Low cobalt limits cyanobacteria heterocyst frequency in culture but potential for cobalt limitation of frequency in nitrogen-limited surface waters is unclear

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Abstract

1. Impacts of three cobalt (Co) concentrations were examined on heterocyst frequency and growth rate in four diazotrophic cyanobacteria species in nitrogen (N)-depleted culture and growth rate in one non-diazotrophic species in N-replete culture. After 11 days in batch culture, heterocyst frequency (HF, % of all cells that are heterocysts) increased from 4.1-5.7% to 5.4-7.4% to 5.9-9.3% at 0.17, 17 and 170 nmol L^{-1} Co, implicating Co in heterocyst differentiation. Growth rate was not significantly affected by Co in any of the species suggesting that the impact of low Co on other metabolic pathways was minimized.
2. Stoichiometric extrapolation of culture results to N-limited natural systems with lower nutrient concentrations infers that HF could be limited by sub-nanomolar Co concentrations.
3. In experimentally fertilized N-limited Lake 227, mean summer HF in 2000-2020 was 3.4% (epilimnion) and 4.0% (metalimnion). However, in 2017 (the only year for which Co data are available) dissolved Co increased from 0.7 to 2.0 nmol L^{-1} during the bloom simultaneously with increasing HF and cyanobacteria biomass, hence, Co probably did not limit HF and biomass. HF was significantly higher after 2015 following a shift in dominant bloom species from *Aphanizomenon schindlerii* to smaller *A. skujae*. The smaller cell size may have required a higher HF in order to maintain a relatively constant supply rate of fixed N per unit biomass.
4. Surveys of ambient Co in over 280 aquatic systems across Canada and elsewhere indicate that Co is sometimes low enough to theoretically limit HF in N-limited waters. However, numerous variables influence HF so a clear understanding of relationships between Co and HF in natural systems remains elusive.

Keywords: heterocyst frequency, cyanobacteria, cobalt, Lake 227

1. INTRODUCTION

Although the productivity of most freshwaters waters is most often limited by nitrogen (N) and phosphorus (P), phytoplankton are occasionally limited by metabolically essential trace metals (iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), molybdenum (Mo), and cobalt (Co)) (Wurtsbaugh and Horne, 1982, 1983; Wurtsbaugh, 1988; Evans and Prepas, 1997; Twiss et al., 2000; Downs et al., 2008; Romero, et al., 2013; Schmidt, 2018; Facey et al., 2021, 2022; Halac et al., 2023). Many of these metal-limited systems are eutrophic, which increases biological demand for these elements, and tend to be located in relatively unurbanized watersheds with low levels of industrialization, presumably with low metal loading rates from anthropogenic sources. Low weathering rates may also result in low metal loading to aquatic ecosystems in very dry watersheds or watersheds subjected to long periods of sub-zero temperatures.

Metabolically essential trace metals are critical components for cells, acting as enzyme cofactors, electron transfer agents and protein structure stabilizers (Barton et al., 2007; Schoffman et al., 2016; Barber-Zucker et al., 2017; Andresen et al., 2018). Fe, Mn and Zn are used as cofactors by approximately 30% of all enzymes with Co, Mo, Ni and Cu less widely used (Ho et al., 2003; Foster et al., 2014; Reich et al., 2020). Hence, their availability and chemical speciation have the potential to influence microbial productivity as well as species composition and physiology. For example, low levels of Mo and Fe have the potential to exacerbate N limitation by limiting synthesis of the cofactor for nitrogenase, the N fixing enzyme (Howarth et al., 1988; Burgess, 1990).

Co, the focus of this study, is an essential nutrient for N-fixing cyanobacteria (Holm-Hansen et al., 1954). Cyanobacteria require inorganic Co to synthesize pseudocobalamin, a variant of the enzyme cofactor cobalamin (Helliwell et al., 2016). Co has been found to occasionally limit cyanobacteria productivity in freshwaters (Downs et al., 2008; Facey et al., 2022) even though cyanobacteria cellular Co content is relatively low (Hawco et al., 2020).

While Co is an essential micronutrient for filamentous cyanobacteria (Holm-Hansen et al., 1954), studies have not demonstrated that it is directly involved in N fixation or the processing of its end products, H_2 and superoxide (O_2^-) although other metals are involved, e.g., Fe, Mo and Ni (Burgess, 1990; Ho, 2013; Ogata et al., 2016; Søndergaard et al., 2016). However, evidence appears to suggest that low Co can limit heterocyst frequency (percentage of all cells in filaments that are heterocysts, also called heterocytes) which implies at least an indirect role in N fixation (Kelly et al., 2021) such as heterocyst differentiation. Heterocysts, the site of N-fixation in filamentous cyanobacteria species in the order Nostocales, are

specialized cells derived from vegetative cells with thick walls and high respiration rates designed to minimize O_2 deactivation of the N-fixing enzyme, nitrogenase (Kangatharalingam et al.,1992). Heterocyst differentiation which is a complex multi-step (and multi-enzyme) process (Zhao and Wolk, 2006; Kumar et al., 2010; Videau et al., 2016; Xu et al., 2020; Harish and Seth, 2020). If Co is involved in heterocyst differentiation, low Co could limit N fixation in heterocystous species through resource limitation of the differentiation process.

There are a few studies of heterocyst frequency in surface waters, estimates vary widely and our understanding of heterocyst frequency regulation in natural systems is poor aside from the effect of inorganic N in suppressing heterocyst formation (Cmiech et al., 1984; Riddolls, 1985; Anagnostidis et al., 1988; Wood et al., 2010; Zakrisson and Larsson, 2014). Thus, we do not have a good understanding of how heterocyst frequency is regulated at the ecosystem level or the consequences for N fixation rate in N-limited systems although some modeling progress on heterocyst frequency has been made (Brown and Rutenberg, 2012). The objectives of this study were to determine (1) whether heterocyst differentiation is dependent on the availability of inorganic Co in cyanobacteria cultures, and (2) ambient Co concentrations and thus the potential for Co limitation of heterocyst frequency in Canadian freshwaters. Heterocyst frequencies of several species of freshwater cyanobacteria were measured in N-deficient laboratory cultures in three Co concentrations and the results were compared to a detailed 20-year data set of heterocyst and vegetative cell abundance and biovolumes in experimentally eutrophic Lake 227 and surveys of dissolved Co concentrations in lakes and reservoirs across Canada.

2. MATERIALS AND METHODS

2.1 Cyanobacteria culture experiment

Cultures of the cyanobacteria *Dolichospermum flos-aqaue* (CPCC67), *Aphanizomenon flos-aqaue* (NIES 81), *Aphanizomenon skujae* (isolated from Lake 227) and *Dolichospermum lemmermanii* (isolated from Lake Erie) were grown in $BG11_0$ media (BG11 without inorganic N) containing an equivalent amount of $FeCl_3$ instead of ferric ammonium citrate (Rippka et al.,1979). A culture of the non-N fixing cyanobacteria *Microcystis aeruginosa* (PCC7005) was grown in BG11 with $NaNO_3$ as a reference species. The phosphorus concentration in BG11 and $BG11_0$ was 172 µmol L^{-1} (5.33 mg L^{-1}).

Cultures were grown at 20°C on a 12:12 hr light/dark cycle at 100 µmol m^{-2} s^{-1}. All reagents used were trace metal grade, and all flasks and bottles were soaked in 10% HCl over

48 hours and then in deionized water (Milli-Q) for another 24 hours. Only acid-washed clear pipette tips were used throughout this experiment. All media, glassware and supplies were UV sterilized under a laminar flow hood for 15 minutes.

1 mL of exponentially growing cells from starter cultures was transferred to duplicate plastic tubes of $BG11_0$ or BG11 containing three concentrations of inorganic Co added as $CoSO_4$ spanning three orders of magnitude, 0.17, 17 or 170 nmol L^{-1}, and incubated for 11 days. Each treatment is referred to by its nominal concentration, i.e., the total Co added, regardless of speciation and phase which changes with time. Before inoculation, all of the Co was dissolved and primarily complexed to an organic chelator (EDTA) which would have partitioned into particulate (cellular) and perhaps colloidal phases as cultures grew.

Biomass was assayed as absorbance at 750 nm (A_{750}) using a Cary 100 spectrophotometer. At 750 nm, interference from photosynthetic pigments is minimal and can be used as a proxy for population biomass (Chioccioli et al., 2014). Vegetative cells and heterocysts were counted on the 11^{th} day in late exponential/early stationary phase using a haemocytometer under the microscope at 40X magnification. Heterocysts were stained with alcian blue (0.015% weight/volume) for 10 minutes (Maldener et al., 2003). A minimum of five squares of the haemocytometer field were counted for each culture tube. Heterocyst frequency was calculated by dividing the number of heterocysts by the total number of heterocyst and vegetative cells.

The R package *growthcurver* (version 0.3.0) was used to estimate the growth rate of each sample (Sprouffske and Wagner, 2016) by finding the best fit of a given dataset to the logistic growth equation (Eq. 1),

$$N_t = \frac{K}{1+\left(\frac{K-N_0}{N_0}\right)e^{-rt}} \qquad (1)$$

where N_t is the A_{750} at a given time, K is the carrying capacity (maximum cell biomass), N_0 is the initial A_{750}, t is time and r is the growth rate that would occur if there were no restrictions imposed on total population size (Sprouffske and Wagner, 2016). We interpret this to mean that r is the maximum instantaneous growth rate which is located to the right of the inflection point in the N_t versus time curve. Statistical differences between mean heterocyst frequencies were determined with two-way ANOVA followed by Tukey's HSD. Growth rate was also calculated as the slope of $\ln(A_{750})$ versus time during the linear phase of the semi-logarithmic curve (μ_{sl}) and thus represents an averaged value for a multi-day period rather than an

instantaneous growth rate like r.

2.2 Lake 227 heterocyst enumeration

Lake 227 is a small (5 ha, mean depth 4.4 m, maximum depth 10 m), headwater lake located at the IISD-Experimental Lakes Area in northwestern Ontario, Canada. The lake is dimictic, with thermal stratification in the summer occurring at 1-3 m. Lake 227 was fertilized with N and P (27:1 molar N:P) from 1969 to 1974, with reduced N loading from 1975 to 1989 (9:1 molar N:P) and with only P from 1990 to present (Findlay et al., 1994; Molot et al., 2010; Higgins et al., 2018). A bloom of N-fixing cyanobacteria *Aphanizomenon* typically occurred in early summer of each year since 1990, lasting about one month (Schindler et al., 2008; Higgins et al., 2018).

Phytoplankton in integrated epilimnetic and metalimnetic samples were enumerated by the same person via microscopy during the ice-free seasons in 2000-2021 for cell counts and cell sizes at the species level allowing estimates of population abundance as cell density and biovolume (Findlay and Kasian, 1987). Biovolumes were converted to biomass wet weight by assuming a cell density of 1 g mL^{-1}. Heterocyst frequency was calculated as the ratio of heterocyst cells/(heterocyst cells + vegetative cells) of Nostocales species and expressed as a percentage.

Surveys of dissolved Co in Canadian freshwaters

Water samples were collected in 2017 from 40 lakes in five provinces (New Brunswick, Quebec, Ontario, Manitoba, and Saskatchewan) ranging in size from 5 ha (Lake 227 in northwestern Ontario) to 24,514 km^2 (Lake Winnipeg in Manitoba). Site locations, morphometry and basic water quality are presented in Table S1 with references and links to watershed geology in Table S2. Epilimnetic samples were collected according to each research group's sampling protocols. While collection methods differed, all groups used vials, syringes and syringe filters provided by York University, Toronto, Ontario and all samples were analyzed at the Trent Water Quality Centre in Peterborough, Ontario. Vials and syringes were acid-washed in 10% trace metal grade HCl before shipping to participants. Plastic syringes were used to collect 20 mL from well-shaken samples. A syringe filter cartridge (0.45 µm cellulose acetate with GF pre-filter, Sartorius Minisart) was placed on the end of the syringe, 5 mL were discarded and the remaining 15 mL were filtered into a 15 mL Falcon polypropylene vial. Vials were labeled with date, lake name, depth, and 'filtered' and shipped to York University where they were acidified to pH < 2 with concentrated trace

metal grade nitric acid. Dissolved Co in this survey is operationally defined as Co passing through a 0.45 µm filter pore size. All samples were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS). Each sample run consisted of 3 repeated measurements and each repeat consisted of 25 measurements (0.1 sec dwell time). Hence, the overall mean value for each sample was based on 75 individual measurements of each isotope peak. The Co detection limit was 0.017 nmol L^{-1} (0.001 µg L^{-1}).

Co data from two other projects were provided to the authors for this study. Sampling and analytical methods for surveys of 94 lakes conducted by the Northwest Territories Geological Survey in 2012 and 2014 (Palmer et al., 2015) and nine lakes in central Ontario conducted by the Ontario Ministry of Environment, Conservation and Parks (MECP) between 2010 and 2017 (unpublished data) are presented in Appendix 1.

3. RESULTS

3.1 Batch cultures

Co concentration did not significantly affect instantaneous (r) or semi-logarithmic growth rates (μ_{sl}) of the five cyanobacterial species examined in batch culture (ANOVA $p > 5\%$) with the exception of *Aphanizomenon skujae* which had significantly higher μ_{sl} at 0.17 nmol L^{-1} at the 1% level (Figure 1, Table 1). Semi-logarithmic growth rates (μ_{sl}) were consistently lower than instantaneous logistic growth rates (r) which is not surprising since r is the slope of the population size versus time curve at one point in time, probably after the inflection point where the maximum slope typically occurs, and μ_{sl} is the slope over several days. Growth rates at 0.17 nmol L^{-1} were higher than growth rates at 17 and 170 nmol Co L^{-1} for three of the five species, suggesting that 0.17 nmol L^{-1} was not growth-limiting and, therefore, the concentration thresholds for membrane transport and Monod growth (i.e., maximum Co concentrations where transport and growth rates are zero) were much lower than 0.17 nmol L^{-1}.

All four filamentous cyanobacterial species showed increasing heterocyst frequency with increasing Co concentration (Figure 2). Heterocyst frequencies ranged from 4.1 to 9.3% with lowest frequencies observed at 0.17 nmol L^{-1} Co (4.1-5.7%, mean 4.6%), intermediate frequencies at 17 nmol L^{-1} (5.4-7.4%, mean 6.4%) and highest frequencies at 170 nmol L^{-1} (5.9-9.3%, mean 7.4%). A post-hoc test showed that heterocyst frequencies at 0.17 nmol L^{-1} were significantly lower than frequencies at 170 nmol L^{-1} in three of the four heterocystous species and significantly lower than the frequencies in two of these four species at 17 nmol L^{-1}. Hence, the lowest Co treatment may have limited heterocyst differentiation even though the

concentration did not limit growth over the 11 days of the experiment.

These limiting Co concentrations cannot be directly extrapolated to other systems without applying a stoichiometric correction because of the high concentrations of other elements in BG11 media. If we assume that P limits growth in diluted BG11 rather than light, then we can use the P/Co molar ratios in the three experimental treatments (10^6, 10^4 and 10^3) to estimate limiting concentrations of Co at lower P. In a system with a total P concentration of, say, 1.61 µmol L^{-1} (50 µg L^{-1}), the three Co treatments would be equivalent to 0.0016, 0.16, and 1.61 nmol L^{-1}. This suggests that sub-nanomolar concentrations of dissolved Co could potentially limit heterocyst frequency in N-limited eutrophic waters.

3.2 Lake 227

In Lake 227, the mean (± standard deviation) annual summer (June-September) heterocyst frequencies in the epilimnion and metalimnion (mainly *Aphanizomenon* with some *Dolichospermum*) were not significantly different during 2000-2020, 4.0 ± 1.4% in the epilimnion and 3.4 ± 1.1% in the metalimnion (t-test, $p = 0.12$). Annual summer frequencies exceeded 6% in two of the years and only in the epilimnion (Figure 3). These mean values are similar to the values observed at the lowest Co of 0.17 nmol L^{-1} in the cultures.

However, some differences over time were noted. *Aphanizomenon schindlerii* was the dominant species from 2002-2012, shifting to *Aphanizomenon skujae* from 2015-2020. While individual *A. skujae* cell and heterocyst biovolumes were smaller than *A. schindlerii*, total heterocyst biovolume remained relatively unchanged between the two time periods due to an increase in heterocyst frequency (Table 2). In the metalimnion, the mean annual summer heterocyst frequency increased from 3.0% in 2002-2012 to 4.8% in 2015-2020, a large and significant increase of 60%. While the differences in mean values for June-September are statistically significant, caution is warranted given the size of errors that are sometimes associated with phytoplankton sampling and manual enumeration even though samples were enumerated by the same analyst for the entire study period (Kutkuiin, 1958; Irish and Clarke, 1984).

The increase in mean heterocyst frequency was not associated with significant differences (Student t-test at the 1% level) in ammonia, calcium or temperature between the two periods. Metalimnetic data are too sparse to estimate long term means, however, mean epilimnetic values from May to September for ammonia were 17.5 ± 5.0 µg L^{-1} in 2002-2012 and 12.4 ± 4.6 µg L^{-1} in 2015-2020 ($p = 0.062$), and calcium were 1.6 ± 0.2 mg L^{-1} in 2002-

2012 and 1.5 ± 0.1 mg L^{-1} in 2015-2020 (p = 0.031). The mean temperature at 2 m from May to September was 16.9 ± 1.2°C in 2002-2012 and 16.1 ± 1.4°C in 2016-2019 (p = 0.29). The higher heterocyst frequency associated with *A. skujae* after 2015 could have been influenced by the lower ammonia concentration.

In 2017, the only year in which Co was measured in Lake 227, dissolved Co ranged from 0.7 to 4.0 nmol L^{-1} with a mean and standard deviation of 2.2 ± 0.9 nmol L^{-1} in the top 3 m and was less than 1 nmol L^{-1} until late June. Co was not correlated with heterocyst frequency. Heterocyst frequency paralleled changes in cyanobacteria biomass during the bloom period in 2017 with the timing of maximum heterocyst frequency corresponding to peak cyanobacteria biomass (Figure 4). Heterocyst frequency may not have been Co-limited since a buildup rather than a drawdown of dissolved Co occurred during the cyanobacteria bloom with epilimnetic and metalimnetic Co increasing 3x from 0.7 to 2.0 nmol L^{-1} and 0.8 to 2.1 nmol L^{-1}, respectively, coincident with increases in heterocyst frequency and biomass (Figure 4). The increasing heterocyst frequency during the exponential growth phase suggests that heterocysts were synthesized as needed to meet an accelerating cellular demand for N. Ammonia was low, ranged from 1 to 5 µg L^{-1} from the end of May to late September except for one sample in the metalimnion (Figure 4).

3.3 Surveys of cobalt in Canadian freshwaters

A list of Canadian Co surveys is presented in Table 3 with some pertinent characteristics. The wide range in detection limits reported by accredited laboratories (three orders of magnitude) is attributable to differences in methods and equipment used over the last 40 years. For example, the detection limits were 17 nmol L^{-1} (1 µg L^{-1}) in the Canadian Arctic Archipelago survey (Michelutti et al., 2002a, 2002b; Antoniades et al., 2003a, 2003b), 0.7 nmol L^{-1} (0.01 µg L^{-1}) in the Laurentian Great Lakes (probable detection limit since it was not explicitly stated in the paper) (Rossmann and Barres, 1988) and 0.017 nmol L^{-1} (0.001 µg L^{-1}) in the detection limit in the 2017 survey.

In the 2017 cross-Canada survey, dissolved Co samples ranged from 0.03 to 11.5 nmol L^{-1} in the epilimnia of 40 lakes in Manitoba, Ontario, Quebec, and New Brunswick (n = 167 samples) with all samples above the detection limit of 0.017 nmol L^{-1}). Mean lake concentrations were below 4 nmol L^{-1} in 37 of the lakes (Figure 5). Co was highest in three eutrophic, N-limited lakes in the Qu'Appelle River system in Saskatchewan, ranging from 5.1 to 11.5 nmol L^{-1} (Hall et al., 1999). Co in Hamilton Harbour, Ontario, the most industrialized watershed, was 3.2 nmol L^{-1}, a value similar to several other lakes and

reservoirs in Ontario and Saskatchewan. For reference, the mean (± standard deviation) Co concentration in 16 blanks (syringe filtered deionized water samples) was 0.069 ± 0.112 nmol L^{-1}.

The majority of samples (88 of 115) from 94 lakes in the Yellowknife region of the Northwest Territories in 2012 and 2014 had dissolved Co concentrations that were at or below their detection limit of 1.7 nmol L^{-1} (0.1 µg L^{-1}) (Palmer et al., 2015). The remainder (23%) were 3.4 or 5.1 nmol L^{-1} (0.2 or 0.3 µg L^{-1}; results were reported in 0.1 increments).

Total Co in most of the samples in 161 High Arctic lakes and ponds were below that study's relatively high detection limit of 17 nM (1 µg L^{-1}). Michelutti et al. (2002a, 2002b) did not present details other than to report that more than 50% of the samples were below the detection limit. Antoniades et al. (2003a, 2003b) reported that about 2/3 of the 73 samples in their study were below their detection limit of 17 nmol L^{-1} but the high detection limit precludes knowing if Co was in the sub-nanomolar range relevant to limitation of heterocyst frequency. The remainder of the samples, 21 of 73, had total Co concentrations that were either 34, 51 or 68 nmol L^{-1} (i.e., 2, 3 or 4 µg L^{-1}, results were reported in 1 µg L^{-1} increments). A large majority of the 103 ponds and lakes sampled for nutrients by Michelutti et al. (2002a, 2002b) and Antoniades et al. (2003b) had TN/TP ratios > 23 by weight, suggesting they were P limited while only four the sites were potentially N limited with TN/TP < 9 by weight (Guildford and Hecky, 2000). In contrast, 9 of the 25 sites on Ellef Ringnes Island, all ponds, had TN/TP ratios < 9 (Antoniades et al., 2003a). However, none of these potentially N-limited systems would have been warm enough to support growth of pelagic cyanobacteria.

Co concentrations in 97% of coarse filtered (80 µm mesh), settled epilimnetic samples from eight thermally stratified lakes in central Ontario between 2010 and 2017 in the MECP study (414 samples) were less than or equal to their detection limit of 1.2 nmol L^{-1}. Hence, there appears to be some potential for Co limitation of heterocyst frequency although these lakes are not N-limited.

Median dissolved Co concentrations in near surface waters (1 m depth) in the Laurentian Great Lakes ranged from 0.1 nmol L^{-1} in Lake Superior to 0.4, 0.8 and 1.5 nmol L^{-1} in Lake Ontario, Lake Michigan and Lake Erie, respectively, with 73%, 0%, 0% and 9% of the dissolved Co samples below the detection limit which was probably < 0.4 nmol L^{-1} (the detection limit was inferred from the Lake Ontario median value in 1985) in these lakes, respectively, between 1981 and 1985 (Rossman and Barres, 1988). Hence, there appears to be some potential for Co limitation of heterocyst frequency in the Great Lakes although N-

limitation of the pelagic zones is not widespread, appearing episodically in some locations in the western basin in Lake Erie (Chaffin et al., 2013).

4. Discussion

4.1 Does Co limit heterocyst frequency?

Our batch culture study revealed that heterocyst differentiation was dependent on Co concentration. Heterocyst frequency was limited after 11 days of incubation with Co $\leq$ 17 nmol L^{-1}. Mean heterocyst frequency increased 39% when Co increased from low (0.17 nmol L^{-1}) to intermediate concentration (17 nmol L^{-1}) and the frequency increased 61% when Co increased from low to high concentration (170 nmol L^{-1}).

The impact of low Co on heterocyst frequency implicates Co in heterocyst differentiation, perhaps by limiting production of an unknown Co cofactor required in the multi-step cell differentiation pathway (Zhao and Wolk, 2006; Kumar et al., 2010; Videau et al., 2016; Xu et al., 2020; Harish and Seth, 2020). Interestingly, Co deficiency limits nodule formation and N fixation by symbiotic N-fixing bacteria in legumes and non-leguminous nodular plant roots (Hallsworth et al., 1960; Iswaran and Rao, 1964; Hewitt and Bond, 1966; Dilworth et al., 1979; Riley and Dilworth 1985; Jayakumar et al., 2008). Multicellular nodules in plant roots are analogous to one-celled heterocysts in that both structures are designed to house low O_2 environments to protect the N-fixing enzyme, nitrogenase (Guinel 2009a, 2009b).

The limitation of heterocyst frequency by lower Co in batch culture was not accompanied by a limitation of growth rate. This decoupling suggests that Co was essential to heterocyst differentiation but was not as necessary for other metabolic processes, perhaps due to substitution of Co by other metals, as is the case for replacement of Zn by Cd and Co to some extent in eukaryotic phytoplankton under low Zn conditions (Morel et al., 2020). Our experiments were conducted in full strength culture media with high metal concentrations but the capacity for metal substitution in low-metal natural systems would presumably be more limited.

Membrane transporters specifically for Co have not been reported, instead, Co appears to cross membranes via transporters that also move other divalent metals such as Fe^{2+}, Zn^{2+}, Ni^{2+} and Cd^{2+} (Sunda and Huntsman, 1995; Kobayashi and Shimizu, 1999; Hu et al., 2021). This implies that high concentrations of these metals can competitively limit transport of Co^{2+}. In Co-limited cultures of the pico-cyanobacterium, *Prochlorococcus*, the growth rate decreased when presented with high levels of Zn and Mn (Hawco and Saito

2018). Ni can inhibit Co uptake in bacteria (Kobayashi and Shimizu, 1999) and Fe competes with Co for uptake in the bacterium *Pseudomonas* (Kothamasi and Kothamasi, 2004). If the filamentous cyanobacteria used in this study react the same way as these prokaryotes, then the relatively high levels of divalent trace metals in $BG11_o$ culture media could have exacerbated Co deficiency.

Relatively high growth rates for the five cyanobacteria species at the lowest Co concentration of 0.17 nmol L^{-1} in this study (Table 1) imply that their concentration thresholds for Co membrane transport and Monod growth (i.e., the highest concentration at which transport and growth do not occur) were less than 0.17 nmol L^{-1}, i.e., in the sub-nanomolar range. Facey et al. (2022) found that growth of the non-heterocystous, non-N fixing cyanobacterium *Microcystis aeruginosa* was most severely inhibited in cultures when dissolved Co was less than 0.34 nmol L^{-1} so the threshold for *Microcystis* must have been less than 0.34 nmol L^{-1}. Monod growth thresholds for Fe reported by Shah et al. (2023) for three cyanobacteria (two of which were used in this study) under N-replete and N-deplete conditions and two eukaryotic algae ranged from 0.02 to 1.20 nmol L^{-1}, with three of the seven thresholds below 0.17 nmol L^{-1}. Cyanobacteria thresholds for other metabolically essential trace metals are probably in the sub-nanomomolar range as well.

We found 22 published studies reporting heterocyst frequencies in 24 wild-type filamentous cyanobacteria strains grown in N-free cultures (Table 4) but interpretation of relationships between heterocyst frequency and Co is not straightforward because of the different experimental conditions (e.g., temperature, light, photoperiod) and media used and because most of the studies used very high Co concentrations. While different media recipes tend to be 'variations on a theme', culture media typically have high concentrations of the three major groups of ingredients to ensure high growth rates and yields - trace metals, major cations and anions, and the macronutrients P and N. Hence, it is not surprising that 16 of these 22 studies used 170 nmol L^{-1} (the highest concentration in out batch culture experiment) and 198 nmol L^{-1} which are well above concentrations found in the Swedish, Norwegian and Canadian surveys of surface waters. Based on our culture results, high heterocyst frequencies are expected in severely N-limited culture media with high Co concentrations which explains why many of the studies in Table 4 reported frequencies between 5.9 and 9.2%. However, the results of several published studies cannot be explained using Co as a driver: frequencies in eight taxa were relatively low, ranging between 3.2 and 5.8%, and two taxa grown at 42 nmol L^{-1} Co (higher than our 0.17 and 17 nmol L^{-1}) had relatively low frequencies of 3.2 and 3.8%.

4.2 Potential for Co limitation of heterocyst frequency in Canadian freshwaters

The ambient concentration of Co within Canadian freshwaters appeared related to lake trophic status, local geology and land use. In our 2017 survey, lakes with the highest Co concentrations were typically eutrophic with the highest values found in Qu'Appelle Valley lakes of Saskatchewan where surrounding land use developed on thick quaternary sediment sequences is dominated by agriculture. Lakes with the lowest Co concentrations were oligotrophic lakes on the Precambrian Shield of Ontario and New Brunswick, with forested watersheds, weathering resistant bedrock and minimal disturbance with the exception of experimentally eutrophied Lake 227 at the IISD -ELA in northwestern Ontario which had elevated levels. Any Co impurities in the phosphate fertilizer added to Lake 227 could have raised lake concentrations.

Using the P/Co molar ratio in the $BG11_0$ culture media to extrapolate to eutrophic natural systems with approximately 50 µg P L^{-1}, it appears that sub-nanomolar Co concentrations < 0.2 nmol L^{-1} could potentially limit heterocyst frequency in N-limited eutrophic waters. Concentrations below 0.2 nmol L^{-1} were observed in some lakes across Canada inferring potential Co limitation of heterocyst frequency should they become N-limited. The range of dissolved Co in the 2017 survey was < 0.03 to 11.5 nmol L^{-1}. Concentrations would have to be substantially lower than 0.2 nmol L^{-1} to limit growth.

Most dissolved Co concentrations in the 2017 Canadian survey were < 4 nmol L^{-1} with some in the sub-nanomolar range but it is difficult to predict the importance that Co might have had on heterocyst frequency in N-limited systems since other factors also affect frequency. We know that heterocyst frequency differs among species (perhaps because of differences in cell size as discussed above) and among strains grown under controlled conditions (this study and Nayak et al., 2007; Ahad et al., 2015). Frequency also varies with environmental factors such as incubation time (Vasas et al., 2013; Zulkefli and Hwang, 2020), calcium (Smith et al, 1987; Torecilla et al., 2004), Fe (Aly and Andrews, 2016), Ni (Rai and Raizada, 1986), inorganic N (Fogg, 1949; Ogawa and Carr, 1969; Rother and Fay 1979; Brown and Rutenberg, 2012; Mohlin et al., 2012; Zulkefli and Hwang, 2020), CO_2 (Kulasooriya et al.,1972; Kang et al., 2004; Masukawa et al., 2017), O_2 (Kangatharalingam et al., 1992), light (Fogg, 1949) and temperature (Zakrisson and Larsson, 2014). This large number of known confounding variables (there may be others) makes it very difficult to assign relative importance to variables known to affect heterocyst frequencies.

Co seems not to have limited heterocyst frequency in Lake 227 in 2017 although concentrations were below 1 nmol L^{-1} in June just before the bloom began. Heterocyst

frequency was generally low, however, it increased during the ascending limb of the bloom as did epilimnetic dissolved Co from approximately 0.7 to approximately 2 nmol L^{-1} (Figure 4). The fact that dissolved Co was not drawn down during the bloom but increased along with heterocyst frequency suggests that cyanobacteria were able to synthesize heterocysts as needed.

It is unknown why the dominant species shifted from *A. schindlerii* to *A. skujae* after 2015 although lower ammonia may have been a factor. Co was probably not a factor since concentrations exceeded 0.2 nmol L^{-1} and there was no major difference in epilimnetic and metalimnetic dissolved Co before and after 2015. However, other surveyed lakes at ELA had lower Co, for example, dissolved Co was 0.12 nmol L^{-1} in oligotrophic P-limited Lake 304 in September 2017.

The higher heterocyst frequency associated with the smaller *A. skujae* after 2015 could have been affected by the change in mean heterocyst cell volume relative to mean vegetative cell volume. The ratio of mean heterocyst cell volume to mean vegetative cell volume declined from 1.3 in 2002-2012 to 1.1 in 2015-2020 (Table 2), and while the magnitude of the decline does not seem large, it may have necessitated an increase in heterocyst frequency to maintain a similar fixed N supply rate per unit volume. Consider the following calculations: the mean vegetative/heterocyst cell density ratio declined from 28.2 to 20.6 between 2002-2012 and 2015-2020 which means that newly fixed N diffused down a concentration gradient into approximately 14 vegetative cells on either side of a heterocyst in 2010-2012, and 10 vegetative cells in 2015-2020. At the same, the mean vegetative cell volume declined from 28 to 15 μm^3 per cell so the total vegetative biovolume supplied by each heterocyst cell (V_h) declined 61% from 790 μm^3 in 2002-2012 to 309 μm^3 in 2015-2020. Heterocyst cell size (H) decreased from 36 to 17 μm^3 resulting in V_h/H ratios of 21.9 in 2002-2012 and 18.2 in 2015-2020 which are not markedly different from each other. Thus, while the proportion of cells that were heterocysts increased from 3.0 to 4.8% after 2015, the change in the proportion of biovolume (biomass) that was heterocyst was much smaller (Table 2). The shift from *A. schindlerii* to smaller *A. skujae* also resulted in shorter travel distances for newly fixed N although how this might have affected net N supply rates (N leakage, which is a function of cell surface area/volume ratio and residence time, must be taken into account) is unclear. The mean individual vegetative cell length declined from 5.5 to 5.2 µm so the total travel length for newly fixed N on one side of a heterocyst declined from 77.6 to 53.6 µm. The similar Vh/H ratio and shorter diffusion distance after 2015 may have maintained a similar efficiency of N supply to neighboring cells compared to 2000-

2012. These are variables that have not been previously considered.

This analysis of the potential impact of cell size on heterocyst frequency in Lake 227 suggests that species-specific regressions of fixation rate versus heterocyst abundance (Findlay et al., 1994; Higgins et al., 2018) may not necessarily be transferrable to other filamentous species that differ significantly in cell size. Hence, we recommend augmenting estimates of heterocyst frequency based on cell abundance with estimates based on biovolume or biomass ratios. Frequency estimates based on the number of heterocysts per filament length are sometimes used (Laamanen and Kuosa, 2005; Walve et al., 2014; Zulkefli and Hwang, 2020) but are analogous to frequency estimates based on cell abundance.

We found five published studies of natural freshwater and brackish systems that measured *in situ* heterocyst frequencies (Table 5). Co concentrations were not reported but were probably much lower than full strength culture media. Maximum heterocyst frequencies in these natural systems generally ranged from 3-7%, similar to the frequencies at the two lower Co concentrations in this study and in Lake 227 (Figure 4). However, higher peaks of 10-11% were recorded in the Lower Karori Reservoir in New Zealand in two of the three documented years (Wood et al., 2010). Heterocyst frequency varies with sampling date during blooms (Wood et al., 2010) so sampling date relative to timing of the bloom should be reported along with frequency and biomass. For example, heterocyst frequency increased during the *A. skujae* bloom in Lake 227 in 2017 but the annual peak in frequency preceded a bloom of *Dolichospermum (Anabaena) planktonica* in Lower Karori Reservoir in New Zealand (Wood et al., 2010).

The majority of the total Co concentrations in Canadian High Arctic freshwaters were less than the relatively high detection limit of 17 nmol L^{-1} but the proportion in the sub-nanomolar range relevant to Co limitation of heterocyst frequency is unknown. Low Co in Arctic regions is expected because of low weathering rates caused by long periods of freezing temperatures and low precipitation (Statistics Canada, 2017) and the absence of mining, industrial activities and urbanization in most areas (Aliff et al., 2020). However, Co content in bedrock and overburden can vary regionally which would affect aquatic concentrations. It should be noted that while Co may be low enough to affect heterocyst frequency in cyanobacteria in the Arctic, these would be benthic forms (Vézina and Vincent, 1997; Bonilla et al., 2005) since pelagic filamentous cyanobacteria are absent (Schindler et al., 1974; Schlesinger et al., 1981; Holmgren, 1984; Vincent, 2000; Rautio et al., 2011; Vincent and Quesada, 2012) although this may be changing, at least in the subarctic (Pick, 2016; Sivarajah et al., 2021). In the Northwest Territories (Palmer et al., 2015), dissolved Co in

most of the lakes (77%) were at or below the detection limit of 1.7 nmol L^{-1} with 23% of the samples at 3.4 and 5.1 nmol L^{-1}. The large proportion of samples below 1.7 nmol L^{-1} suggests there is some potential for Co limitation of heterocyst differentiation in N-limited systems in the Yellowknife region of the Northwest Territories.

Despite higher runoff and consequently higher weathering rates, Co is also low in Scandinavia although high runoff can dilute concentrations (European Environment Agency, 1999). Dissolved Co (0.22 µm filter) in one region in northern Sweden ranged from 0.17 to 17 nmol L^{-1} with a median concentration of 0.7 nmol L^{-1} in one local area within the region (Fischer et al., 2020). Skjelkvale et al. (2006) reported a median Co concentration of 0.85 nmol L^{-1} in Norwegian surface waters with a range from less than the detection limit of 0.34 nmol L^{-1} to greater than 3.4 nmol L^{-1} (samples may have been unfiltered). Lenvik et al. (1978) reported a range of 1.5 to 7.8 nmol L^{-1} for settled, decanted samples from 11 Norwegian rivers.

Co concentrations are usually reported as total (unfiltered) or dissolved (filtered), i.e., as size fractions, as we have done in this study but the supply rate to the microbial community is influenced by more than just the concentration within size classes. Co availability is also a function of chemical species which is influenced by within-lake factors, especially dissolved organic carbon (DOC). Co^{2+} is the dominant oxidation state in circum-neutral, oxygenated waters which partitions between free uncomplexed and DOC-bound states (Collins and Kinsela, 2010; Tang et al., 2021). Co^{2+} binding to organic ligands serves to keep it in solution while adsorption to amorphous ferric hydroxides and manganese oxides removes Co to sediments along with settling phytoplankton and particulate organic matter (Esmadi and Simms, 1995; Tang et al., 2021). Since DOC inhibits formation of particulate ferric hydroxide (Moore et al., 1979; Molot and Dillon, 2003) and perhaps manganese oxide which would limit Co removal from the water column, and DOC maintains Co^{2+} in solution through complexation, it follows that DOC potentially helps to meet microbial demand for Co. However, high concentrations of DOC with very strong binding affinities for Co could have the opposite effect (Imai et al., 1999).

There was one exception to the generally low Co levels found in the 2017 survey of Canadian surface waters – 311 nmol L^{-1} was measured in a filtered sample of cyanobacteria surface scum (i.e., a dense population) in Buffalo Pound, Saskatchewan, a concentration that was 97 times higher than a surface sample collected 2 days earlier. The scum also concentrated several other metabolically essential metals but to a much lesser extent. Ni, Fe, Mn, and Cu were concentrated 4 to 8-fold so the elevated metal concentrations may be real.

Metals in surface samples without scum in Buffalo Pound were consistently lower throughout the summer than this one scum sample. Co was also apparently concentrated during the *A. skujae* bloom in Lake 227 in 2017 judging by the 3x increase in dissolved Co from 0.7 to 2 nmol L^{-1} during the exponential growth phase of the bloom in the epilimnion and 0.8 to 2.1 nmol L^{-1} in the metalimnion.

The presence of higher dissolved Co concentrations in the middle of a dense cyanobacteria population suggests that large populations possess a concentration mechanism that limits Co loss from the upper water column such as might occur via competing adsorption of inorganic Co^{2+} to settling Fe and Mn hydroxides (Esmadi and Simm, 1995; Balistrieri et al., 1992). The concentration mechanism might consist of inorganic Co^{2+} adsorption to cell sheaths or complexation to dissolved extracellular ligands (e.g., siderophores) (Sharma and Azeez, 1988; Freire-Nordi et al., 2005; Baptista and Vasconcelos, 2006; Li et al., 2009; Olguín and Sánchez-Galván, 2012; Mona and Kaushik, 2015; Rossi and de Phillipis, 2015; Bishop et al., 2019). Saito et al. (2002) speculated that excretion of organic ligands could account for the higher growth rate of the pico-cyanobacterium, *Prochlorococcus*, in conditioned culture media versus growth in fresh media. Alternatively, Co buildup could be due to loss from senescing cells or excretion by viable cells (Bonnet et al., 2012). Whatever the nature of the concentration mechanism, it follows from Michaelis-Menten transport and Monod growth kinetics (Shah et al., 2023) that a buildup of Co next to cellular membranes could increase Co transport and growth rates although dissociation of Co strongly bound to ligands with high binding affinities would likely have to be biologically facilitated first (Quigg et al., 2006; Worms et al., 2006; Fujii et al., 2010; Rose, 2012).

In summary, we found that (1) Co affects heterocyst frequency in batch cultures which suggests that Co may be involved in differentiation of vegetative cells into heterocysts. (2) Some Co concentrations in natural systems in Canada and Scandinavia were low enough (sub-nanomolar range) to potentially limit heterocyst frequency in N-limited waters based on comparison to stoichiometrically corrected culture results. (3) However, we cannot conclude that sub-nanomolar concentrations of Co will result in low heterocyst frequency in N-limited natural systems because many variables influence frequency. (4) Low heterocyst frequency by itself should not be taken as an indicator of Co limitation in N-limited systems as the Lake 227 analysis demonstrates. (5) The ratio of heterocyst to vegetative cell volume may affect heterocyst frequency. Hence, our understanding of relationships between Co and heterocyst frequency in natural systems is still unclear. (6) All of the Canadian Co surveys listed in Table 3 used analytical methods with relatively high detection limits between 1.2 and 17

562 nmol Co L^{-1} with the exception of the 2017 Canadian survey which had a much lower
563 detection limit. This made it difficult to accurately ascertain the extent to which low (sub-
564 nanomolar) Co might affect filamentous cyanobacteria in N-limited waters. Given that Co
565 concentrations above 0.17 nmol L^{-1} did not affect cyanobacteria growth rates in our cultures,
566 future studies of the impacts of low Co in natural systems would benefit by selecting
567 analytical methods with very low detection limits.
568
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588 The authors declare that they have no conflicts of interest.
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Appendix 1

Water samples for dissolved Co (passing through a 0.45 µm membrane filter) were collected from 94 lakes within a 30 km radius of Yellowknife in the Northwest Territories by the Northwest Territories Geological Survey by helicopter in September 2012 and September 2014 (Palmer et al., 2015). Samples were collected 30 cm below the surface in 250 mL polyethylene containers that had been rinsed three times with lake water. Following collection, samples were stored out of direct sunlight in a cooler with ice packs and immediately delivered to a laboratory accredited by the Canadian Association for Laboratory Accreditation. Samples were filtered immediately upon arrival at the laboratory with a 0.45 µm filter and acidified with high purity nitric acid. Trace metals were measured using ICP-MS following EPA Method 200.8. The detection limit was 1.7 nmol L^{-1} (0.1 µg L^{-1}). Data were provided in digital format by Jennifer Korosi to the authors of this paper.

Integrated epilimnetic samples were collected from nine lakes in central Ontario between June 2010 and July 2017 by the Ontario Ministry of Environment, Conservation and Parks (MECP). These lakes include the eight so-called Dorset 'A' lakes (Blue Chalk, Crosson, Dickie, Plastic, Harp, Heney, Red Main, Red Chalk East (Molot and Dillon, 2003; Arnott et al. 2003) and Ridout. Samples were filtered with 80 µm mesh, allowed to settle, acidified with nitric acid to make the final solution 1% HNO_3, decanted and analyzed via ICP-MS with an inductively coupled argon plasma as the ion source (MECP method MET3474). Hence, Co included the dissolved and all colloidal phases and perhaps some small non-settling particulate matter as well. The method detection limit was 1.7 nmol L^{-1} (0.1 µg L^{-1}).

Table 1. Mean growth rates (day^{-1}, ± standard deviation) of four filamentous cyanobacteria species in $BG11_0$ media without inorganic N and *Microcystis* in BG11 media with inorganic N grown in duplicate at three Co concentrations (nmol L^{-1}). Paired superscript letters indicate significant differences between treatments within a species at the 1% level (ANOVA).

r estimated from r growthcurver logistic growth package

Species	0.17	17	170
Aphanizomenon flos-aquae	1.26 ± 0.15	0.84 ± 0.27	0.79 ± 0.13
Aphanizomenon skujae	0.90 ± 0.12	0.84 ± 0.22	0.79 ± 0.06
Dolichospermum flos-aquae	1.83 ± 0.18	1.25 ± 0.17	1.35 ± 0.09
Dolichospermum lemmermannii	0.79 ± 0.16	1.03 ± 0.31	0.61 ± 0.09
Microcystis aeruginosa	0.84 ± 0.15	0.76 ± 0.19	1.10 ± 0.51

μ_{sl}, estimated as slope of $\ln(A_{750})$ versus time during days 0-7.

Species	0.17	17	170
Aphanizomenon flos-aquae[1]	0.66 ± 0.05	0.55 ± 0.03	0.67 ± 0.22
Aphanizomenon skujae[2]	1.05 ± 0.08[ab]	0.66 ± 0.03[a]	0.67 ± 0.04[b]
Dolichospermum flos-aquae[1]	0.62 ± 0.06	0.64 ± 0.00	0.77 ± 0.07
Dolichospermum lemmermannii[2]	0.57 ± 0.28	0.76 ± 0.03	0.60 ± 0.02
Microcystis aeruginosa[2]	0.73 ± 0.10	0.69 ± 0.04	0.63 ± 0.10

1. maximum μ_{sl} occurred during days 4-7.

2. maximum μ_{sl} occurred during days 0-4 (cultures were sampled on days 0, 4, 5, 7 and 11).

Table 2. Changes in mean summer (June-September) cell abundance, biomass and cell size of cyanobacteria vegetative and heterocyst cells in the metalimnion of Lake 227 between 2002-2012 when *Aphanizomenon schindlerii* dominated and 2015-2020 when *A. skujae* dominated the cyanobacteria community. June-September means are reported with standard deviations. p values are for two-tailed t test for independent means. t-test p values (independent means) are presented; n.s., not significant at the 5% level.

	2002-2012	**2015-2020**	**% change**	**p value**
Heterocyst abundance (10^7 cells L^{-1})	1.0 ± 0.5	1.6 ± 0.4	+68	0.015
Heterocyst biomass (µg L^{-1})	313 ± 115	269 ± 85	-14	n.s.
Heterocyst cell volume (µm^3 per cell)	36 ± 10	17 ± 2	-54	0.0002
Vegetative cell abundance (10^8 cells L^{-1})	3.1 ± 1.1	3.3 ± 0.8	+6	n.s.
Vegetative biomass[1] (µg L^{-1})	7607 ± 2381	4857 ± 1613	-36	0.024
Vegetative cell volume (µm^3 per cell)	28 ± 5	15 ± 3	-46	<0.0001
Vegetative cell length (µm per cell)	5.5 ± 0.42	5.2 ± 0.2	-5.5	n.s.
Cell ratio (%), heterocyst/total cyanobacteria (i.e., heterocyst frequency)	3.0 ± 1.3	4.8 ± 0.9	+60	0.01
Biomass ratio (%), heterocyst/total cyanobacteria[2]	4.1 ± 1.2	5.4 ± 0.1	+32	<0.0001

1. Biovolume was converted to biomass wet weight by assuming a cell density of 1 gm L^{-1}.
2. Caution is warranted when comparing cell ratio to biomass ratio since cell densities are used to estimate biomasses, i.e., spurious correlation is possible.

1224 **Table 3.** Summary of metal surveys of Canadian surface waters. The wide range in detection limit reported by accredited laboratories
1225 is due to differences in method and equipment.
1226

Reference	Location	Number of aquatic systems	Number of samples and Co phase (total, dissolved)	Laboratory and method	Detection limit (DL)
Antoniades et al. (2003a)	Alert, Arctic Archipelago	31	31 total	Environ Canada NLET[1]	17 nM (1 ppb)
"	Mould Bay, Arctic Archipelago	17	17 total	Environ Canada NLET[1]	17 nM (1 ppb)
Antoniades et al. (2003b)	Ellef Ringnes Island, Arctic Archipelago	25	25 total	Environ Canada NLET[1]	17 nM (1 ppb)
Michelutti et al. (2002a)	Victoria Island, Arctic Archipelago	34	34 total	Environ Canada NLET[1]	17 nM (1 ppb)
Michelutti et al. (2002b)	Axel Heiberg Island, Arctic Archipelago	38	38 total	Environ Canada NLET[1]	17 nM (1 ppb)
Rossmann and Barres (1988)	Lake Superior	1	22 dissolved (0.5 µm), 22 particulate	flameless atomic absorption spectrophotometry	DL not stated but Tables 1 and 10 suggest 1.7 nM (0.01 ppb)
"	Lake Huron	1	1 dissolved (0.5 µm), 1 total	"	"
"	Lake Michigan (USA)	1	11 filtered (0.5 µm), 11 total	"	"

"	Lake Erie	1	11 filtered (0.5 µm), 11 total	"	"
"	Lake Ontario	1	23 dissolved (0.5 µm), 23 particulate	"	"
Palmer et al. (2015)	Northwest Territories, Yellowknife region	94	115 dissolved (0.45 µm)	ICP-MS following EPA Method 200.8 revision 5.4 (Creed et al., 1994)	1.7 nM (0.1 ppb with 0.1 ppb increments above DL)
2017 cross Canada survey (this study)	Saskatchewan, Manitoba, Ontario, Quebec, New Brunswick	40	167 dissolved (0.45 µm) excluding Buffalo Pound surface scum and Ottawa and Toronto municipal stormwater facilities	Trent Water Quality Centre; ICP-MS	0.017 nM (0.001 ppb)
Ontario Ministry of the Environment, Conservation and Protection (unpublished)	Central Ontario near Dorset	9	414 settled	Ontario Ministry of Environment, Conservation and Protection Laboratory, ICP-MS Method MET3474	1.2 nM (0.07 ppb with 0.1 ppb increments above DL)

1. Method not described but cited as Environment Canada (1994). Manual of Analytical Methods. National Laboratory for

1229 Environmental Testing, Canadian Centre for Inland Waters.

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Table 4. Summary of heterocyst frequencies (HF) and Co concentration in published culture studies. Only wild-type species are included here. The genus names listed here are the names reported in the publications but pelagic *Anabaena* has been renamed *Dolichospermum*.

Species	Co (nmol L^{-1})	HF (%)	Reference
Anabaena cylindrica	170	4.1	Jewell and Kulasooriya (1970)
Anabaena cylindrica	170	4.7	Kulasooriya et al. (1972)
Anabaena cylindrica	170	5.2	Ogawa and Carr (1969)
Anabaena cylindrica	170	9.0	Bradley and Carr (1976)
Anabaena cylindrica	170	5.8	Nayak et al. (2007)
Anabaena fertilissima	170	7.4	Nayak et al. (2007)
Anabaena flos-aquae	170	3.2	Ogawa and Carr (1969)
Anabaena flos-aquae	170	9.2	Kangatharalingam et al. (1992)
Anabaena inequalis	170	5.4	Ogawa and Carr (1969)
Anabaena iyengarii	170	7.6	Nayak et al. (2007)
Anabaena laxa	170	5.1	Nayak et al. (2007)
Anabaena oryzae	170	8.5	Nayak et al. (2007)
Anabaena oscillarioides	170	4.3	Nayak et al. (2007)
Anabaena PCC7108	170	7.8	Nayak et al. (2007)
Anabaena PCC7120	170	6.5	Nürnberg et al. (2015)
Anabaena PCC7120	170	7.2	Chaurasia and Apte (2011)
Anabaena PCC7120	170	7.5	Berendt et al. (2012)
Anabaena PCC7120	170	8.0	Videau et al. (2016)
Anabaena PCC7120	170	8.7	Borthakur et al. (2005)
Anabaena PCC7120	170	8.9	Rivers et al. (2018)
Anabaena PCC7120	21	11	Masukawa et al. (2017)
Anabaena PCC7122	170	8.0	Nayak et al. (2007)
Anabaena sp.	170	6.3	Ahad et al. (2015)
Anabaena sphaerica	170	5.5	Nayak et al. (2007)
Anabaena spiroides	170	4.3	Nayak et al. (2007)
Anabaena vaginicola	170	6.3	Nayak et al. (2007)
Anabaena variabilis	170	4.3	Ogawa and Carr (1969)
Anabaena variabilis	170	5.9	Nayak et al. (2007)
Dolichospermum lemmermannii	0, 1.7, 17	<2	Kelly et al. (2021)
Dolichospermum planctonicum	0, 1.7, 17	>6	Kelly et al. (2021)
Aphanizomenon aphanizomenoides	42	3.2	de Figueiredo et al. (2011)
Aphanizomenon flos-aquae	16	4.4	Rother and Fay (1979)
Aphanizomenon			

ovalisporum	170	8.4	Vasas et al. (2013)
Aphanizomenon sp.	42	3.8	Mohlin et al. (2012)
Nodularia spumigena	198	8.5	Vintila and El-Shehawy (2007)
Nostoc muscorum	84	5.9	Rai and Raizada (1986)

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1237 **Table 5.** Summary of published heterocyst frequencies in natural freshwater and brackish
1238 systems. Co concentrations are not available (n/a) except for Lake 227.

Dominant species	**Study site**	**Heterocyst frequency (%)**	**Co (nmol L^{-1})**	**Reference**
Aphanizomenon flos-aquae	Lough Neagh, Northern Ireland	~5-7% in 1970 and 1971 when nitrate was low; <0.2% when nitrate was high	n/a	Riddolls (1985)
Aphanizomenon sp.	Baltic Sea	peaked at 4.5%, range 1.5-4.5% at 7 stations, oscillated with time	n/a	Zakrisson and Larsson (2014)
various	Sawley Dene, North Yorkshire, UK	peaked at 4% in 1976 and 1977	n/a	Cmiech et al. (1984)
Aphanizomenon sp.	Lake Trichonis, Greece	peaked at 3% in 1985-86 when nitrate was low	n/a	Anagnostidis et al. (1988)
Dolichospermum (Anabaena) planktonica	Lower Karori Reservoir, New Zealand	several annual peaks between 5.3 and 9.3%	n/a	Wood et al. (2010)
Aphanizomenon schindlerii 2002-2012, *Aphanizomenon skujae* 2015-2020	Lake 227, northwestern Ontario, Canada	mean 3.0% 2002-2012; mean 4.8% 2015-2020	0.7-4.0	this study

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Figure 1. Growth curves of five cyanobacteria species at three nominal concentrations of Co. A_{750} is absorbance at 750 nm. Lines connect mean absorbance of duplicate cultures and bars indicate standard deviations.

Figure 2. Heterocyst frequencies as a percentage of total cell number of four cyanobacteria species at three nominal Co concentrations. Each bar represents the mean heterocyst percentage of five counts, bars indicate standard deviation and letters above indicate statistically different means as found by a two-way ANOVA followed by Tukey's HSD.

Figure 3. Mean summer (June-September) ($\pm$ standard deviation) heterocyst frequency based on cell abundance in the epilimnion and metalimnion of Lake 227, 2000-2020. Error bars are standard deviations *Aphanizomenon schindlerii* was the dominant cyanobacteria 2002-2012 and *Aphanizomenon skujae* 2015-2020.

Figure 4. Cyanobacteria and total phytoplankton biomass, heterocyst frequency (HF, %) and dissolved cobalt concentration (nmol L^{-1}) in Lake 227 in 2017.

Figure 5. Mean epilimnetic dissolved Co concentrations in Canadian lakes and reservoirs during the summer of 2017. Error bars indicate standard deviation when multiple samples were analyzed for a particular location during the year. Colors indicate the province of the lake. Lake Winnipeg is large enough to be divided into three distinct parts: North Basin, South Basin and the Narrows which separates the basins.

Figure 1. Growth curves of five cyanobacteria species at three nominal concentrations of Co. A_{750} is absorbance at 750 nm. Lines connect mean absorbance of duplicate cultures and bars indicate standard deviations.

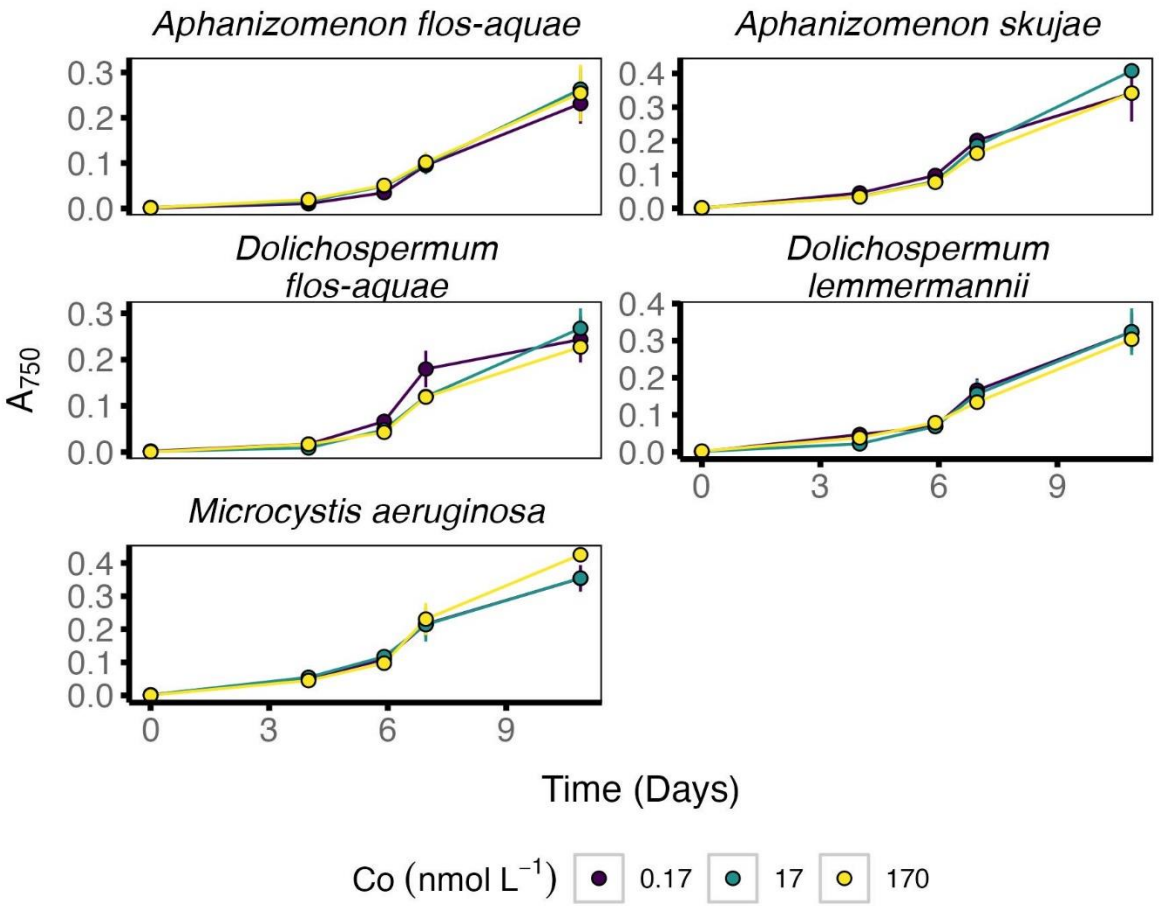


Figure 2. Heterocyst frequencies as a percentage of total cell number of four cyanobacteria species at three nominal Co concentrations. Each bar represents the mean heterocyst percentage of five counts, bars indicate standard deviation and letters above indicate statistically different means as found by a two-way ANOVA followed by Tukey's HSD.

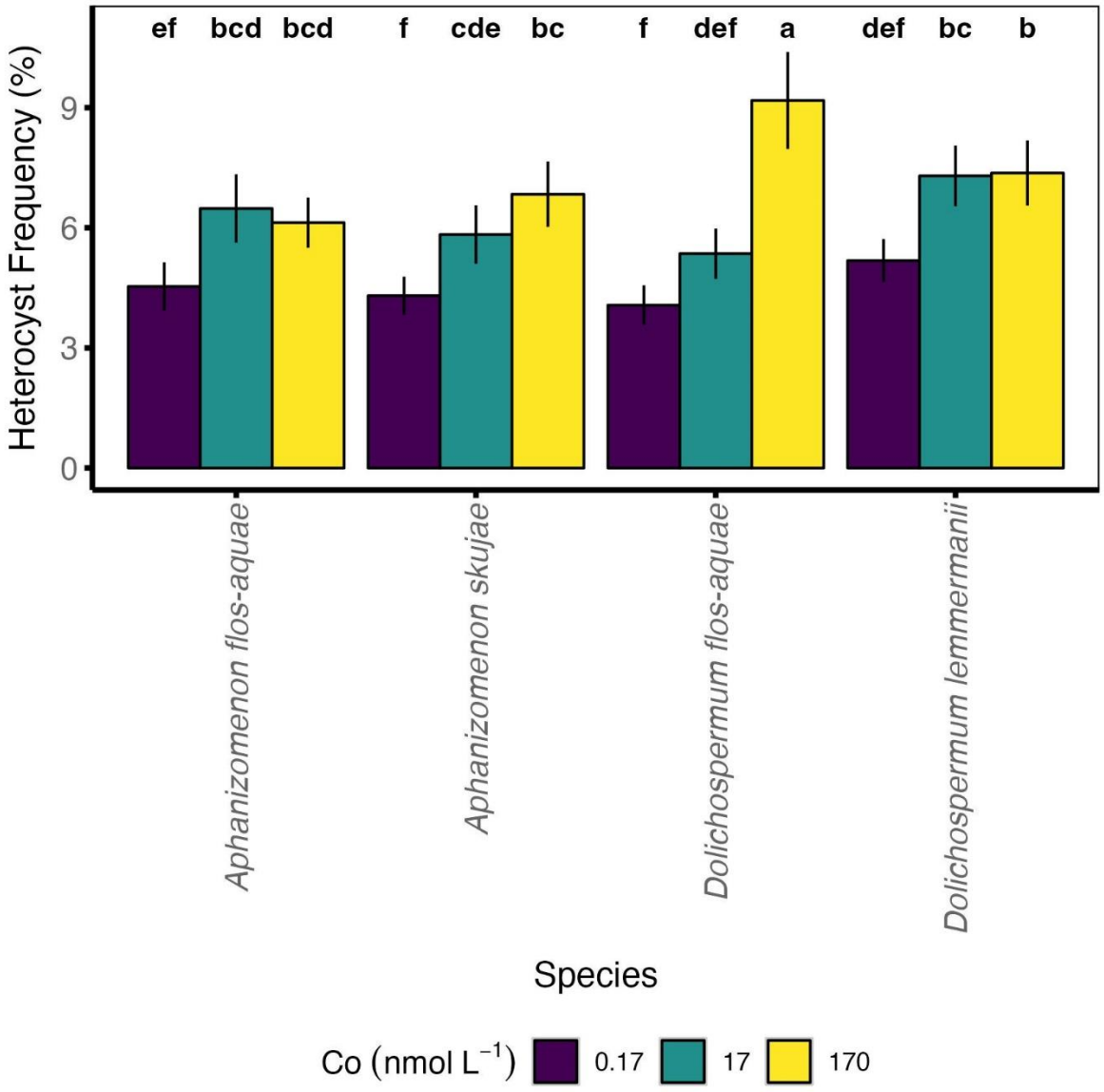


Figure 3. Mean summer (June-September) (± standard deviation) heterocyst frequency based on cell abundance in the epilimnion and metalimnion of Lake 227, 2000-2020. Error bars are standard deviations. *Aphanizomenon schindlerii* was the dominant cyanobacteria in 2002-2012 and *Aphanizomenon skujae* in 2015-2020.

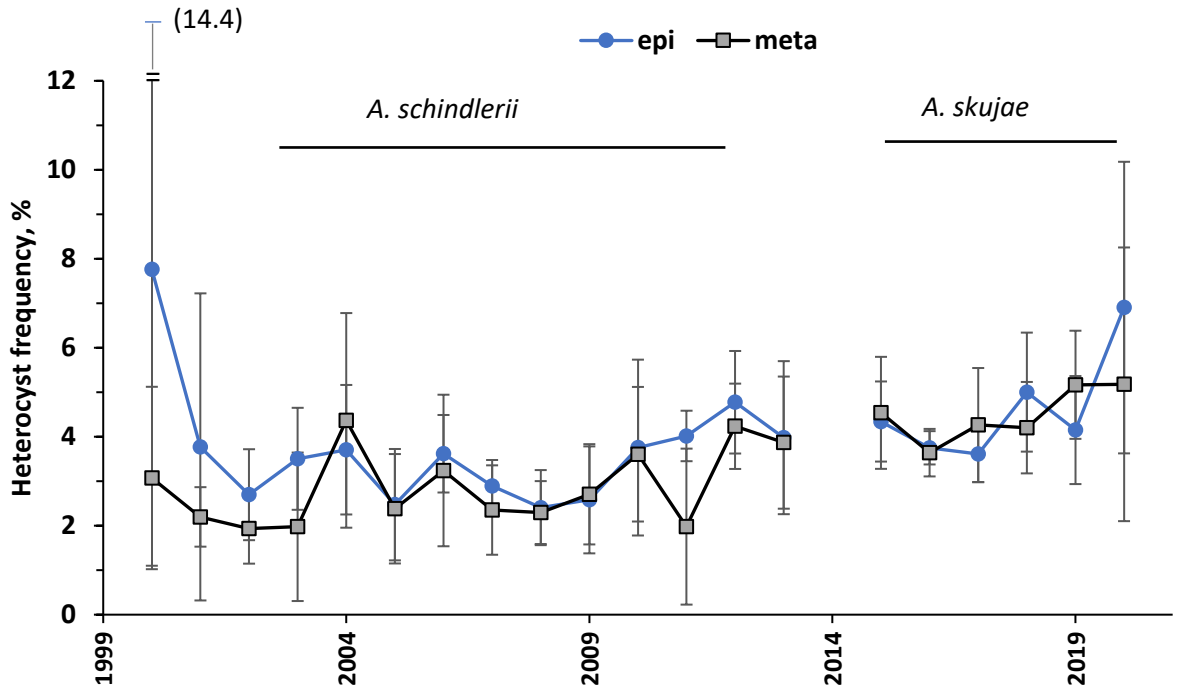


Figure 4. Cyanobacteria and total phytoplankton biomass, heterocyst frequency (HF, %), ammonia concentration (µmol L^{-1}) and dissolved cobalt concentration (nmol L^{-1}) in Lake 227 in 2017. Note the different vertical scales on the right side.

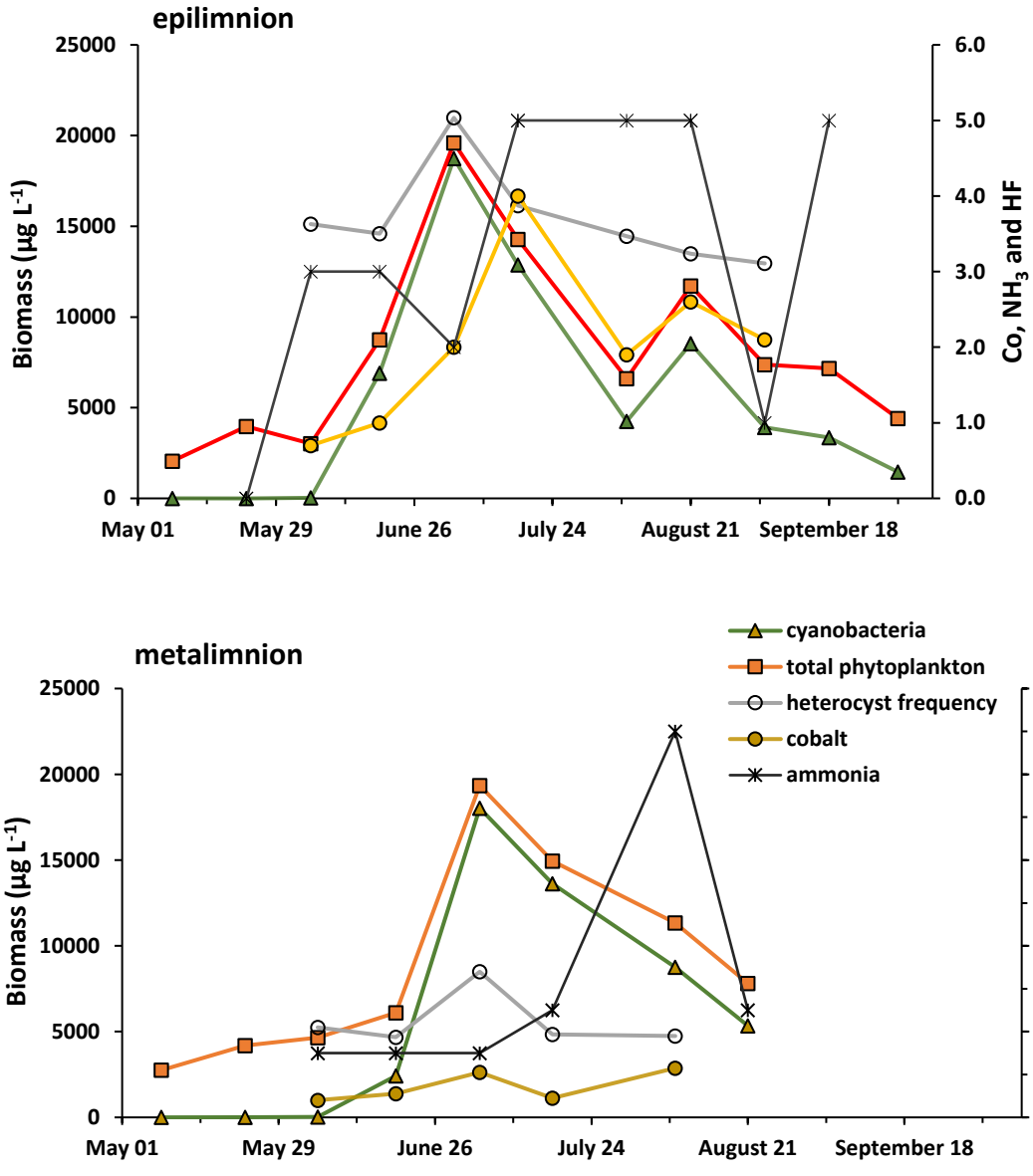
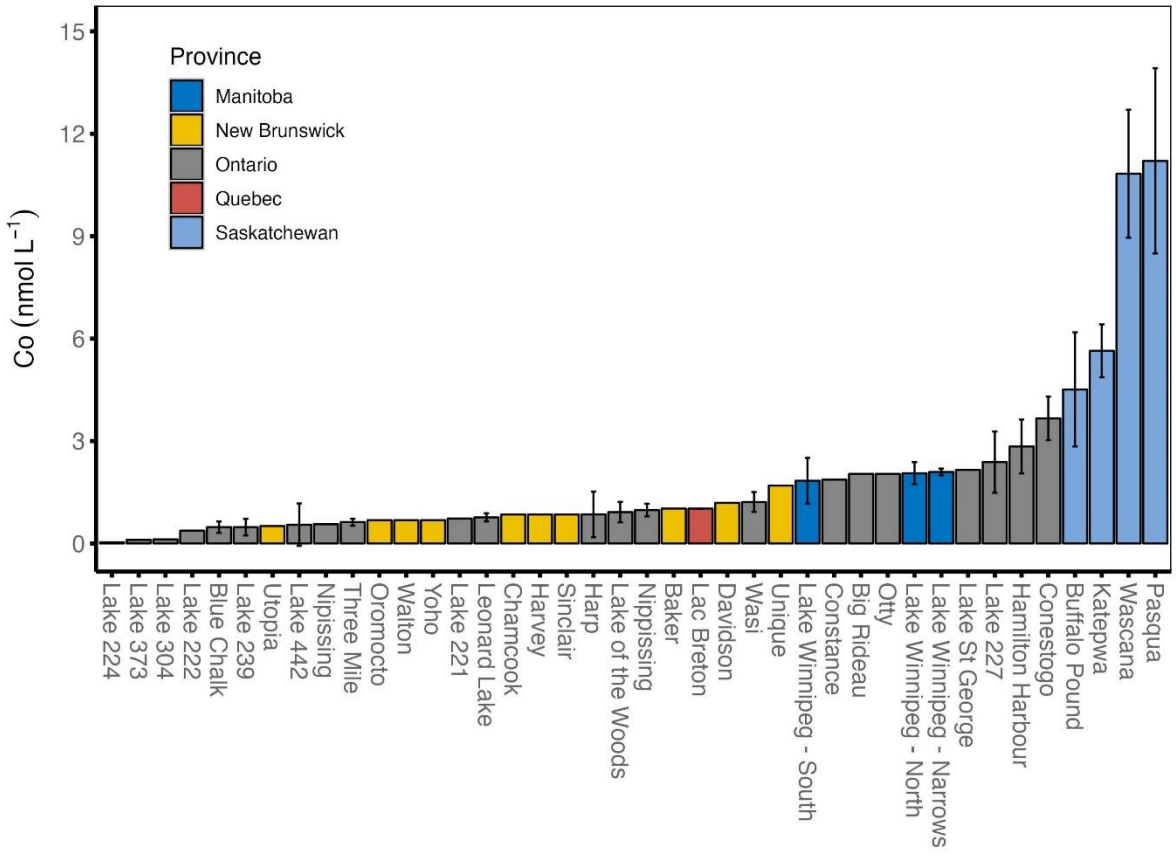


Figure 5. Mean epilimnetic dissolved Co concentrations in Canadian lakes and reservoirs during the summer of 2017. Error bars indicate standard deviation when multiple samples were analyzed for a particular location during the year. Colors indicate the province of the lake. Lake Winnipeg is large enough to be divided into three distinct parts: North Basin, South Basin and the Narrows which separates the basins.



Supplementary Information

Table S1. Locations of lakes and reservoirs in 2017 metals survey with basin morphometry, land use (forestry, grassland, agriculture, urban) and surface layer pH, conductivity, total N (TN) and total P (TP). For Lake Winnipeg, data are given for the north and south basins. Chemistry varies with season, depth and station (in large lakes) and these data are meant for approximate characterization purposes only.

	Latitude longitude	Province	Mean depth (m)	Maximum depth (m)	Surface area (ha)	Catchment area (km^2)	pH	Conductivity ($\mu S\ cm^{-1}$)	TP ($\mu g\ L^{-1}$)	TN ($mg\ L^{-1}$)	Land use
Buffalo Pound[1] Lake	50.577 N 105.360 W	Saskatchewan	3	5.5	2910	1282	8.4	480	99	1.2	Qu'Appelle River basin: 75% agriculture, 12% grasslands, urban
Wascana Lake[2]	50.431 N 104.589 W	Saskatchewan	1.5	3.4	50	1248	9	1000	108	0.4-1.6 (dissolved)	See Buffalo Pound; in large urban centre
Pasqua Lake	50.785 N 103.961 W	Saskatchewan	5.8	15.5	2020	11×10^3	8.5	2100	615	4.23	See Buffalo Pound

Katepwa Lake	50.723 N 103.657 W	Saskatchewan	14.3	23.2	1620	12.4×10^3	8.5	1150	380	1.80	See Buffalo Pound
Lake Winnipeg South basin North basin	52.606 N 98.495 W	Manitoba	 9 13	 14 19	23,750 km^2	1.0×10^6	8.2	 378[4] 390[4]	 104 39	 0.85 0.63	Forested, agriculture, urban
Lake of the Woods[6]	49.560 N 94.502 W	Ontario	10.7	64.0	4350 km^2	69.8×10^3	7.4-8.2	80-120	20-29	0.3-0.64	mostly forest, 6.4% agriculture, some urban
Lake 221	49.702 N 93.727 W	Ontario	2.1	5.7	9.0	82	6.4	13.5[5]	10.1	0.48	forest
Lake 222	49.696 N 93.723 W	Ontario	3.7	5.8	16.4	204.3	6.8	20.6[5]	9.5	0.45	forest
Lake 224	49.690 N 93.718 W	Ontario	11.6	27.4	25.9	97.5	7.1	13.8[5]	5.4	0.23	forest
Lake 227	49.688 N 93.689 W	Ontario	4.4	10.0	5.0	34.4 ha	7.0	13.0[5]	29.2	0.80	forest
Lake 239	49.664 N 93.724 W	Ontario	10.5	30.4	54.3	393.3 ha	7.1	21.6[5]	6.4	0.30	forest
Lake 304	49.660 N 93.749 W	Ontario	3.2	6.7	3.6	26.4 ha	6.5	11.0[5]	10.6	0.41	forest
Lake 373	49.745 N 93.800 W	Ontario	11.0	20.8	27.3	80.6	7.2	20.1[5]	5.3	0.24	forest
Lake 442	49.776 N 93.817 W	Ontario	9.0	17.8	16.0	161	7.0	16.6[5]	6.4	0.34	forest
Lake Nipissing	46.205 N	Ontario	4.5	10.5	296	12.1	7.3	74	19	0.45	forest

(Callander Bay)	79.398 W										63%; agricultu re 16%; some urban
Wasi Lake	46.140 N 79.228 W	Ontario	2.7	5.5	126	6.3	6.8-7.1	73.5	27-31	0.44	forest 86%; agricultu re 12%
Blue Chalk[3]	45.199 N 78.939 W	Ontario	8.5	23	52.4	105.9 ha	6.7	22[4]	5.9	0.15	forest
Harp Lake[3]	45.380 N 79.135 W	Ontario	13.3	38	71.4	470.7 ha	6.5	30[4]	6.0	0.30	Forest, moderat e shoreline develop ment
Leonard Lake[3]	45.077 N 79.447 W	Ontario	6.8	17.5	195	430 ha	5.5-6.7	33-35[4]	6-8	0.16-0.28	Forest,m oderate shoreline develop ment
Three Mile Lake[3] Hammell's Bay Main basin	45.190 N 79.465 W	Ontario	3.4 (entire lake)	12 4	240 630	1505 ha 12030 ha	6.9-7.1 6.8-7.1	12-23[4] 19-30[4]	12-23 19-30	0.47 0.31-0.42 0.33-0.53	mostly forest, some agricultur e and shoreline developm ent
Lake St. George (west basin)	43.956 N 79.429 W	Ontario	4.9	15.3	10.3		7.0	367	25	0.6	mixed forest, urban,

											agricultu re
Hamilton Harbour	43.290 N 79.842 W	Ontario	13	23	2150	500	8.5	700	40	3-4	mostly urban & industria l
Conestogo Lake	43.684 N 80.680 W	Ontario		18	7.35 km^2	563	7.7-8.3	425-470[4]	14-25	2.0-5.8	Mostly agricultu ral with some urban
Constance Lake	45.410 N 75.979 W	Ontario	1.9	3.4	N/A	1.315	8.6	358	28	623	Wetland and pasture lands, shoreline residenti al develop ment,
Big Rideau Lake	44.724 N 76.231 W	Ontario	12	110	407	100	8.3	196	13	299	Woodlan d and wetland (57%), agricultu ral (37%), shoreline residenc es
Otty Lake	44.843 N 76.225 W	Ontario	9	27	52.8	6.4	8.0	209	13.2	470	Woodlan d and wetland

											(62%), Agricultural (13%), Shoreline residential development
Lac Breton	45.873 N 74.229 W	Quebec	1.4	2.6	0.737	0.119	7.9	84	9.1	440	Mainly woodland with some wetlands; dense shoreline residential development
Lac Baker	47.360 N 68.687 W	New Brunswick		20			7.7-7.9	101	5-10	≤0.3	
Chamcook Lake	45.146 N 67.093 W	New Brunswick		34			7.1	34	4	≤0.3	
Davidson Lake	45.940 N 67.158 W	New Brunswick		7			7.2	33	7	≤0.3	
Lake George	45.819 N 67.047 W	New Brunswick		4.5			7.1	22-33	3-16	≤0.3	
Harvey Lake	45.743 N 67.032 W	New Brunswick		5			7.1	27	5	≤0.3	
Lake	45.707 N	New		10			7.2	22	5	≤0.3	

Magaguadavic	67.210 W	Brunswick									
Oromocto Lake	45.585 N 67.003 W	New Brunswick		14			7.1	22	5	≤0.3	
Sinclair Lake	47.053 N 66.575 W	New Brunswick		7			7.1	22	3	≤0.3	
Lac Unique	47.333 N 68.745 W	New Brunswick		6.7	111.2		7.5-8.9	82-88	4-17	≤0.3	
Lake Utopia	45.195 N 66.791 W	New Brunswick		23			6.8-7.3	34-43	5-14	≤0.3	
Walton Lake	45.612 N 65.321 W	New Brunswick		25			7.5	40	8	≤0.3	
Yoho Lake	45.780 N 66.858 W	New Brunswick		8			7.1	48	5	≤0.3	

1. Buffalo Pound is a reservoir, with two major water sources. The indicated catchment area is the estimated effective drainage area of the local catchment. The effective area is the area contributing to flow in an average year. (In this semi-arid region, the gross drainage area can be much larger). In addition to flow from this local catchment, the lake receives managed flow from Lake Diefenbaker, which has a vast catchment area.
2. Effective drainage area (see #1).
3. Chemistry data are for ice-free season in 2017.
4. Specific conductance at 25ºC.
5. In situ conductivity
6. Lake of the Woods is morphometrically complex lake with five sub-basins. Chemistry data are ranges of mean values in the mixed layer across the lake.

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Table S2. Provincial and federal Geological Survey and related websites and references describing geological characteristics of watersheds in the 2017 metals survey.

Geological Survey and related websites:

Canada	natural-resources.canada.ca/science-and-data/research-centres-and-labs/geological-survey-canada/17100 www.geologicalsurveys.ca search.open.canada.ca/opendata https://geoscan.nrcan.gc.ca/images/geoscan/1860a.jpg https://openpress.usask.ca/geolmanual/chapter/overview-of-canadian-geology/
Saskatchewan	www.saskatchewan.ca/business/agriculture-natural-resources-and-industry/mineral-exploration-and-mining/saskatchewan-geological-survey esask.uregina.ca/entry/geology.jsp#:~:text=The%20province%20is%20underlain%20throughout,unmetamorphosed%20younger%20Phanerozoic%20sedimentary%20rocks http://saskmining.ca/ckfinder/userfiles/files/97534-ResourceMap2018_English.pdf
Manitoba	www.manitoba.ca/iem/geo/index.html https://www.gov.mb.ca/iem/info/libmin/bgcms/bgcms_winnipeg.pdf
Ontario	www.ontario.ca/page/geology-and-geoscience https://www.hub.geologyontario.mines.gov.on.ca www.geologyontario.mndm.gov.on.ca/ogsearth.html www.geologyontario.mndm.gov.on.ca/mndmfiles/pub/data/records/M2518.html www.geologyontario.mndm.gov.on.ca/mndmfiles/pub/data/records/M2541.html

	open.canada.ca/data/en/dataset/d22354e8-cb01-5262-aed5-1de48d1ffb0a
Quebec	mrnf.gouv.qc.ca/en/mines/geology sigeom.mines.gouv.qc.ca/signet/classes/I1102_indexAccueil?l=a profils-profiles.science.gc.ca/en/research-centre/geological-survey-canada-quebec-division
New Brunswick	www2.gnb.ca/content/gnb/en/departments/erd/energy/content/minerals.html www2.gnb.ca/content/gnb/en/departments/erd/energy/content/minerals/content/bedrock_mapping.html www2.gnb.ca/content/gnb/en/departments/erd/energy/content/minerals/content/Surficial_mapping.html www2.gnb.ca/content/gnb/en/departments/erd/energy/content/minerals/content/GeologicalZonation.html#:~:text=The%20Maritimes%20Basin%20includes%20Late,shales%2C%20and%20subaerial%20volcanic%20rocks https://www2.gnb.ca/content/dam/gnb/Departments/en/pdf/Minerals-Minerales/Bedrock_Geology_MapNR1-e.pdf

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