metric tons per year until the 1980s, after which stringent emission regulations were enacted in North America and Europe (Stern 2006). Today, the nature of the sulfur problem has changed quite dramatically. The world's coal, petroleum, metallurgy, and mining industries currently mobilize an estimated 190 million tons of sulfur compounds per year (Rappold and Lackner 2010). Less than 30% of this sulfur is released to the atmosphere, and the remainder is reclaimed for use mainly as a chemical leaching or catalytic reagent in industrial processes or an active ingredient in agricultural fertilizers. The flux of sulfur to the environment from discharges of industrial wastes and runoff of fertilizers from agricultural fields (ca. 80 million tons yr^{-1}) now exceeds anthropogenic sulfur

emissions on a global scale (Rappold and Lackner 2010). Notably, as a result of the reduction in atmospheric sulfur emissions during the 1990s, sulfur deficiencies in crops are more common and the use of sulfur fertilizers is increasing (Till 2010). Furthermore, the supply of sulfur reclaimed from industrial processes exceeds the demand for sulfur products, so excess sulfur is stockpiled as element sulfur on land or injected as hydrogen sulfide underground. In other words, efforts to reduce acid rain by capturing an ever increasing proportion of the sulfur in fossil fuels has resulted in a global surplus of sulfur compounds whose environmental fate and ecological consequences are poorly understood.

Unfortunately, it is not known to what extent sulfur pollution over the last century has depleted natural stores of reactive iron in the environment. Nor do we know how today's growing dependence on sulfur fertilizers and disposal of massive amounts of sulfur wastes into rivers, lakes, and underground aquifers is affecting the pool of available iron in freshwater lakes. In this study, we demonstrated how the delicate balance between sulfate and iron in a nutrient-rich lake has far-reaching consequences for aquatic ecosystem health. Sulfur pollution holds the potential to push some lakes over the delicate tipping point to a more eutrophic state, but scientific evidence to corroborate – or refute – this hypothesis is urgently needed.

Materials and Methods

Fifteen mesocosms were installed in Nakamun Lake, Alberta Canada (Figure S5-10, a). Each mesocosm (manufactured by Currie Industries, Ltd., Canada)

consisted of a floating, hexagonal ring (vinyl-covered Styrofoam; 2-m in diameter) reinforced with an aluminum frame, and a flexible, clear, woven plastic wall (Nova-thene®; 6-m in height) externally supported by four rigid plastic pipes (Figure S5-10, b). Mesocosms were anchored in a bay over a depth of 4.5 m, and the bottoms were firmly sealed (ca. 0.5 m) into the sediments with sandbags (Figure S5-10, c). Twelve mesocosms were each treated with a different dose of iron (2 – 225 g Fe m^{-2} sediment, on equal log_{10}-scale increments) and three "control" mesocosms were left untreated. Treatments were assigned using a random, spatially-stratified scheme (Figure S5-10, c). Treatments were determined based on the results of pilot studies (Chapter 4). Iron (standard liquid grade ferric chloride, 13.8 ± 0.7%; Kemira Water Solutions, Inc., Canada) was added to mesocosms incrementally over 3 days (6-9 June 2009) by slowly delivering the dose into a stream of lake water being pumped into surface waters of each mesocosm for 10 min. Every 2 weeks from June to October 2009 (and every month from January to March 2010) integrated water samples (0 – 4 m) were collected from each mesocosm and a reference site in the lake adjacent to the mesocosms. Samples were processed and analyzed for a suite of water chemistry parameters, phytoplankton pigments, algal species enumeration, and microcystin concentrations as described in Chapter 4. Sediments were sampled only in March 2010 so as not to disturb the mesocosms during the experiment. Element concentrations in solid phase and porewaters of sediments from each mesocosm were determined as described in Chapter 4. Strips of wall material (10 cm x 3 m) were hung in the mesocosms for periphyton colonization, and harvested monthly

to estimate phosphorus accumulation and algal biomass (chlorophyll *a*) on mesocosms walls. Data analyses were performed using SigmaPlot for Windows (Version 12.3). Dose-response relationships were fitted using linear regression models and tested using analysis of variance. Normality tests and equal variance tests were used to confirm assumptions of parametric tests, and data was $\log_{10}$-transformed as required.

Figures

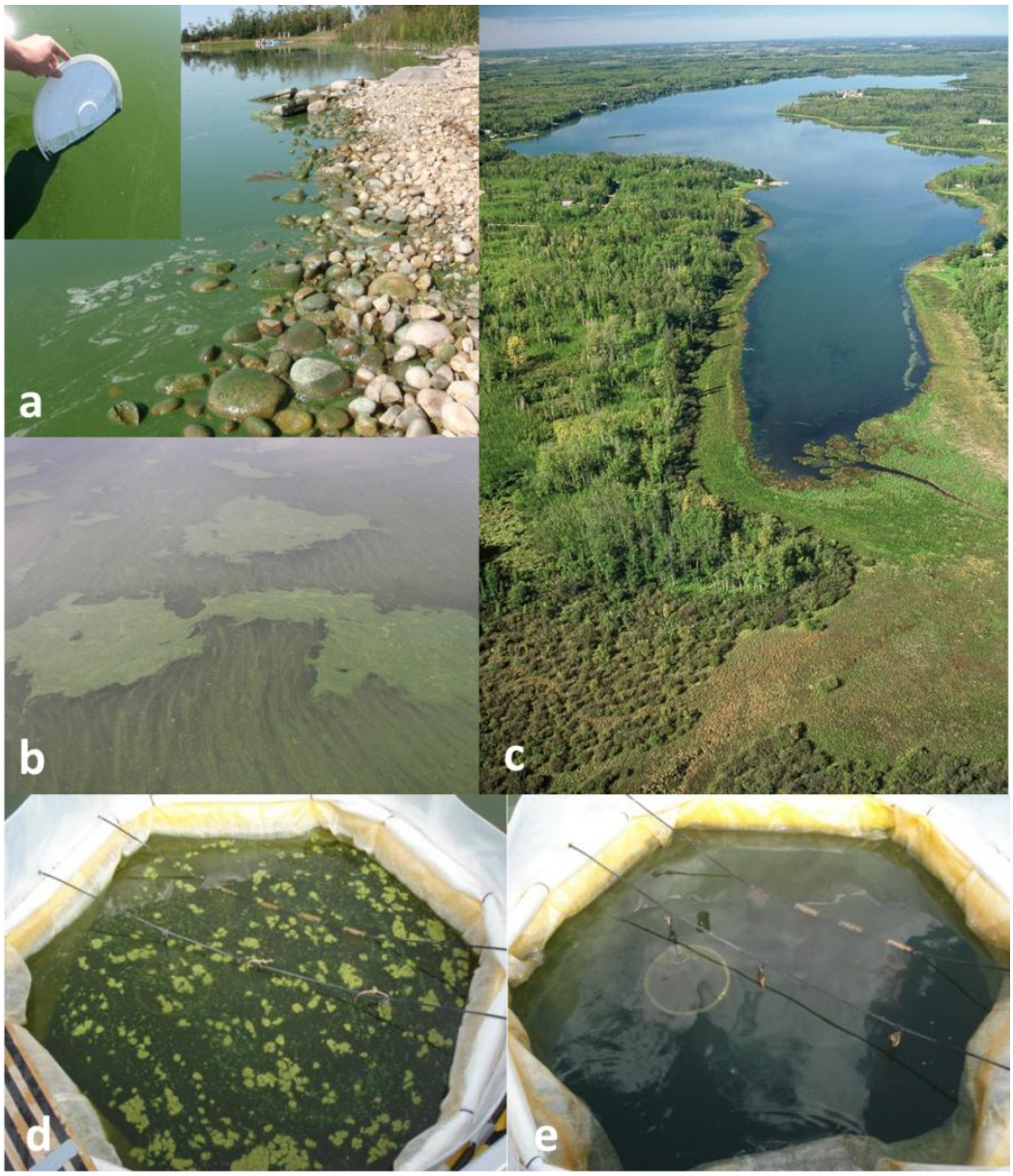

Figure 5-1. Like many nutrient-rich shallow lakes, Nakamun Lake in Alberta, Canada experiences intense cyanobacterial blooms in summer that foul its shoreline with green noxious scums (a) and form unsightly floating mats on the lake surface (b). Aerial view of Nakamun Lake (Credit: Cows and Fish)(c). Experimental control mesocosm (d) and iron-treated mesocosm (e) in late summer.

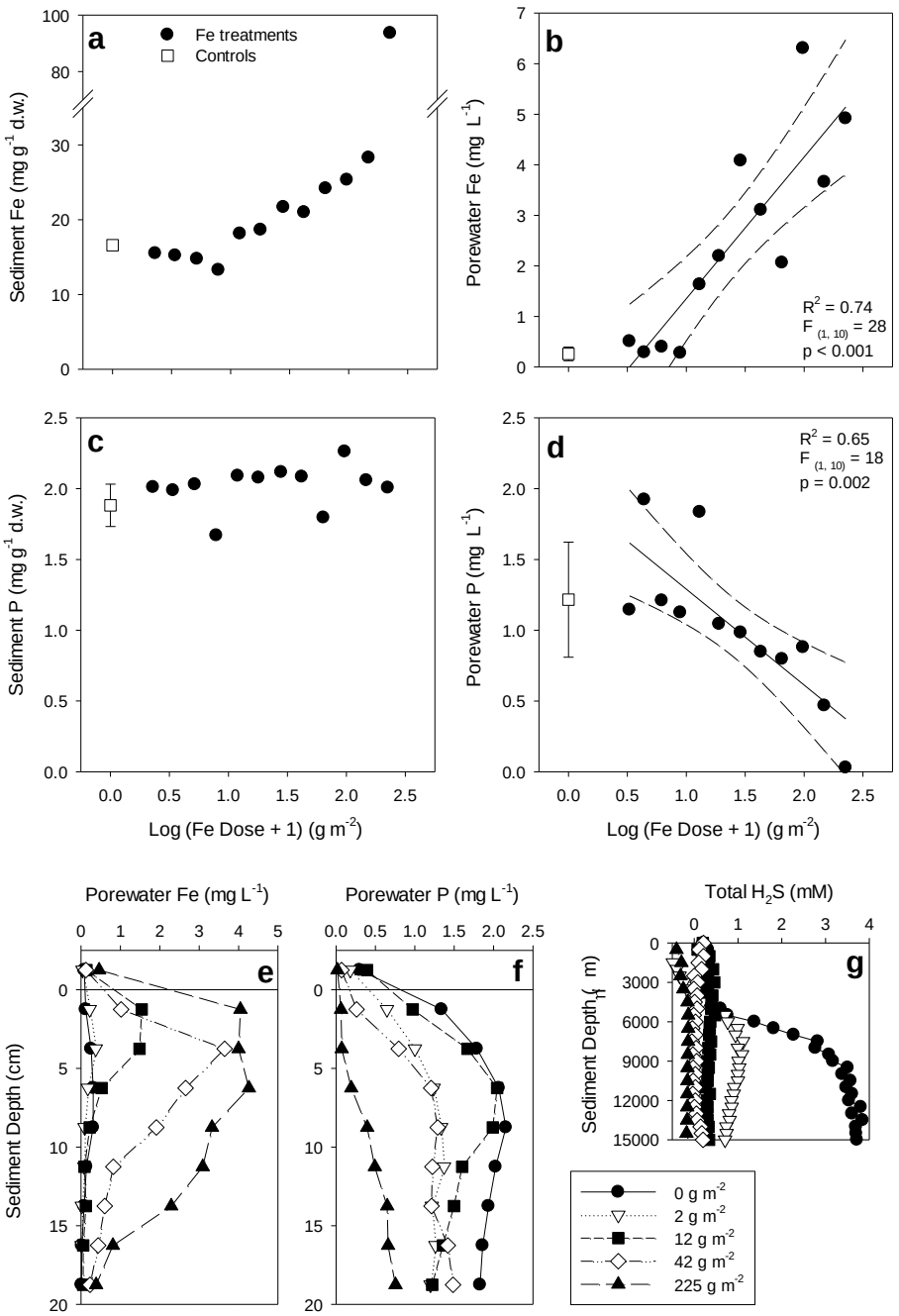

Figure 5-2. Chemistry of sediments in iron-treated and control mesocosms. Iron concentrations in surface sediments (a) and porewaters (b). Phosphorus concencentrations in surface sediments (c) and porewaters (d). Values for control mesocosms (n = 3) are shown as mean ± SE (a-d). Vertical concentration profiles of porewater iron (e), phosphorus (f), and total sulfide (g) in sediments of five mesocosms.

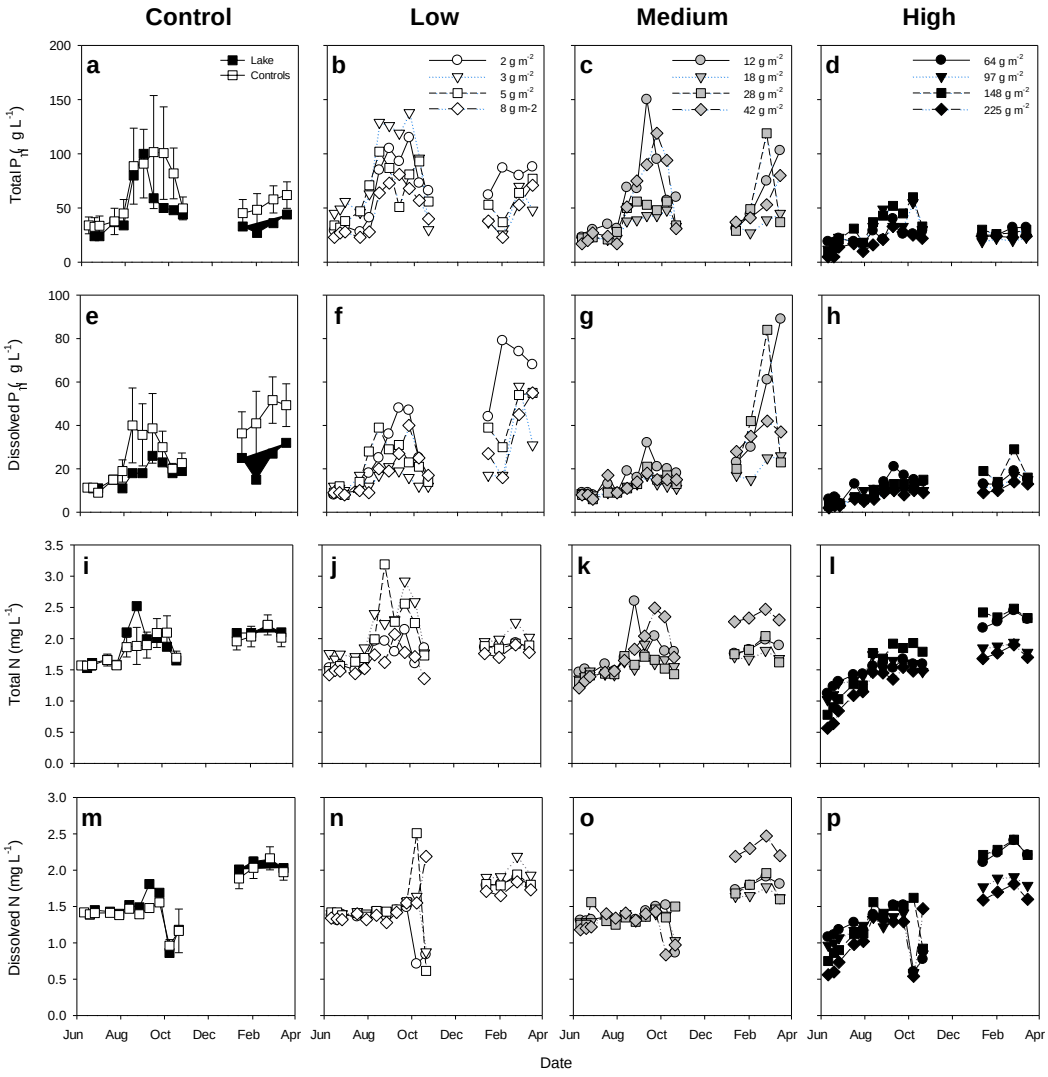

Figure 5-3. Concentrations of the important algal nutrients phosphorus and nitrogen in Nakamun Lake, control mesocosms (mean ± SE, n = 3), and 12 mesocosms treated with different doses of iron (2 – 225 g Fe m^{-2} sediment). Total phosphorus (a-d), dissolved phosphorus (e-h), total nitrogen (i-l), and dissolved nitrogen (m-p) in the water column from June to October, and under ice in January to March.

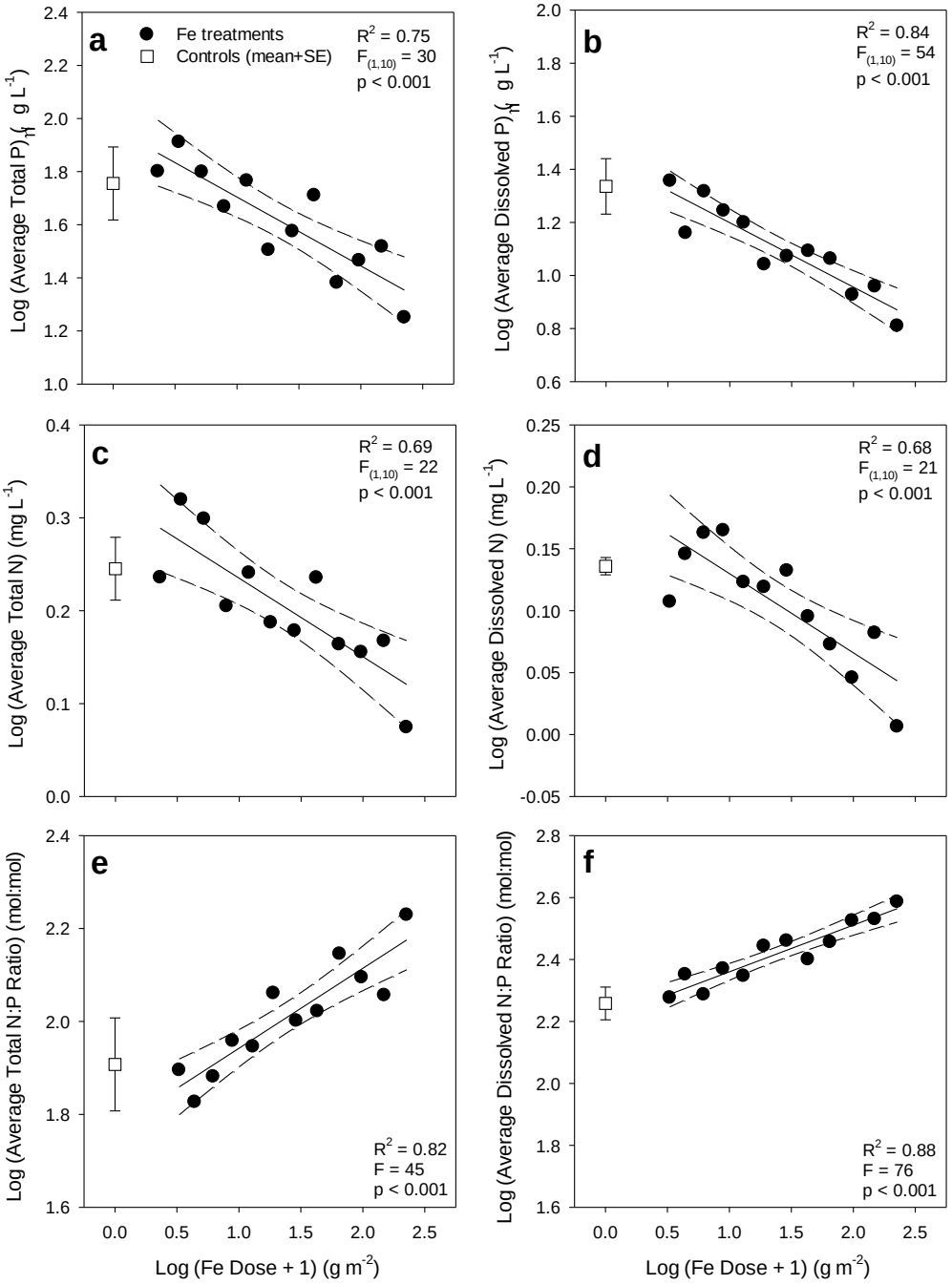

Figure 5-4. Relationships between iron dose applied to mesocosms and concentrations of total phosphorus (a), dissolved phosphorus (b), total nitrogen (c), and dissolved nitrogen (d), or ratios of total nitrogen to total phosphorus (e) and dissolved nitrogen to dissolved phosphorus (f). Each value represent the mean of measurements in individual mesocosms from June to October (n = 11). The line of best fit and 95% confidence intervals of the linear regression model are shown on each panel, along with associated test statistics. Shown for comparison is the mean (± SE) of three control mesocosms.

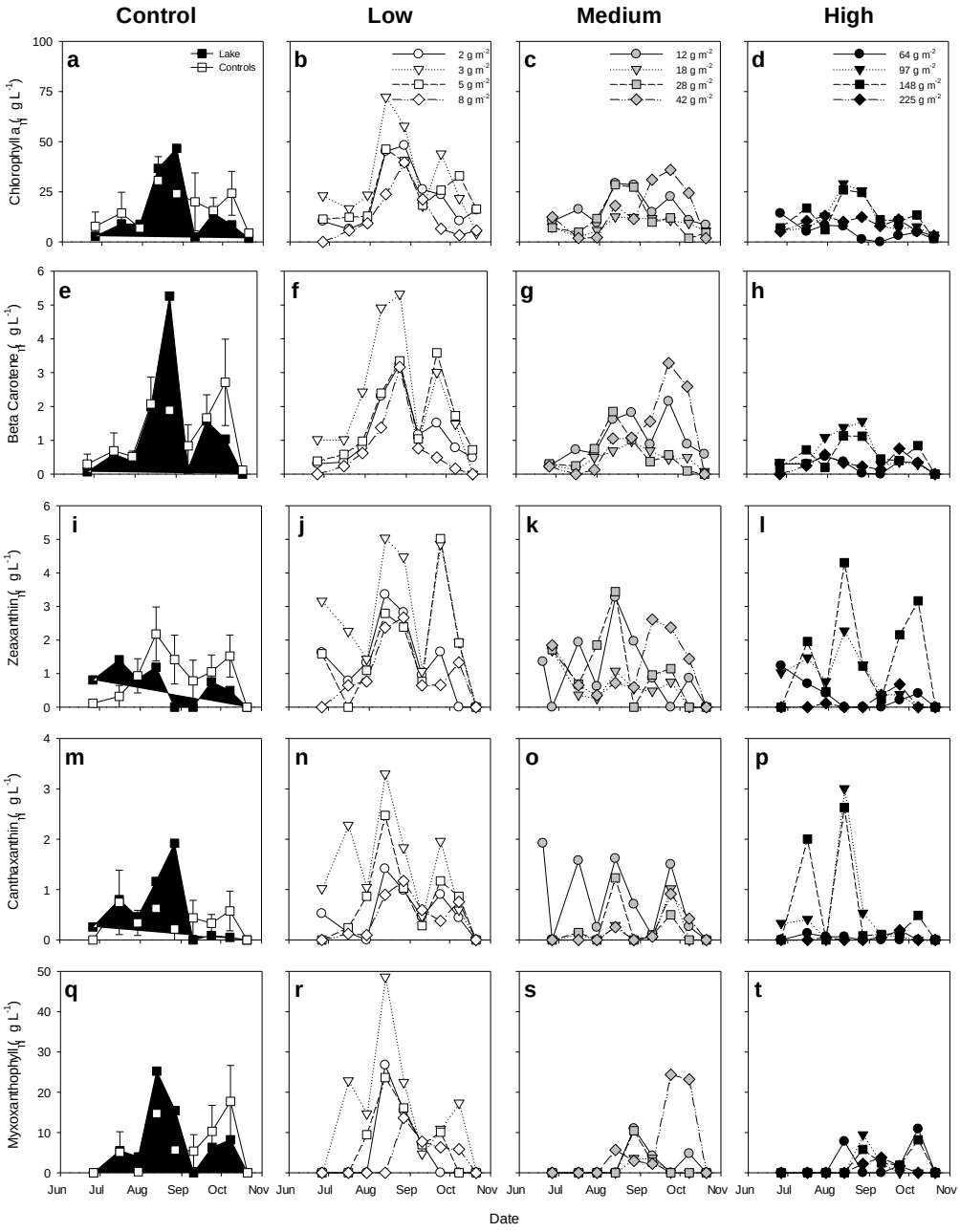

Figure 5-5. Concentrations of algal pigments in Nakamun Lake, control mesocosms (mean ± SE, n = 3), and twelve mesocosms treated with different doses of iron (2 – 225 g Fe m^{-2} sediment). Algal pigments are indicators of the biomass of various algal groups: chlorophyll *a* (a-d) and beta carotene (e-h) are produced by all algae; zeaxanthin is produced by all cyanobacteria (i-l); and canthaxanthin (m-p) and myxoxanthophyll (q-t) are produced by filamentous and colonial cyanobacteria, respectively. Concentrations of the hepatotoxin microcystin, produced by certain cyanobacteria, are shown in panels (u-x). Values represent integrated samples of the water column (0-4 cm) measured by high performance liquid chromatography.

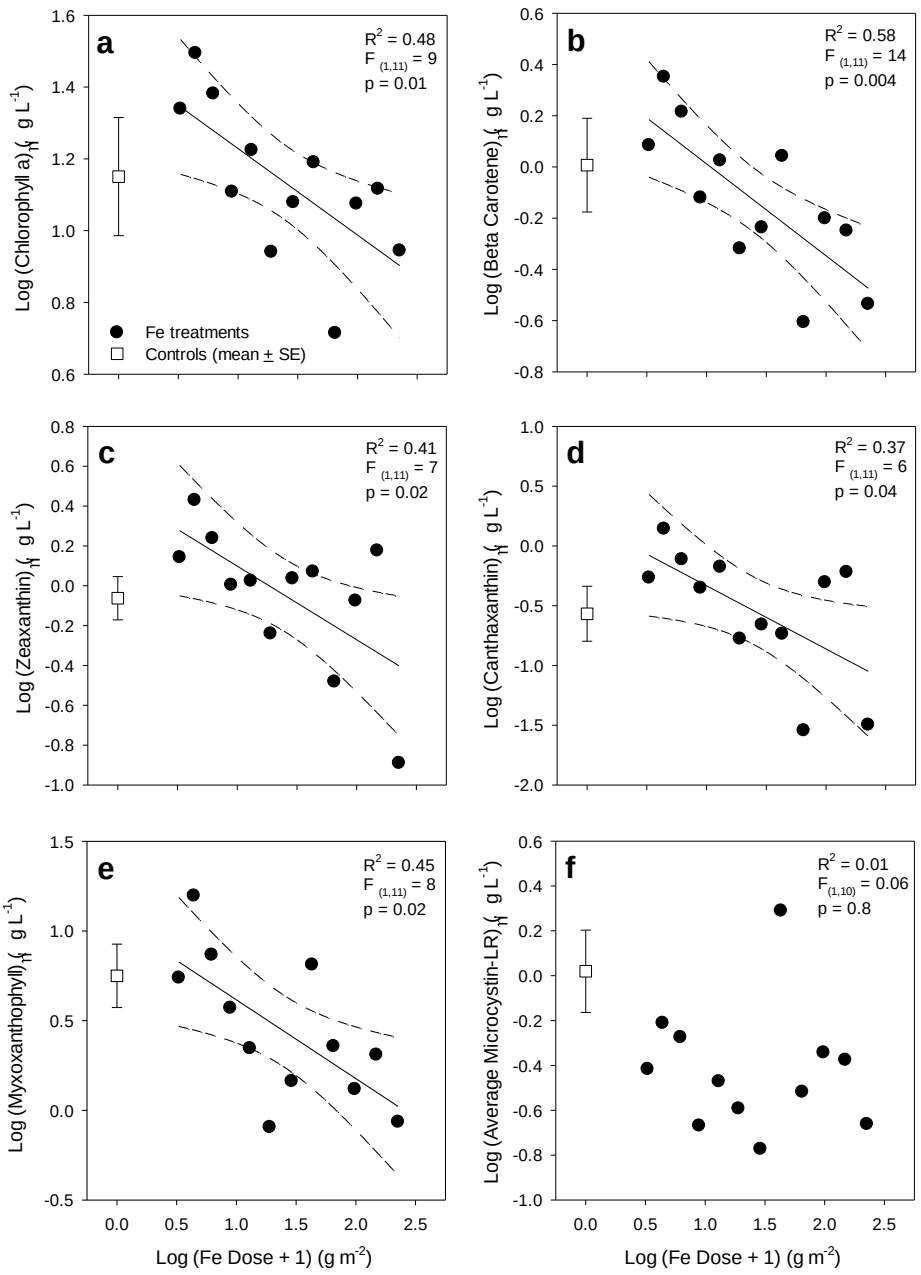

Figure 5-6. Relationships between iron dose applied to mesocosms and average concentrations of the algal pigments chlorophyll *a* (a), beta carotene (b), zeaxanthin (c), canthaxanthin (d), myxoxanthophyll (e) and the cyanobacterial toxin microcystin (f). Statistics as in Figure 5-3.

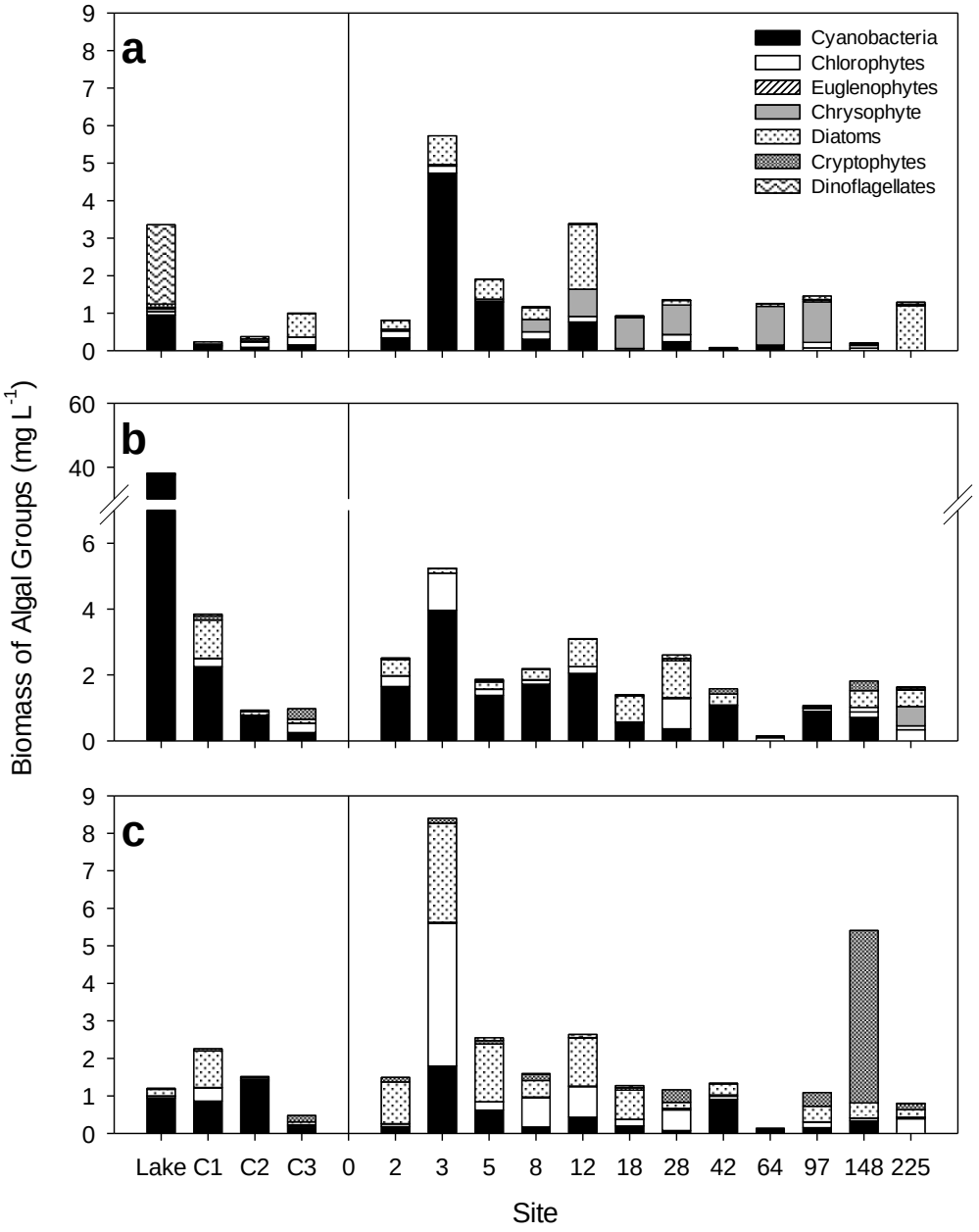

Figure 5-7. Biomass of major phytoplankton groups in Nakamun Lake, control mesocosms (C1-C3), and iron-treated mesocosms (2 – 225 g Fe m^{-2} sediment). Biomass was estimated from species enumeration and biovolume measurements of integrated samples (0-4 m) on 30 July (a), 27 August (b), and 24 September (c).

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Chapter 6. Conclusions

The main conclusions from my thesis research are:

i. Microcystins, a family of mammalian hepatotoxins produced by cyanobacteria, are now prevalent in lakes across Canada, making microcystins an issue of national concern for human health. Elevated levels of microcystins in Canada occur in nutrient-rich lakes and only at low ratios of nitrogen-to-phosphorus.

ii. Under certain conditions, lake sediments release bioavailable nutrients that support the growth of toxigenic cyanobacteria. Environmental factors may indirectly influence microcystin concentrations in lakes by increasing the pool of available nutrients, which in turn, fuels the production of microcystins.

iii. Toxic blooms of cyanobacteria in shallow lakes on the Canadian prairies may result from the synergy between iron-deficient sediments and discontinuous polymixis. Phosphorus released from sediments during stratification is delivered to surface water during mixing, priming conditions for potentially toxic cyanobacterial blooms.

iv. Iron may act as a master variable controlling the trophic status of lakes. Iron loading to lakes inhibits internal phosphorus loading, decreases algal biomass, discourages the dominance of cyanobacteria, and reduces microcystin concentrations.

These conclusions have several implications for the management of eutrophied lakes. First, the finding that high microcystin concentrations occur

only at low nitrogen-to-phosphorus ratios raises questions about how proposals for nitrogen loading reductions to eutrophic lakes will impact microcystin concentrations in lakes. Second, as internal phosphorus loading can stimulate blooms of cyanobacteria and increase levels of microcystins and potentially other cyanotoxins, measures to control internal phosphorus loading should be considered, alongside with efforts to reduce external nutrient loading, as part of overall lake management strategies. Third, the findings of this research substantiate the use of iron as a potential in-lake treatment to inhibit phosphorus loading in iron-deficient eutrophied lakes. Finally, the fundamental role of iron in controlling internal phosphorus loading and cyanobacterial blooms suggests that management efforts to protect natural inputs of iron to freshwater lakes are warranted.

Finally, this research encourages a number of avenues for future research, including: (i) experiments to establish whether a cause-and-effect relationship exists between nitrogen-to-phosphorus ratios and microcystin concentrations; (ii) mechanistic studies to elucidate the biogeochemical processes by which iron may reduce microcystin concentrations in lakes; (iii) whole-lake manipulation experiments to corroborate the hypothesis that sulfur pollution exacerbates eutrophication in lakes; (iv) models to quantify the importance of various pathways by which contemporary sulfur pollution may be depleting natural stores of iron in lakes; and (v) research to investigate if global climate change may be altering the flux of iron to lakes from their watersheds.

Appendix

S2. Supporting Information for Chapter 2

Table S2-1. Information on sources of microcystin data.

Source[a]	Province(s)	No. of Lakes	No. of Samples	Date Range	Sampling Depth	Sample Type	Analytical Method[b]	LOD[c]
[1]	QC	22	23	June - Aug. 2001	integrated epilimnion	particles	PPIA	0.10
[2]	QC	4	47	May - Oct. 2001	integrated epilimnion	particles	PPIA	0.10
[3]	MB	1	87	July - Oct. 2007	integrated euphotic zone	whole water	PPIA	0.10
[4]	BC	3	171	Jan. 2006 - Oct. 2011	discrete depth	whole water	HPLC	0.30
[5]	SK	7	256	May 2005 - Sept. 2009	surface water	whole water	ELISA	0.16
[6]	AB	23	23	Sept. 2010	integrated water column	whole water	PPIA	0.05
[7]	NB	6	24	Oct. 2008 - Aug. 2010	surface water	whole water	ELISA	0.20
[8]	ON	1	66	July 2006 - Sept. 2007	integrated euphotic zone	particles	HPLC	0.05
[9]	AB, MB, ON, PEI	14	20	Aug. 2001 - Sept. 2005	surface water	whole water	PPIA	0.02
[10]	NL	7	32	May - Sept. 2008	surface water	whole water	PPIA	0.22
[11]	NB	1	16	July - Oct. 2008	surface water	whole water	ELISA	0.20
[12]	NS	10	39	Aug. 2008 - Oct. 2010	surface water	whole water	ELISA	0.20

Table S2-1. (continued)

Source[a]	Province(s)	No. of Lakes	No. of Samples	Date Range	Sampling Depth	Sample Type	Analytical Method[b]	LOD[c]
[13]	AB	90	793	May 2004 - Sept. 2009	integrated euphotic zone	whole water	PPIA	0.07
[14]	AB, MB, ON, NB	29	719	July 2003 - Oct. 2010	surface water or integrated epilimnion	whole water	ELISA	0.10-0.16
[15]	MB	22	535	July 2007 - Oct. 2011	surface water	whole water	ELISA	0.10-0.20
[16]	QC	22	22	June - Nov. 2009	surface water or integrated epilimnion	whole water	ELISA	0.005
[17]	ON	9	602	May 2004 - Sept. 2010	discrete depth	whole water	LC-(ESI) MS/MS	0.05

[a] List of data sources:

[1] Giani, A., Bird, D.F., Prairie, Y.T., and Lawrence, J.F. 2005. Emperical study of cyanobacterial toxicity along a trophic gradient of lakes. Can. J. Fish. Aqaut. Sci. **62**(9): 2100–2109. doi:10.1139/F05-124.

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[3] Kotak, B.G. 2009. Occurrence of the cyanobacterial toxin, microcystin, in Lake Winnipeg in 2007. Department of Water Stewardship, Government of Manitoba, Winnipeg, MB.

[4] North Salt Spring Waterworks District, , unpublished data. (Contact: Bob Watson, 761 Upper Ganges Road, Salt Spring Island, BC, V8K 1S1)

[5] Donald, D.B., Bogard, M.J., Finlay, K., and Leavitt, P.R. 2011. Comparative effects of urea, ammonium, and nitrate on phytoplankton abundance, community composition, and toxicity in hypereutrophic freshwaters. Limnol. Oceanogr., **56**(6), 2161–2175. doi:10.4319/lo.2011.56.6.2161.

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[7] New Brunswick Department of Environment, unpublished data. (Contact: Erin Douthwright, Marysville Place, P. O. Box 6000, Fredericton, NB, E3B 5H1,)

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[13] Zurawell, R.W. 2010. Alberta Environment Cyanotoxin Program Status Report. Report # W1001, Alberta Environment, Government of Alberta, Edmonton, AB; Alberta Department of Environment and Water, unpublished data (Contact: Ron Zurawell, Water Policy Branch, Alberta Environment, 7th Floor Oxbridge Place, 9820-106 Street, Edmonton, AB, T5K 2J6).

[14] Watson, S.B.,unpublished data (contact: Sue Watson, Aquatic Ecosystem Management Research Division, Environmental Canada, National Water Research Institute, 867 Lakeshore Road, Burlington, ON, L7R 4A6,)

[15] Manitoba Conservation and Water Stewardship, unpublished data. (Contact: Elaine Page, Water Quality Management Section, Water Stewardship Division, 200 Saulteaux Crescent, Winnipeg, MB, R3J 3W3)

[16] Bird, D.F., unpublished data (contact: David Bird, Département des Sciences Biologiques,Université du Québec à Montréal, C.P. 8888, Succursale Centre-ville, Montréal, QC, H3C 3P8).

[17] Ontario Drinking Water Surveillance Program, unpublished data. (Contact: Jillian Kingston, Environmental Monitoring and Reporting Branch, Ministry of the Environment, 125 Resources Road, Etobicoke, ON, M9P 3V6)

[b] ELISA = enzyme-linked immunosorbent assay (polyclonal antibodies); HPLC = high performace liquid chromatography; LC-(ESI) MS/MS = liquid chromatography-(electrospray ionization) tandem mass spectrometry; PPIA = protein phosphatase inhibition assay.

[c] LOD = lower limit of detection for microcystin analysis.

Table S2-2. Minimum thresholds for N and P concentrations and probabilities of exceedance for N:P ratios in Canadian water bodies.

Microcystin Concentration ($\mu g \bullet L^{-1}$)	Minimum Thresholds[a]		Probabilities of Exceedance[b]			
	Total P ($\mu g\ L^{-1}$)	Total N ($\mu g\ L^{-1}$)	N:P < 20	20 ≤ N:P < 40	40 ≤ N:P < 60	N:P > 60
1	26	658	0.19	0.14	0.06	0.00
2	45	677	0.10	0.07	0.02	0.00
5	83	1460	0.05	0.04	0.00	0.00
10	129	1536	0.03	0.01	0.00	0.00

[a] Thresholds were calculated as the total N or total P concentration above which 95% of values exceeding a specific toxin level occurred.

[b] Probabilities were calculated by dividing the number of samples above a specific toxin level by the total number of samples in each N:P category.

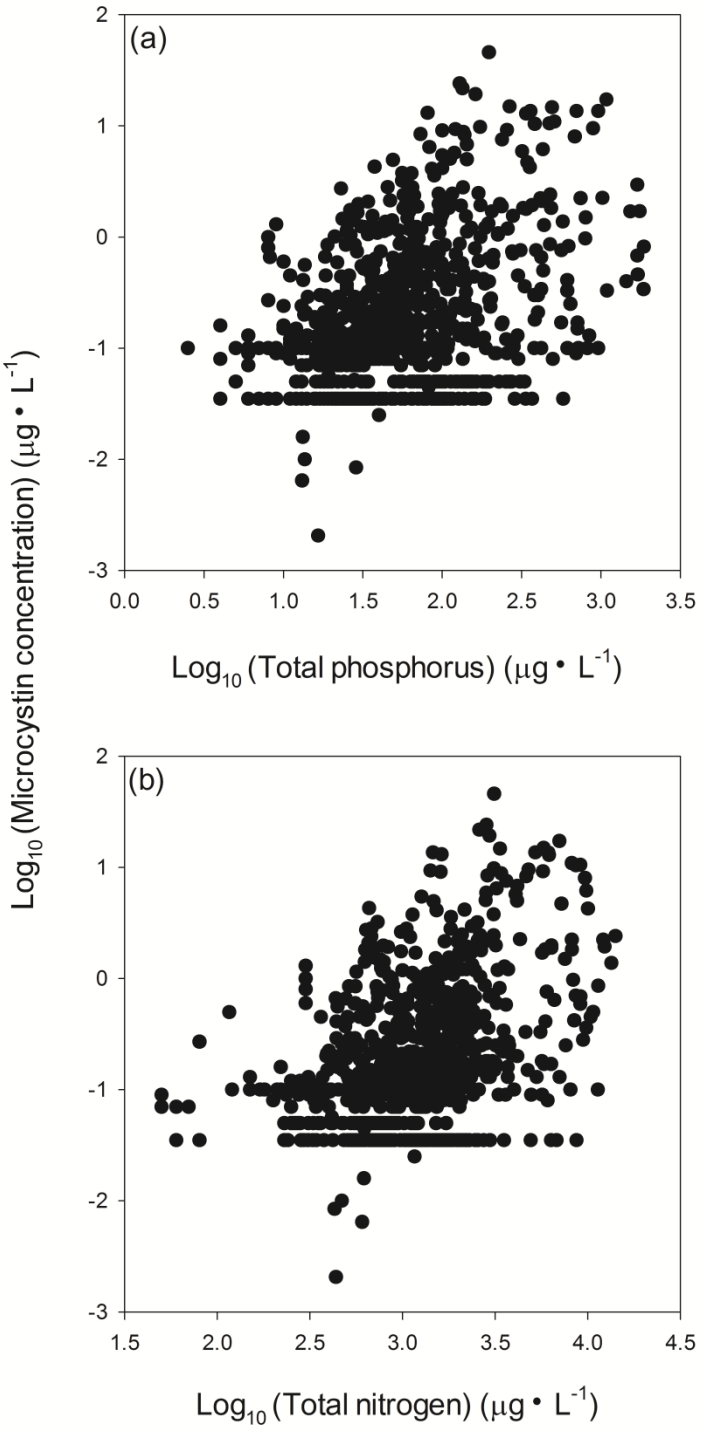

Figure S2-1. Relationship between microcystin concentrations in Canadian water bodies and concentrations of total phosphorus concentrations (a) or total nitrogen concentrations (b).

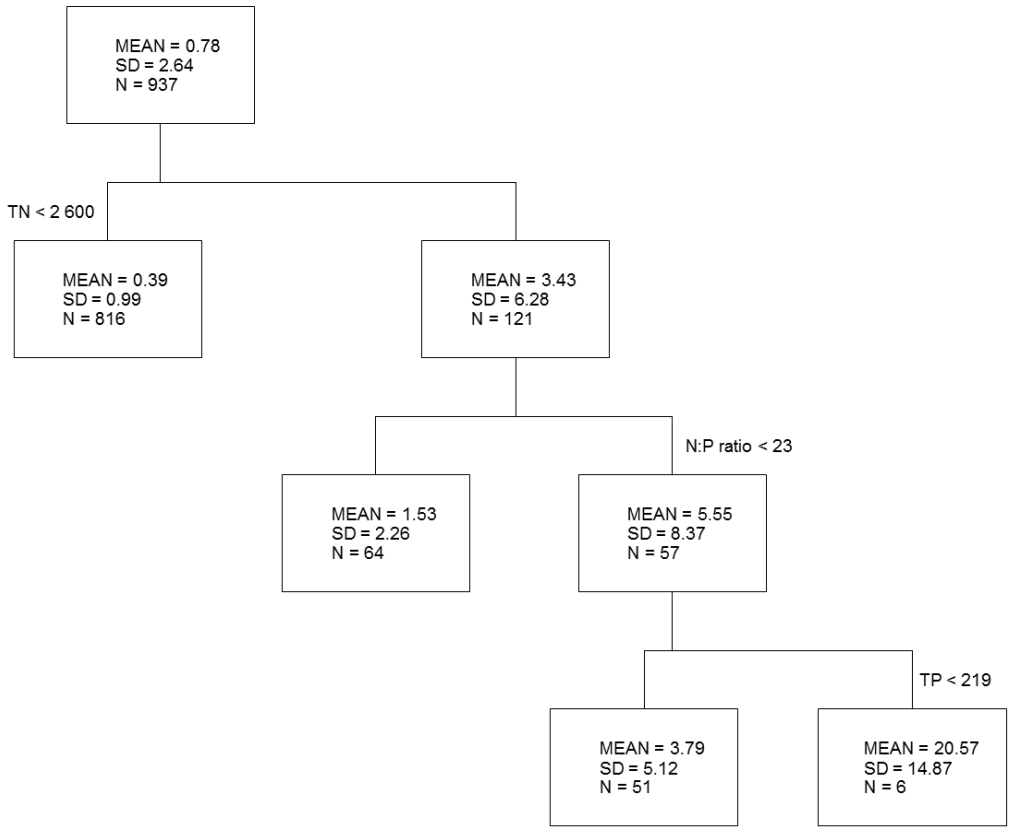

Figure S2-2. Regression tree to explain the variation in microcystin concentrations in Canadian waterbodies based on total phosphorus concentration (TP; µg L^{-1}), total nitrogen concentration (TN; µg L^{-1}), and mass ratio of TP to TN (N:P ratio). The model was computed using the TREES module in SYSTAT 13 (model specifications: fitting method = least squares; minimum split index value = 0.05; minimum improvement in proportional reduction in error = 0.05; and minimum count allowed in each node = 5). Boxes show the mean and standard devation (SD) of microcystin concentrations (µg L^{-1}) and the number of samples (N) in each grouping. Criteria for groupings are indicated at each split. One extreme outlier was excluded from this analysis.

S3. Supporting Information for Chapter 3

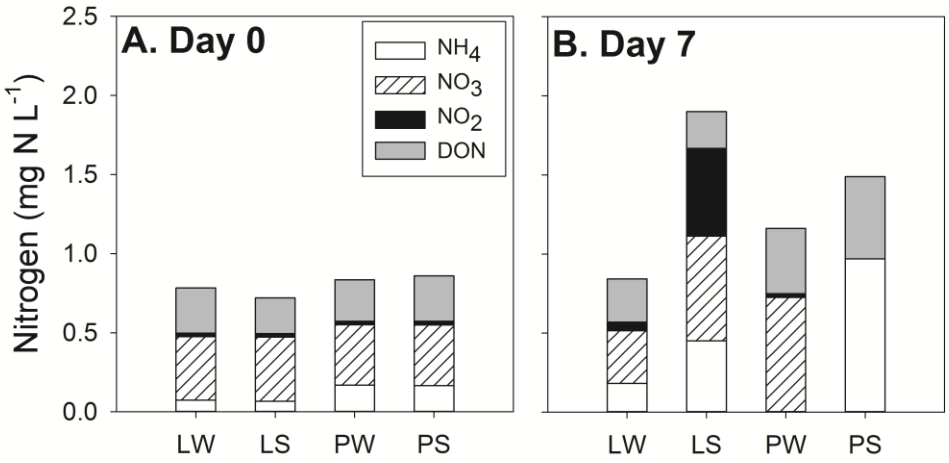

Figure S3-1. Concentrations of dissolved inorganic and organic nitrogen species in overlying water of the sediment cores at the start and end of the incubation experiment.

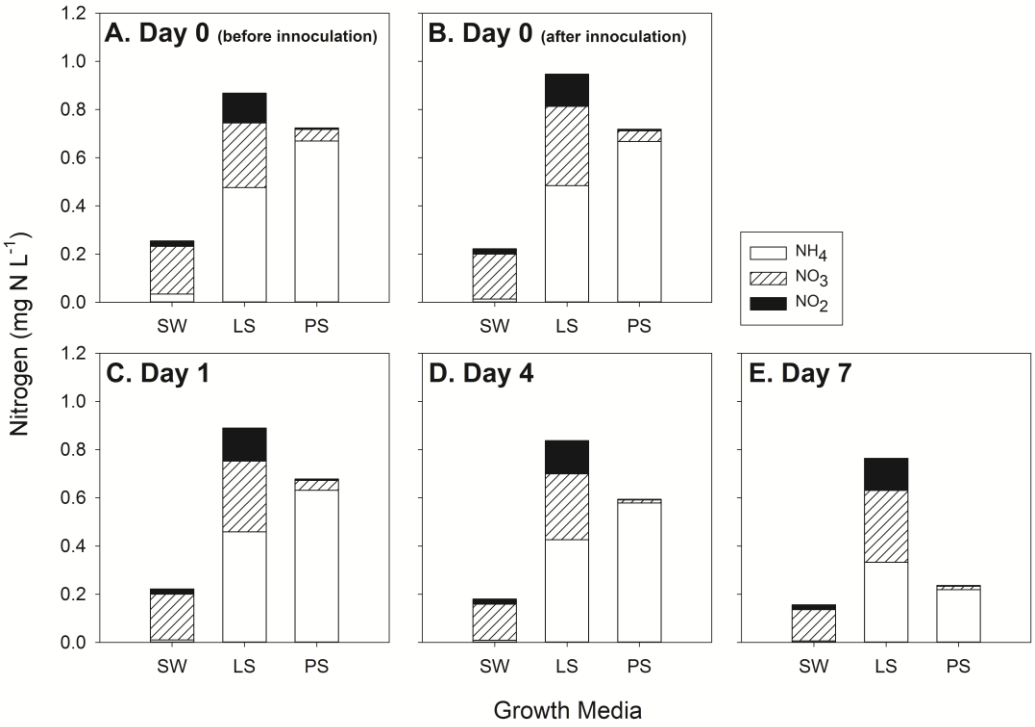

Figure S3-2. Concentrations of dissolved inorganic nitrogen species in the growth media of the *Microcystis* experiment..

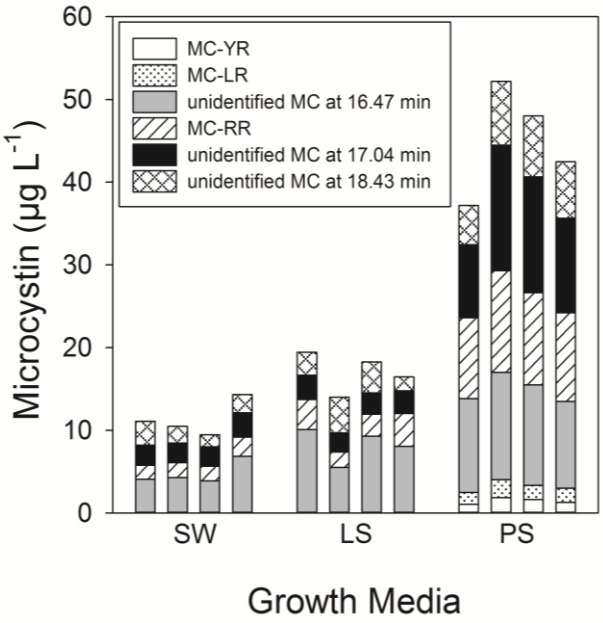

Figure S3-3. Concentrations of microcystin analogues in each *Microcystis* culture at the end of the experiment.

S4. Supporting Information for Chapter 4

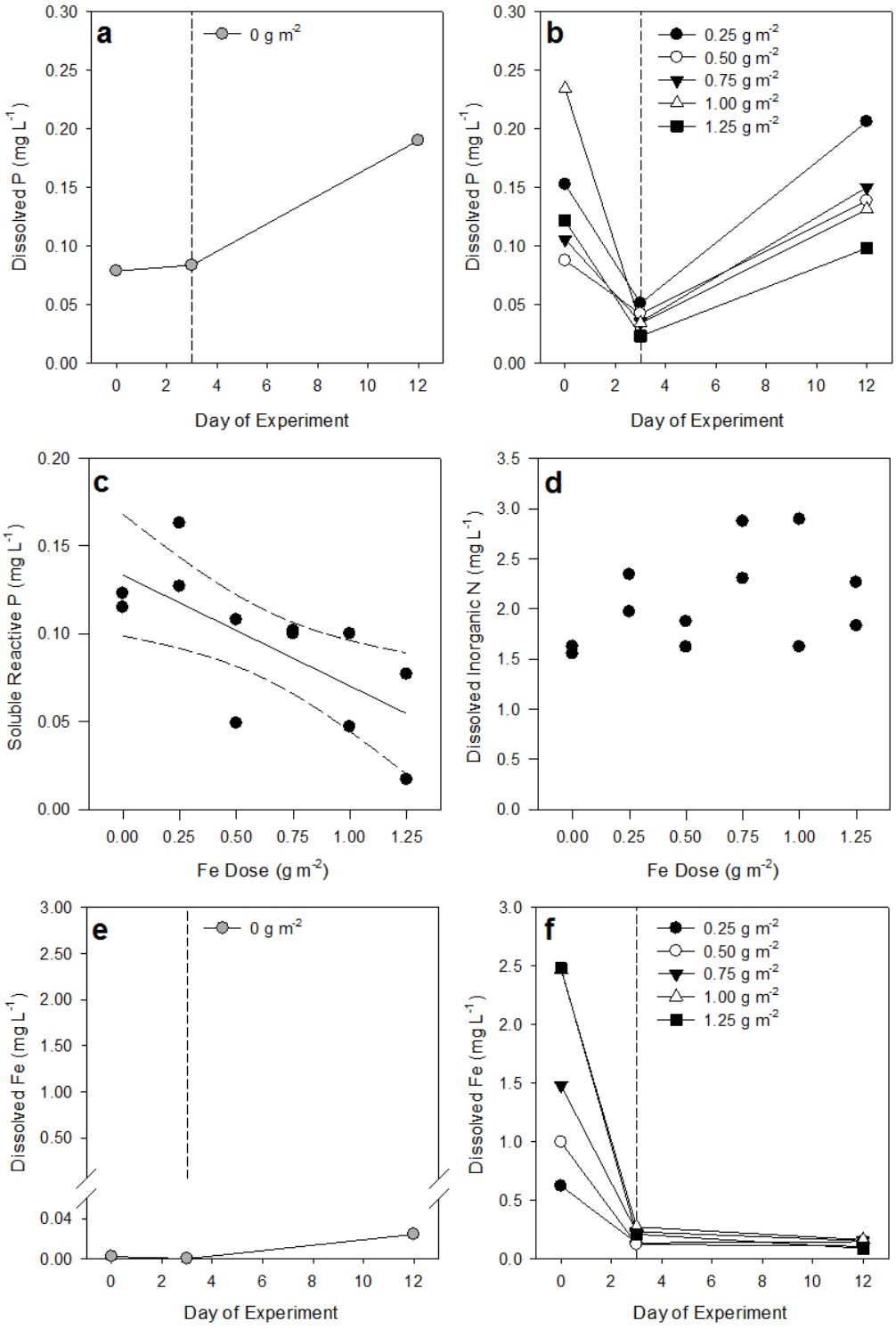

Figure S4-1. Results from Experiment 1. Temporal changes in dissolved phosphorus concentrations in control (a) and iron-treated sediments (b).

Relationships between iron dose and soluble reactive phosphorus (c) and dissolved inorganic nitrogen (d) at end of experiment. Temporal changes in iron concentrations in control (e) and iron-treated sediments (f).

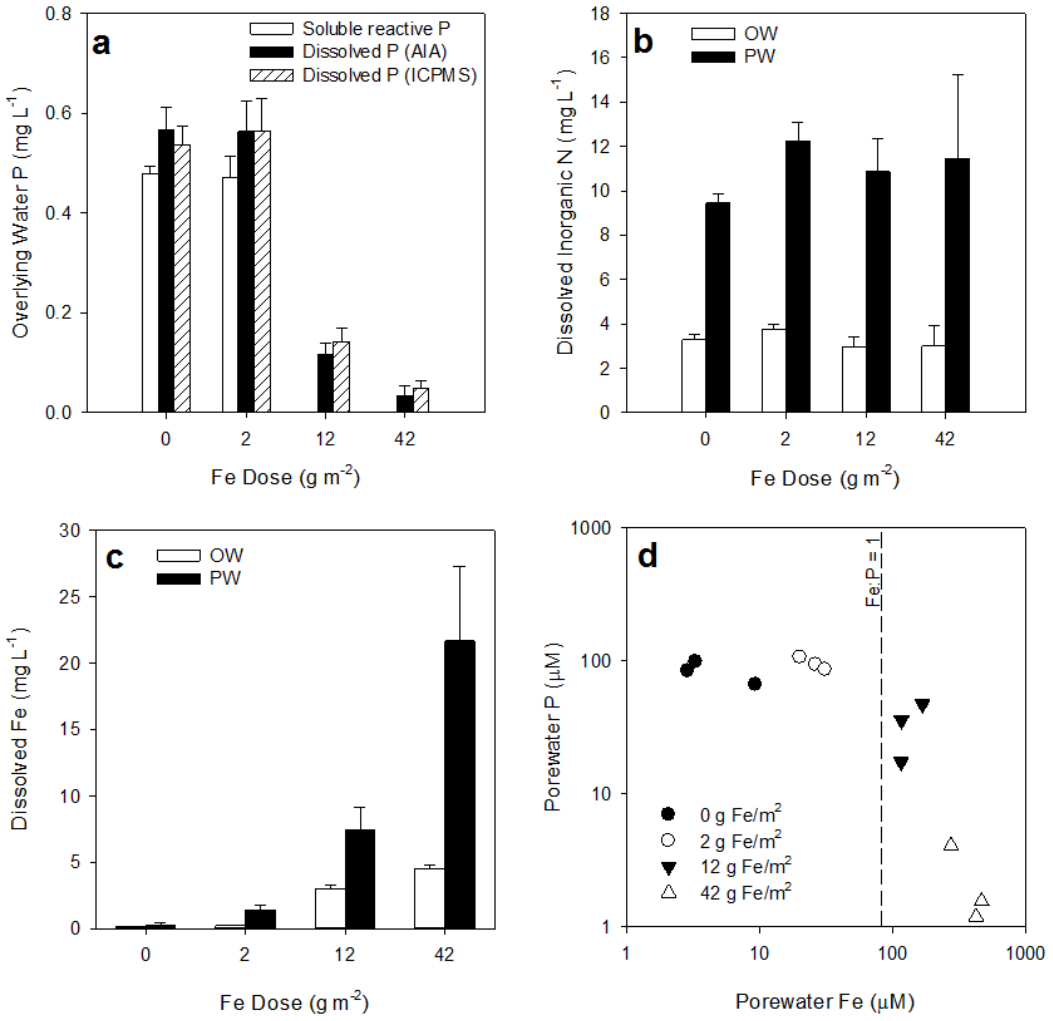

Figure S4-2. Results from Experiment 2. Average (±SD) concentrations of soluble reactive phosphorus and dissolved phosphorus, measured by automated ion analyzer (AIA) or inductively coupled plasma mass spectrometry (ICPMS), in overlying water (a). Concentrations of dissolved inorganic nitrogen (b) and dissolved iron (c) in overlying water (OW) and porewater (PW). Relationship between porewater iron and phosphorus, with dashed line indicating the theoretical Fe concentration required to achive a molar ratio of 1 at the start of the experiment (e).

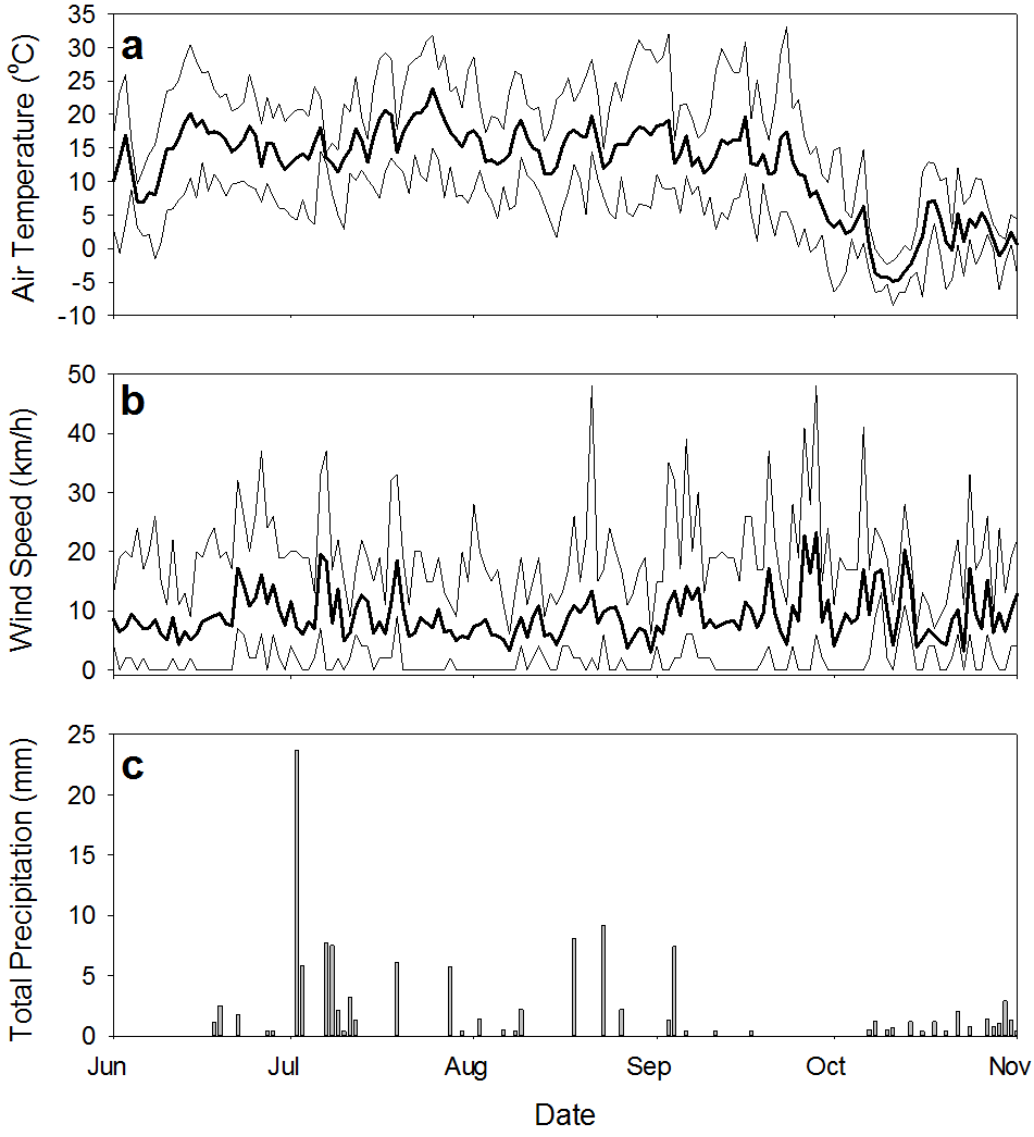

Figure S4-3. Minimum, average, and maximum daily temperature (a) and wind speed (b), and total daily precipitation (c) from June to November 2009 in Busby, Alberta (53°55'51" N, 113°55'18"W). Data obtained from the National Climate Data and Information Archive, Environment Canada [accessed 20 April 2012].

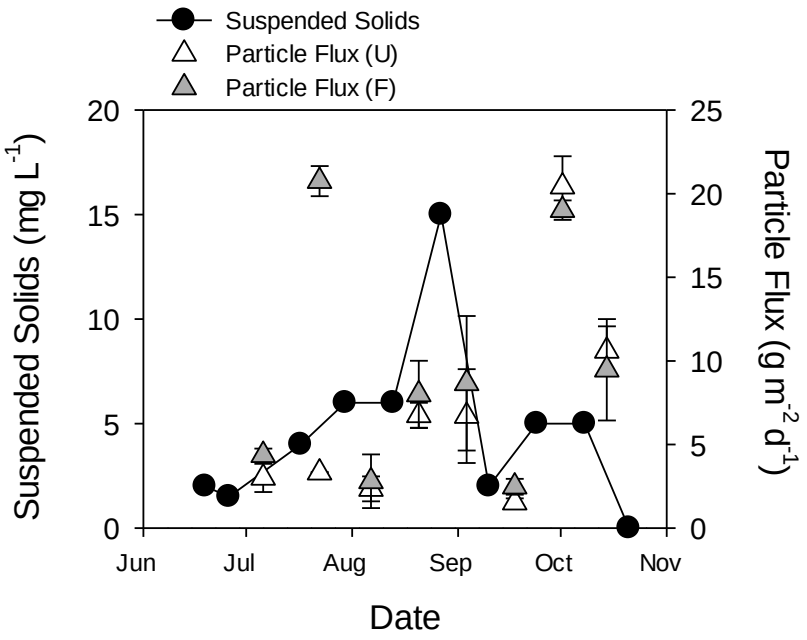

Figure S4-4. Concentration of total suspended solids in Nakamun Lake in 2009 (left axis), and mean (± SD, n = 3) particle sedimentation rates measured by unpoisoned (U) and formalin-poisoned (F) sedimentation traps (right axis).

S5. Supporting Information for Chapter 5

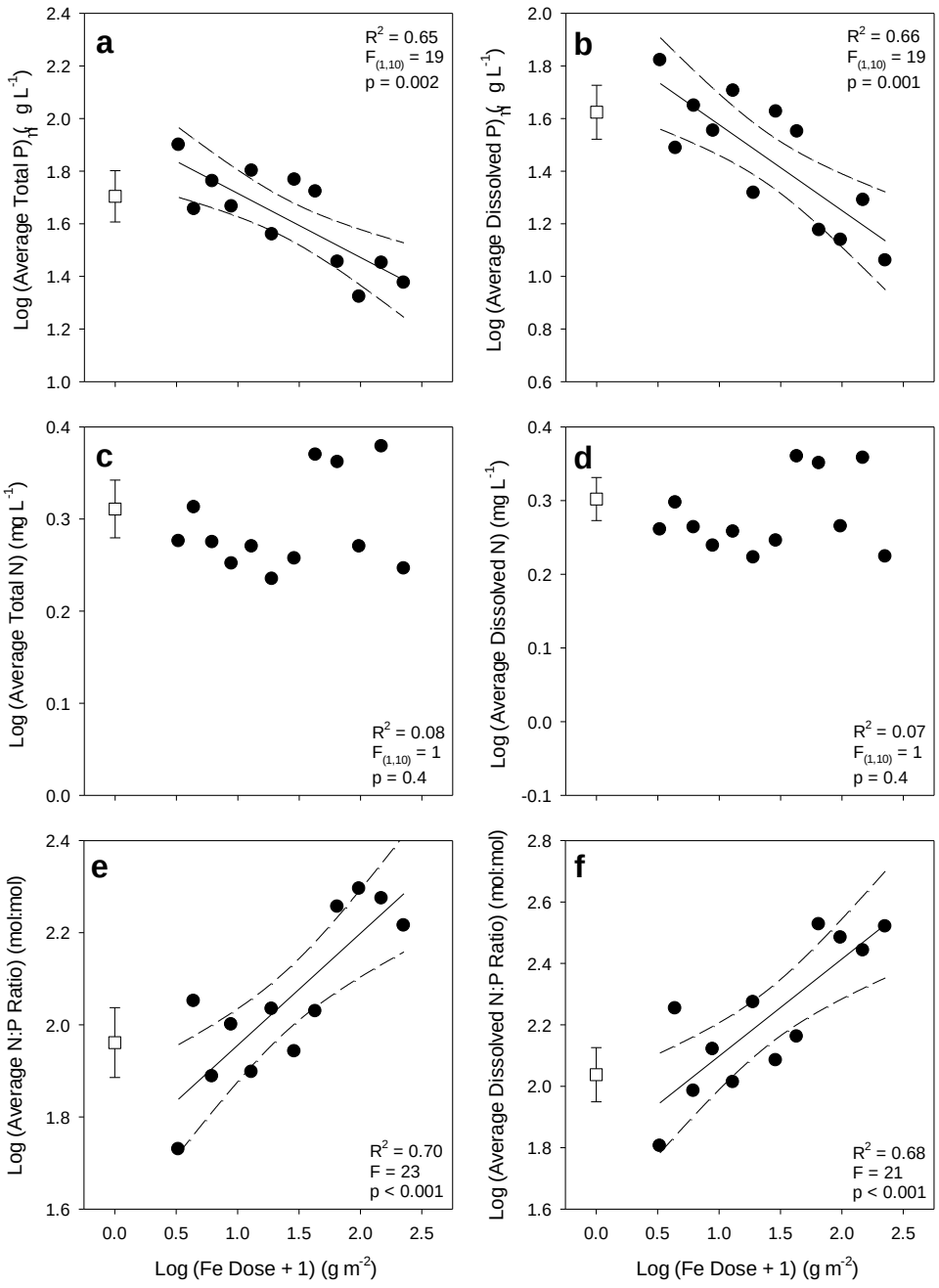

Figure S5-1. Relationships between iron dose applied to mesocosms and concentrations of total phosphorus (a), dissolved phosphorus (b), total nitrogen (c), and dissolved nitrogen (d), or ratios of total nitrogen to total phosphorus (e) and dissolved nitrogen to dissolved phosphorus (f). Each value represent the mean of measurements in individual mesocosms from January to March (n = 4). The line of best fit and 95% confidence intervals of the linear regression model are shown on each panel, along with associated test statistics. Shown for comparison is the mean (± SE) of three control mesocosms.

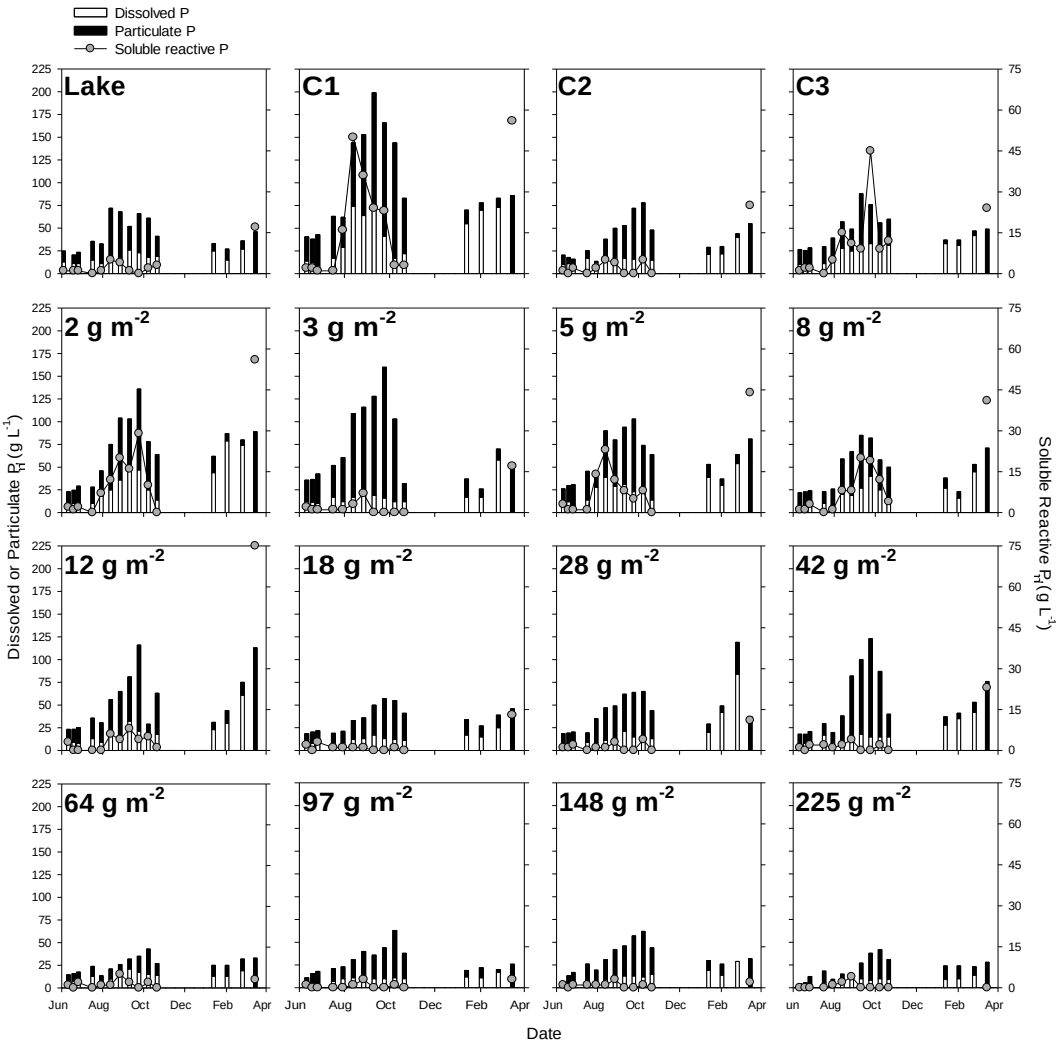

Figure S5-2. Concentrations of dissolved and particulate phosphorus (bars) and soluble reactive phosphorus (circles) over time in Nakamun Lake, control mesocoms (C1-C3), and iron-treated mesocosms (2 -225 g Fe m^{-2}).

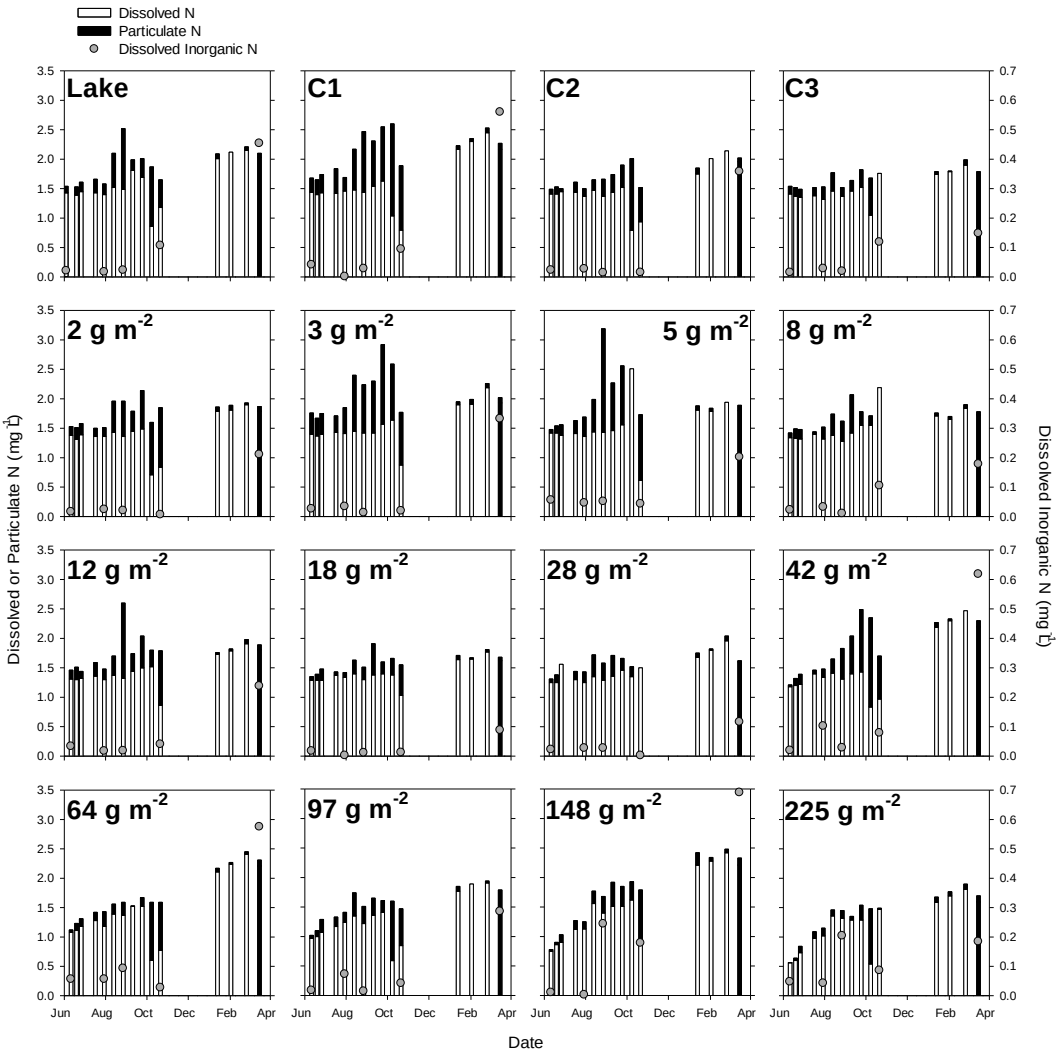

Figure S5-3. Concentrations of dissolved and particulate nitrogen (bars) and dissolved inorganic nitrogen (circles) over time in Nakamun Lake, control mesocoms (C1-C3), and iron-treated mesocosms (2 -225 g Fe m^{-2}).

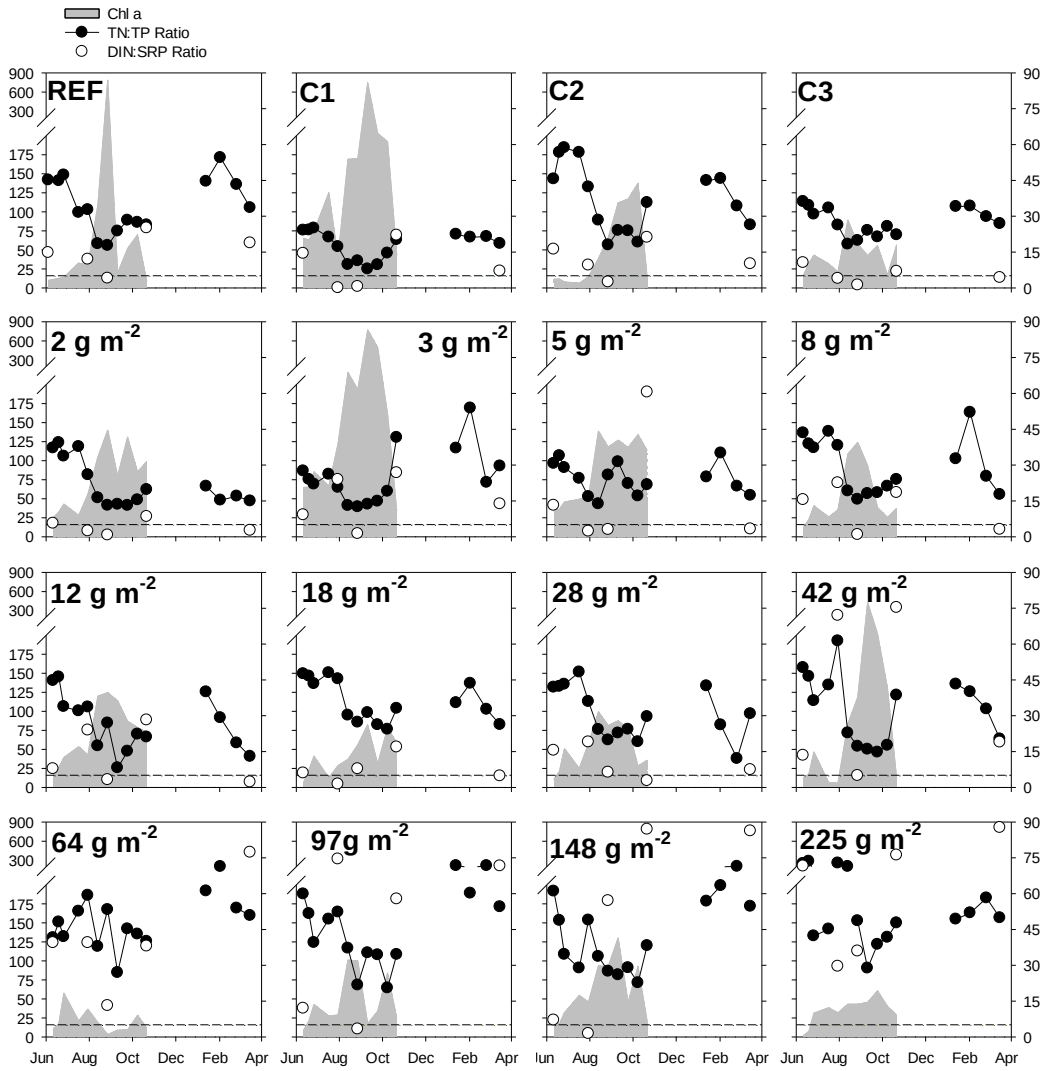

Figure S5-4. Ratios of total nitrogen to total phosphorus, ratios of dissolved inorganic nitrogen to soluble reactive phosphorus, and concentrations of chlorophyll *a* over time in Nakamun Lake, control mesocoms (C1-C3), and iron-treated mesocosms (2 -225 g Fe m^{-2}).

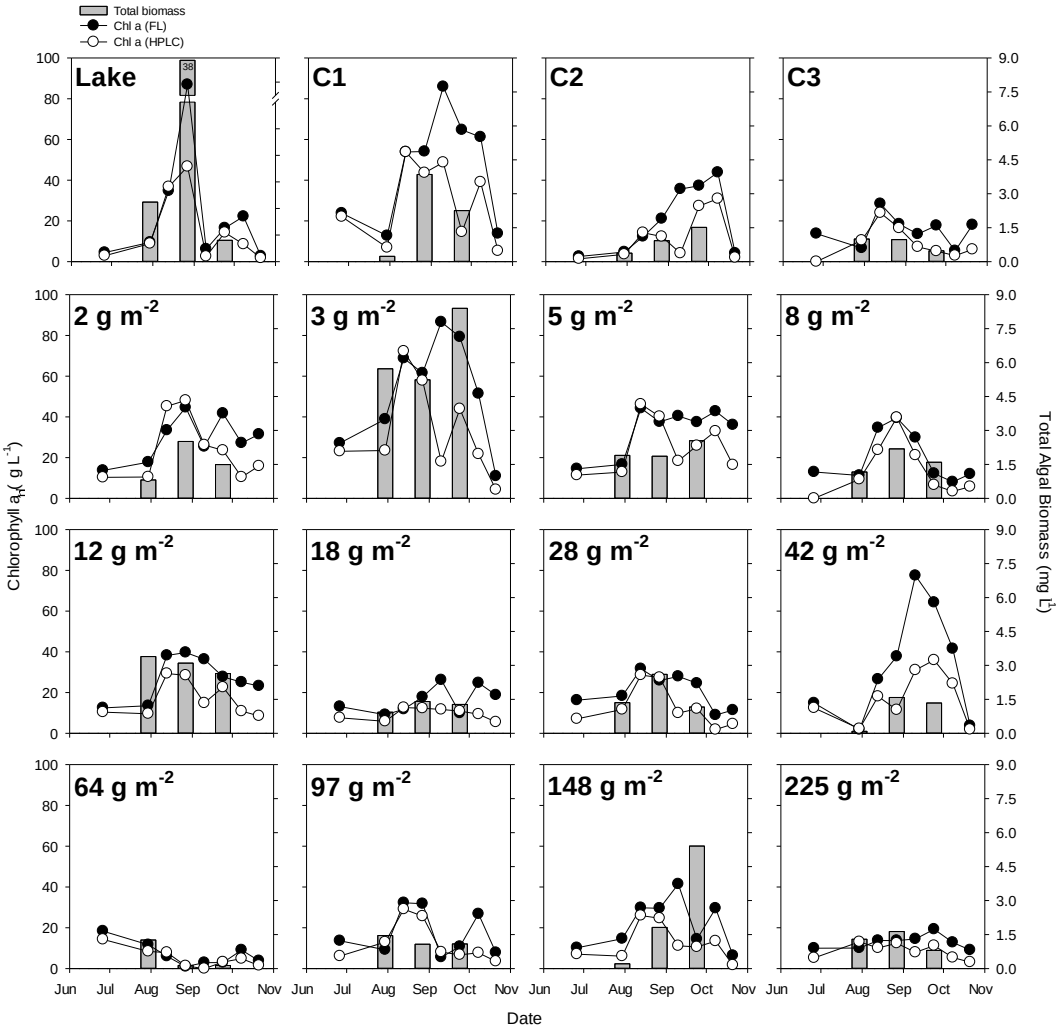

Figure S5-5. Concentrations of chlorophyll *a* (circles) and total algal biomass (bars) over time in Nakamun Lake, control mesocoms (C1-C3), and iron-treated mesocosms (2 -225 g Fe m^{-2}). Chlorophyll *a* was measured by high performance chromatorgraphy (HPLC) and standard fluorometry (FL), and total algal biomass was estimated from cell counts and biovolume measurements.

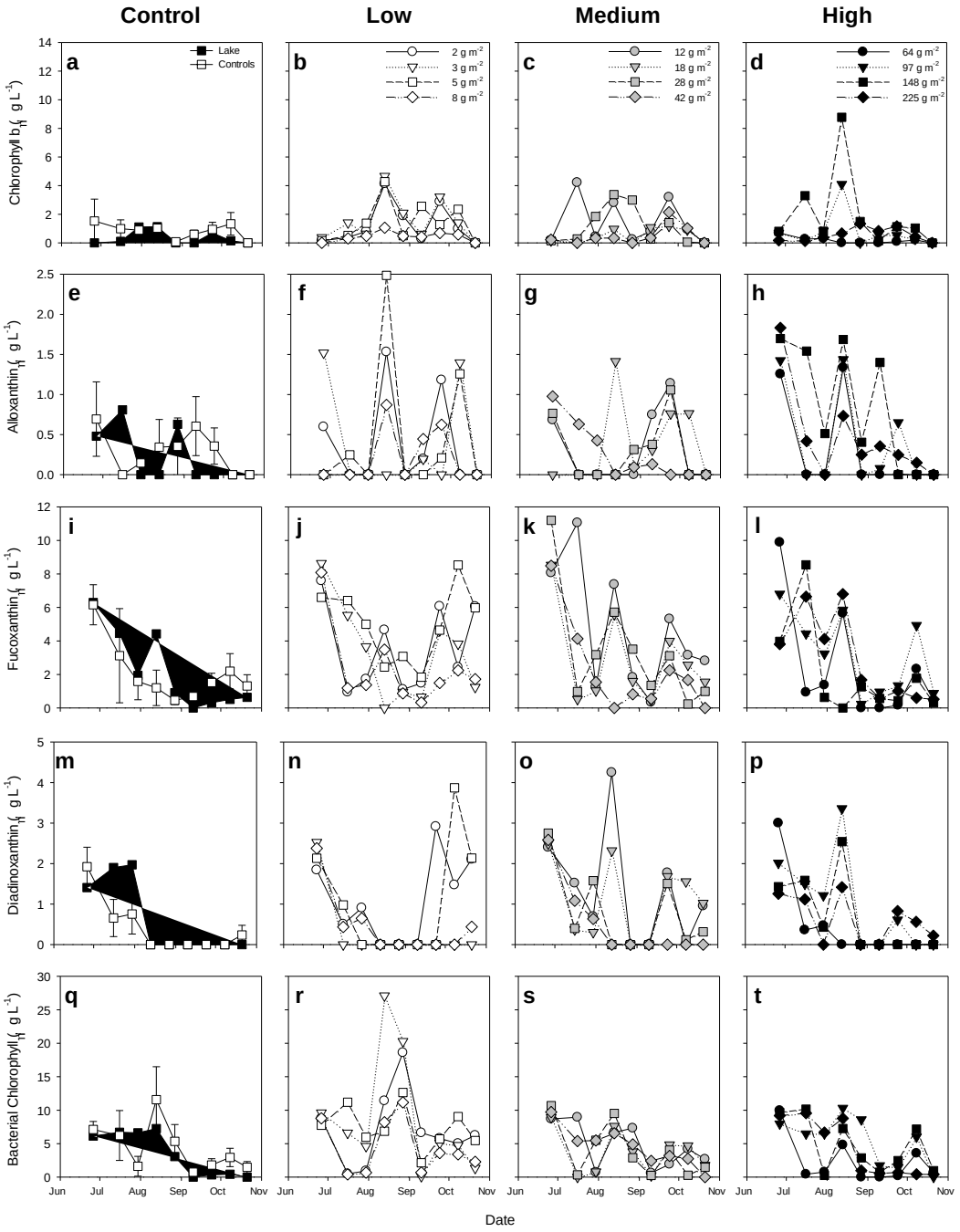

Figure S5-6. Concentrations of algal pigments over time in Nakamun Lake, control mesocosms (mean ± SE, n = 3), and twelve mesocosms treated with different doses of iron (2 – 225 g Fe m^{-2} sediment). Algal pigments are indicators of the biomass of various algal groups: chlorophyll b (a-d) is produced by chlorophytes; alloxanthin is produced cryptophytes (e-h); fucoxanthin (i-l) and diadinoxanthin (m-p) are produced by chrysophytes, diatoms, and dinoflagellates; and bacterial chlorophyll is produced by photosynthetic bacteria (q-t). Values represent integrated samples of the water column (0-4 cm) measured by high performance liquid chromatography.

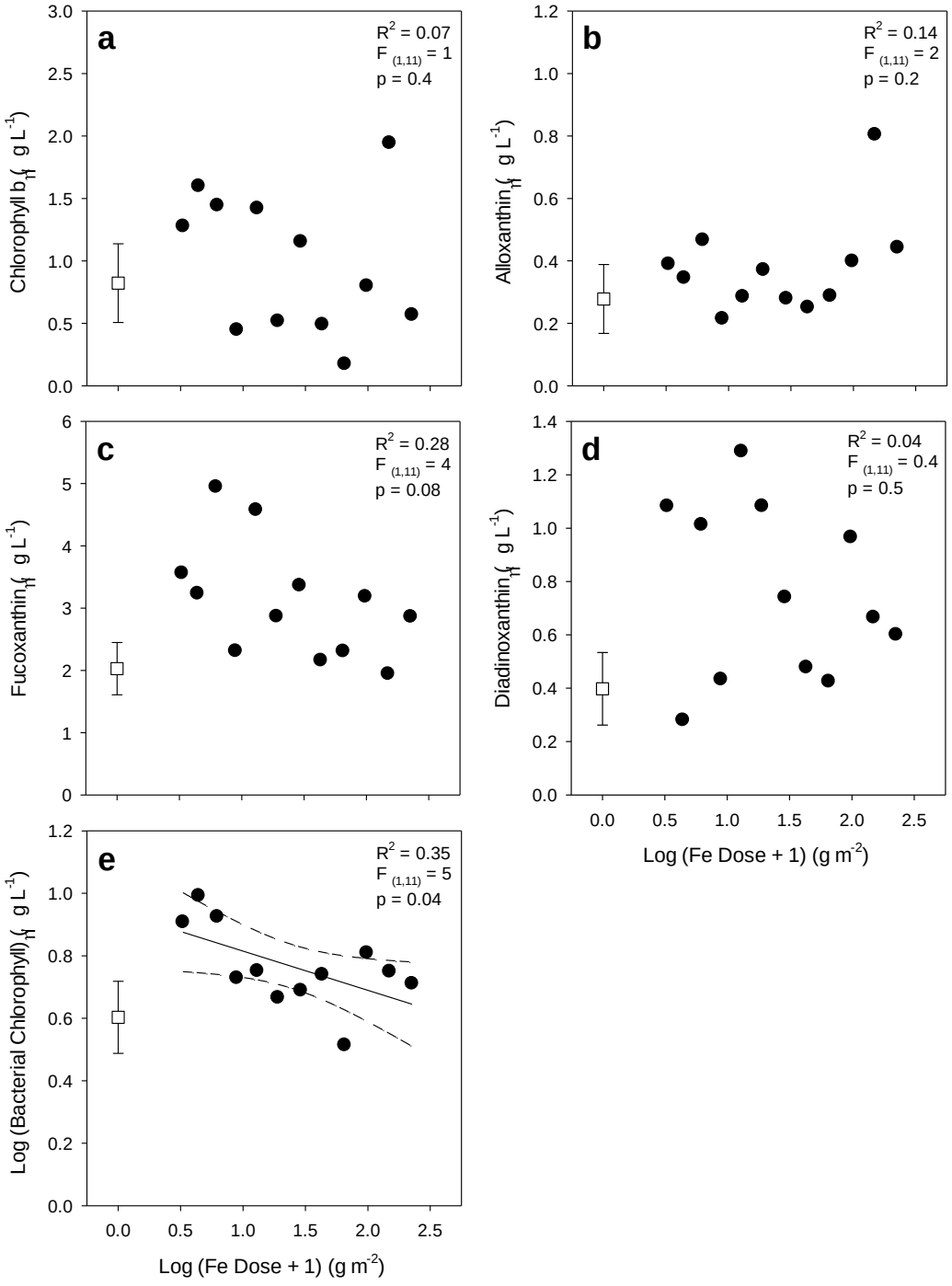

Figure S5-7. Relationships between iron dose applied to mesocosms and average concentrations of the algal pigments chlorophyll b (a), alloxanthin (b), fucoxanthin (c), diadinoxanthin (d), bacterial chlorophyll (e). Statistics as in Figure S5-1.

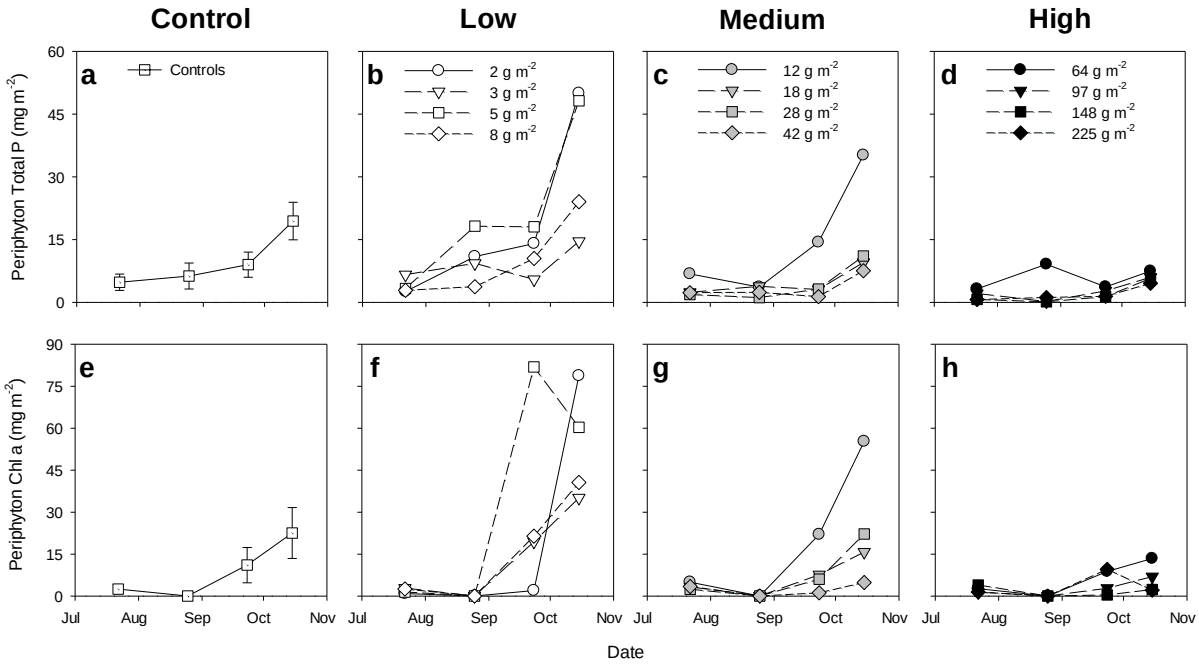

Figure S5-8. Concentrations of total phosphorus (a-d) and chlorophyll *a* (e-h) in periphyton on strips of wall material suspended in the control mesocosms (mean ± SE, n = 3) and twelve mesocosms treated with different doses of iron (2 – 225 g Fe m^{-2} sediment).

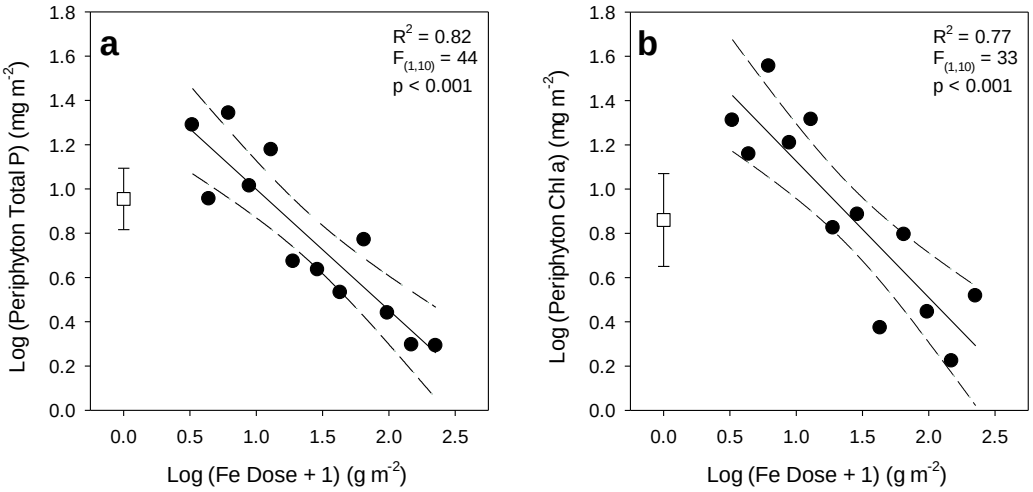

Figure S5-9. Relationships between iron dose applied to mesocosms and average concentrations of total phosphorus (a) and chlorophyll *a* (b) in periphyton colonizing the mesocom walls. Statistics as in Figure S5-1.

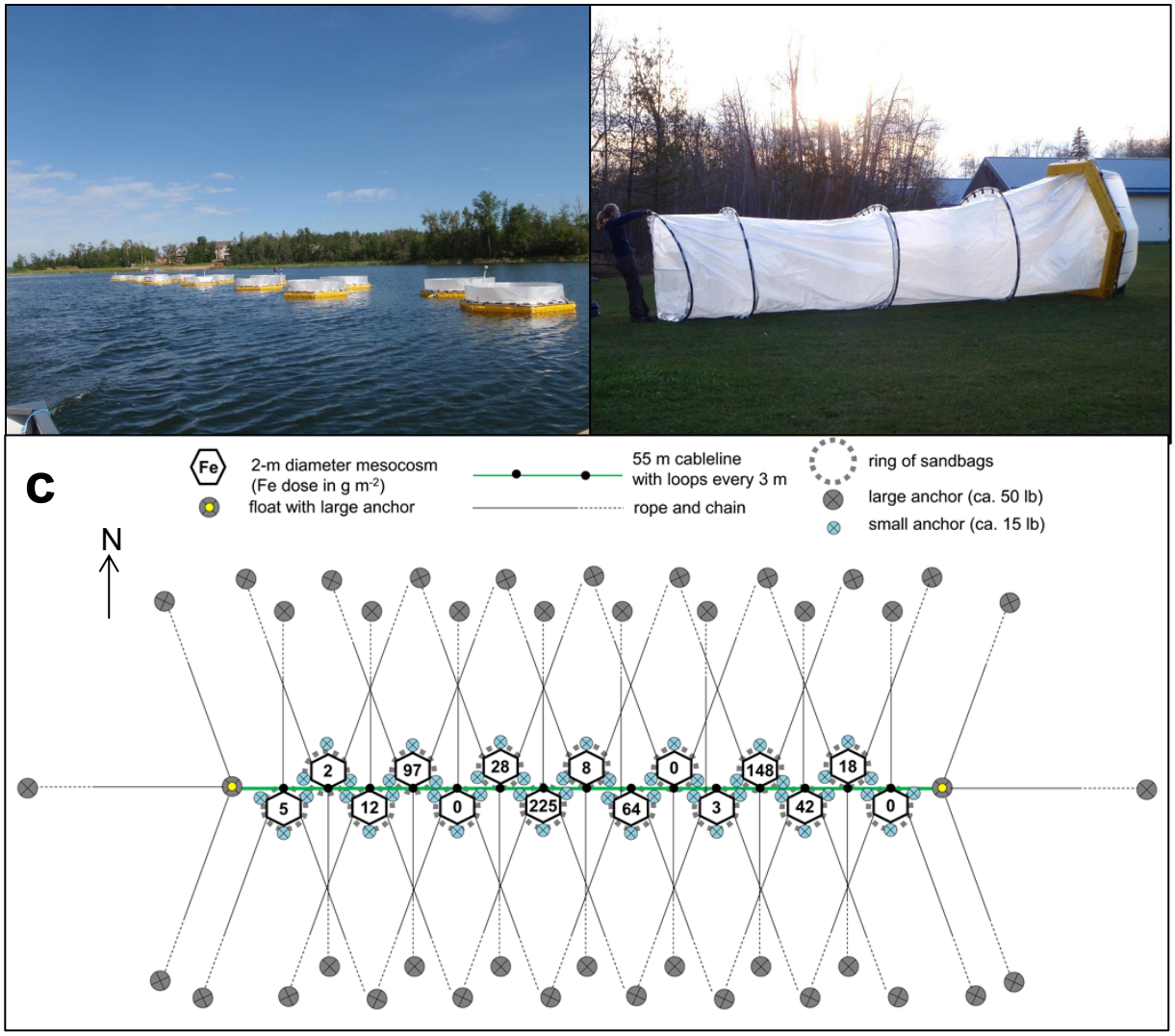

Figure S5-10. Photograph of mesocosms installed in Nakamun Lake (a), and a single mesocosm on shore (b). Graphic of mesocosm installation set-up and random assignment of iron doses (c).