

# Amplicon-based and metagenomic approaches provide insights into toxigenic potential in understudied Atlantic Canadian lakes

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## Abstract

Cyanobacterial blooms and their toxigenic potential threaten freshwater resources worldwide. In Atlantic Canada, despite an increase of cyanobacterial blooms in the last decade, little is known about the toxigenic potential and the taxonomic affiliation of bloom-forming cyanobacteria. In this study, we employed polymerase chain reaction (PCR) and metagenomic approaches to assess the potential for cyanotoxin and other bioactive metabolite production in Harvey Lake (oligotrophic) and Washademoak Lake (mesotrophic) in New Brunswick, Canada, during summer and early fall months. The PCR survey detected the potential for microcystin (hepatotoxin) and anatoxin-a (neurotoxin) production in both lakes, despite a cyanobacterial bloom only being visible in Washademoak. Genus-specific PCR associated microcystin production potential with the presence of *Microcystis* in both lakes. The metagenomic strategy provided insight into temporal variations in the microbial communities of both lakes. It also permitted the recovery of a near-complete *Microcystis aeruginosa* genome with the genetic complement to produce microcystin and other bioactive metabolites such as piricyclamide, micropeptin/cyanopeptolin, and aeruginosin. Our approaches demonstrate the potential for production of a diverse complement of bioactive compounds and establish important baseline data for future studies of understudied lakes, which are frequently affected by cyanobacterial blooms.

**Key words:** Toxic cyanobacteria, toxic blooms, cyanotoxins, microcystin, *Microcystis aeruginosa*, bioactive metabolites

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## Introduction

Cyanobacteria have the capacity to produce a myriad of secondary metabolites, some of them with potent toxic effects (i.e., microcystin, anatoxin-a, nodularins, cylindrospermopsins, and saxitoxins) affecting humans and diverse types of animals (Calteau et al. 2014; Chorus and Welker 2021). These toxic compounds, generically called cyanotoxins, become a major concern when produced by high-density blooms or dense benthic mats, as they threaten aquatic ecosystems, human health, and activities (Codd et al. 2005).

In recent years, reports of cyanobacterial blooms in freshwater systems have been increasing across Canada, although their detection relies mostly on provincial agencies that use dissimilar approaches, complicating direct comparisons (Pick 2016; Smith 2019). Blooms dominated by species of *Microcystis* are the most concerning due to their large-scale occurrence and their potential to produce microcystins, which have been detected in freshwater systems of every Canadian province at concentration levels of concern (Orihel et al. 2012). Most cyanobacteria and cyanotoxin studies have focused on conspicuous blooms occurring in large inland waters on the edge of the Canadian Shield (Pick 2016), leaving more than one million lakes distributed across Canada understudied.

In New Brunswick (Atlantic Canada), the provincial government (via the Office of the Chief Medical Officer of Health) reported that 19 water bodies have experienced cyanobacterial blooms, with 14 of them first reported in the last 5 years. However, the vast majority of these provincial reports have relied on morphological identification via microscopy (New Brunswick Department of Environment and Local Government 2019), which complicates accurate taxonomic identification and makes it impossible to determine the toxigenic potential of the cyanobacteria forming the blooms (Vezie et al. 1998; Al-Tebrineh et al. 2012). The increased reporting of cyanobacterial blooms and lack of toxicity data in this region highlights the necessity for further studies into the toxigenic potential of cyanobacteria in these freshwater systems. In addition to surface blooms, the proliferation of benthic cyanobacteria has recently been associated with toxicity in New Brunswick freshwaters. The ingestion of anatoxin-producing cyanobacterial mats from the Wolastoq/Saint John River has caused the intoxication and death of at least three dogs in Fredericton (McCarron et al. 2019).

Polymerase chain reaction (PCR)-based methods have been used extensively to detect and evaluate the abundance of toxigenic cyanobacteria in freshwater systems by amplifying sequences of genes involved in toxin biosynthesis (Baker et al. 2002; Humbert et al. 2010; Weller 2011). The inherent specificity of PCR is key for sensitivity, but it is also a disadvantage because it requires prior knowledge of the target sequence for efficient primer design (Weller 2011). Additionally, PCR-based surveys are not adequate to explore and predict broad toxigenic potentials (i.e., genetic capacity to produce a wide-range of bioactive secondary metabolites) of cyanobacteria and other microorganisms present in environmental samples. The use of shotgun DNA sequencing of environmental samples (i.e., metagenomics) and the development of bioinformatic tools have enabled the recovery of almost complete microbial genomes (McMahon 2015; Chen et al. 2020; Frioux et al. 2020) and detection of complete biosynthetic gene clusters (Cuadrat et al. 2018; Hannigan et al. 2019), overcoming the limitations of PCR-based methods (Dittmann et al. 2015; Micallef et al. 2015; Zhang and Zhang 2015).

Harvey and Washademoak Lakes (New Brunswick, Canada) have experienced seasonal cyanobacterial blooms for the past 6 years. These lakes represent different trophic regimes; Harvey Lake is categorized as oligotrophic, whereas Washademoak Lake is mesotrophic (New Brunswick Department of Environment and Local Government 2019). In this study, we aimed to determine if the cyanobacterial populations we observed during the summer–fall period of 2018 have the potential to produce cyanotoxins. We used PCR-based detection to investigate the presence of cyanobacterial genes associated with the biosynthesis of both microcystin and anatoxin-a in these lakes. We also used metagenomics to explore the toxigenic potential in both lakes and were able to assemble a near-complete genome of a *Microcystis aeruginosa* strain from the Washademoak with the gene repertoire to produce microcystin and other bioactive secondary metabolites.

## Materials and methods

### Field sampling and processing

Samples were collected bi-weekly from three sites around each of Harvey Lake and Washademoak Lake from 30 July to 10 November 2018 (Fig. 1). Harvey Lake is located in York County, New Brunswick, and is approximately 6 km long and 1.25 km wide, with a maximum depth of 13.6 m. It is classified as an oligotrophic lake, based on a median total phosphorus concentration of  $5 \mu\text{g}\cdot\text{L}^{-1}$  from 2005 to 2016 (New Brunswick Department of Environment and Local Government 2019). It is included in the Government of New Brunswick fish-stocking program, receiving landlocked Atlantic salmon. Washademoak Lake is a widening of the Canaan River upstream of the Saint John River in Queen's County, New Brunswick. It has a maximal depth of 28.7 m, and is approximately 22 km long and 2.5 km at the widest point. It is mesotrophic, based on a median total phosphorus concentration of  $17 \mu\text{g}\cdot\text{L}^{-1}$  from 2005 to 2016 (New Brunswick Department of Environment and Local Government 2019).

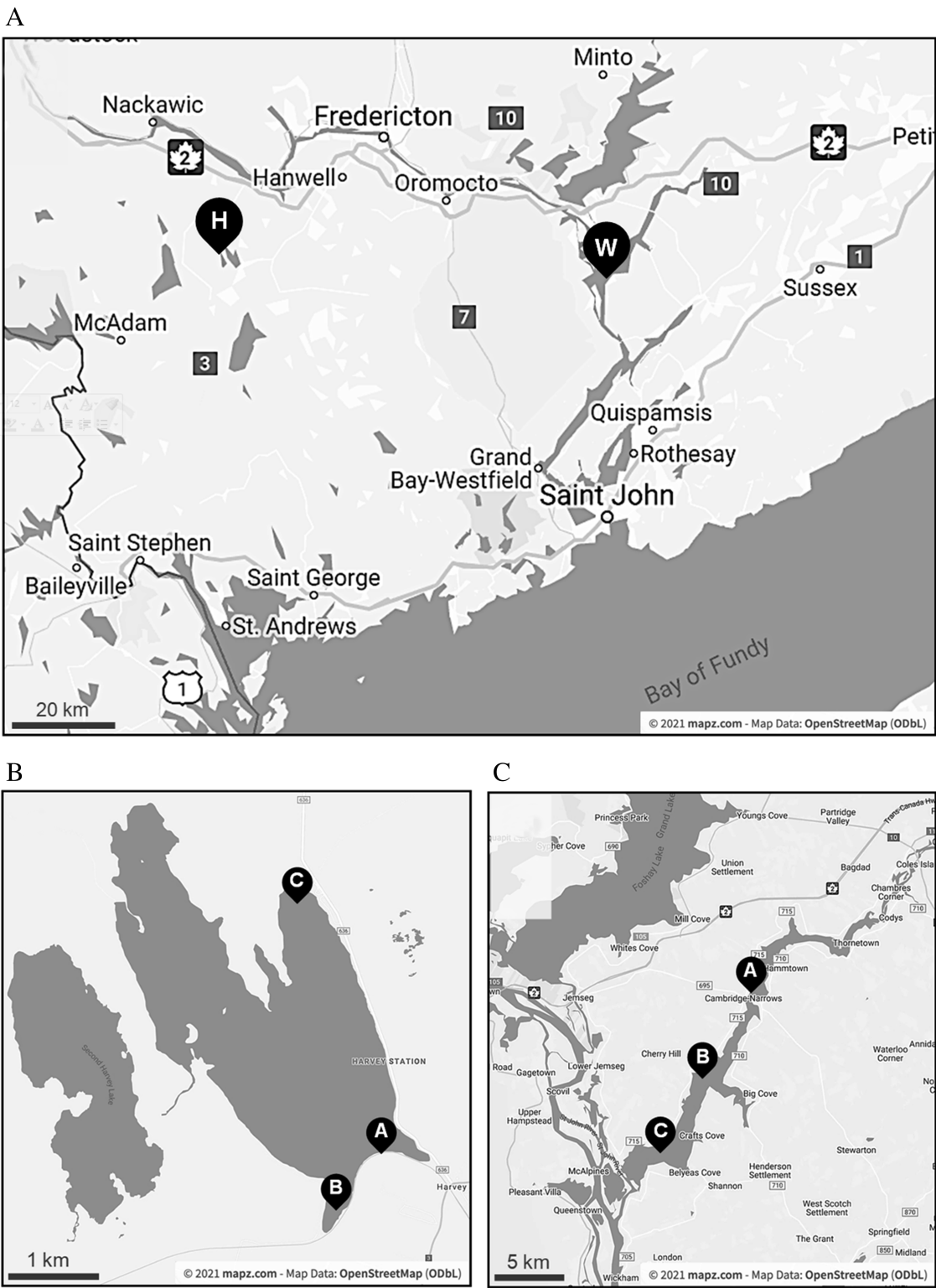
Surface water temperature was recorded, and surface samples were collected in 4-L jugs by wading, then transported back to the lab at *in situ* temperatures for immediate processing. Maximal volumes of each sample were filtered through 3- $\mu\text{m}$  nominal pore-size polycarbonate filters (MilliporeSigma, Burlington, MA, USA) (Table S1). Total DNA was extracted from filters using MoBio's PowerWater DNA Isolation Kit (now QIAGEN, Germantown, MD, USA) following the manufacturer's instructions. Extracts were eluted in 100  $\mu\text{L}$  Tris and stored at  $-20^\circ\text{C}$  until further analysis.

### Culturing toxic strains for PCR controls

Three strains of cyanobacteria were used as positive controls for the PCR-based assays. *Microcystis aeruginosa* CPCC 300 (Canadian Phycological Culture Centre, Waterloo, Canada) was grown on a 14:10 light–dark cycle in BG11 medium at  $20^\circ\text{C}$ . *Dolichospermum lemmermanii* NIVA-CYA83/1 (syn. *Anabaena lemmermanii* NIVA-CYA83/1) and *Planktothrix agardhii* NIVA-CYA126 (Norwegian Institute for Water Research, Oslo, Norway) were grown on a 14:10 light–dark cycle in Z8 medium at  $18^\circ\text{C}$ . Cultures were either extracted using MoBio's Microbial DNA Isolation Kit and eluted in 100  $\mu\text{L}$  of TrisEDTA (*Microcystis*), or MilliporeSigma's Gene Elute Bacterial Extraction Kit and eluted in 200  $\mu\text{L}$  of TrisEDTA (*Dolichospermum* and *Planktothrix*). DNA extracts were stored at  $-20^\circ\text{C}$  until further analysis.

### Detection of the microcystin synthetase E and anatoxin-a C genes

The presence/absence of the general microcystin synthetase E gene (*mcyE*) was determined using PCR-based detection with *mcyE*-F2 (Vaitomaa et al. 2003) and *mcyE*-R4 (Rantala et al. 2004) primers (Table S2). All reactions were performed in 20- $\mu\text{L}$  volumes with 1  $\mu\text{L}$  of DNA template per reaction, and positive (appropriate culture extracts) and negative ( $\text{ddH}_2\text{O}$ ) controls were included in each PCR run. For the initial screening with *mcyE*-F2 and *mcyE*-R4, the PCR mixture contained  $1 \times \text{Mg}$  (+) PCR buffer, 250  $\mu\text{M}$  dNTPs, 0.5  $\mu\text{M}$  of forward and reverse primers, 0.5 U of Ex Taq DNA (Takara Bio, San Jose, CA) polymerase and sterile water. An MJ Mini Personal Thermal Cycler (BioRad, Hercules, CA, USA) was used for the PCRs cycles with initial denaturation step at  $95^\circ\text{C}$  for 3 min, followed by 24 cycles of  $94^\circ\text{C}$  for 30 s,  $60^\circ\text{C}$  for 30 s, and  $72^\circ\text{C}$  for 1 min, and a final extension at  $72^\circ\text{C}$  for 10 min. Samples that tested positive for *mcyE* were then assessed with genus-specific assays to identify the source of the toxin genes. For *Microcystis*-specific *mcyE*, 20- $\mu\text{L}$  PCRs were set-up with 1X Ex Taq Buffer  $\text{Mg}^{2+}$  plus (Takara Bio), 250  $\mu\text{M}$  dNTPs, 0.5  $\mu\text{M}$  of *mcyE*-F2 and *MicmcyE*-R8 primers (Table S2), 1.25 U of Ex Taq polymerase (Takara Bio), and sterile water (Vaitomaa et al. 2003). The PCR amplification included an initial denaturation step at  $95^\circ\text{C}$  for 3 min, followed by 38 cycles of  $94^\circ\text{C}$  for 30 s,  $60^\circ\text{C}$  for 30 s,  $72^\circ\text{C}$  for 60 s, and a final extension at  $72^\circ\text{C}$  for 10 min.



**Fig. 1.** Maps of (A) the province of New Brunswick, Canada, indicating the location of Harvey (H) and Washademoak (W) Lakes, and sampling sites (A–C) on (B) Harvey and (C) Washademoak lakes.

(Vaitomaa et al. 2003). For *Planktothrix*-specific *mcvE*, PCRs were set-up with 1X Standard Taq Reaction Buffer (New England Biolabs, Ipswich, MA, USA), 62.5  $\mu$ M dNTPs, 0.5  $\mu$ M of *mcvE*-F2 and *PlamcyE*-R3 primers (Table S2), 1.25 U of Taq DNA polymerase (New England Biolabs), and sterile water to a final volume of 20  $\mu$ L (Rantala et al. 2006). The PCR amplification was as follows: initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s, 68 °C for 90 s, and a final extension at 68 °C for 5 min (Rantala et al. 2006). The *Dolichospermum*-specific *mcvE* reaction was set-up in 20  $\mu$ L 1X Ex Taq Buffer Mg2+ plus (Takara Bio Clontech), 250  $\mu$ M dNTPs, 5.0  $\mu$ M of *mcvE*-F2 and 0.5  $\mu$ M *AnamcyE*-R12 primers (Table S2), 1.25 U of Ex Taq polymerase (Takara Bio), and sterile water (Doblin et al. 2007). A touchdown PCR program was used, consisting of an initial denaturation step at 98 °C for 3 min, followed by 25 cycles of 94 °C for 30 s, 65 °C for 30 s decreasing by 0.5 °C per cycle, 72 °C for 60 s, and a final extension at 72 °C for 5 min (Doblin et al. 2007). PCR products were run at 70 V for 40 min on 1–1.5% TrisBorateEDTA agarose gels, stained with 1X SYBR Safe Gel stain, (Invitrogen, Waltham, MA, USA), and visualized using a BioRad gel documentation system.

The presence/absence of the anatoxin-a biosynthesis C gene (*anaC*) was assessed by PCR amplification with the *anaC*-genF and *anaC*-genR primers (Table S2; Rantala-Ylinen et al. 2011) as follows: a 20  $\mu$ L PCR was set-up with 1  $\mu$ L of DNA, 1X Ex Taq Buffer Mg2+ plus (Takara Bio), 200  $\mu$ M dNTPs, 0.5  $\mu$ M of *anaC*-genF and *anaC*-genR primers (Table S2), 1.25 U of Ex Taq polymerase (Takara Bio), and sterile water (Rantala-Ylinen et al. 2011). PCR conditions for this reaction started with an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55.5 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min (Rantala-Ylinen et al. 2011).

All samples negative for *mcvE* and *anaC* were assessed for PCR inhibition using 16 S rRNA gene amplification with the universal bacterial primers 27 F and 1492 R (Lane 1991). The PCR reaction mix (25  $\mu$ L) consisted of: 1  $\mu$ L of extracted genomic DNA, 1x reaction buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 1  $\mu$ M of each primer, Taq polymerase (1 U, New England Biolabs) and sterile water. The cycling conditions were as follows: initial denaturation at 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min. The final cycle was followed by extension at 72 °C for 5 min.

## Shotgun sequencing of total DNA from environmental samples

To investigate seasonal changes in the microbial community composition and its potential to produce cyanotoxins and other bioactive metabolites, we selected 8 DNA samples from each lake (Harvey (site A) and Washademoak (site B)) for shotgun Illumina sequencing (see Table S1 for sampling dates details). The 16 libraries (~450 base pairs insert length) were prepared with the Illumina Nextera Flex kit (Illumina, San Diego, CA, USA) for MiSeq+NextSeq platforms and sequenced (150 base pairs paired-end reads) at the Integrated Microbiology Resource of Dalhousie University ([imr.bio/](http://imr.bio/)) using the Illumina NextSeq 550 system.

## Read annotation and community composition analysis

Raw Illumina reads were uploaded to MG-RAST 4.0.3 (Meyer et al. 2008) ([mg-rast.org](http://mg-rast.org)) for quality control. Then, reads were assigned to taxonomic units using BLAT (Kent 2002) and the M5NR database (Wilke et al. 2012) as reference, considering 60% sequence similarity in a protein sequence (conceptual translations) of at least 15 amino acids and considering an E-value cut-off  $\leq 1e-5$ .

## Read processing and assembly variants

We processed the raw Illumina reads for quality control with bbdduk ([jgi.doe.gov/data-and-tools/bbtools/](http://jgi.doe.gov/data-and-tools/bbtools/)) considering the parameters ktrim = r k = 23 mink = 11 hdist = 1. Sanitized reads were then



initially assembled with the metaSPAdes pipeline (SPAdes v3.12.0) (Bankevich et al. 2012) considering the k-mer options -k21,33,55,77,99. Then we used SqueezeMeta v1.1.0 (Tamames and Puente-Sánchez 2019) to identify and retrieve individual metagenome-assembled genomes (MAGs) from each DNA sample. The SqueezeMeta pipeline first bins the assembled sequences with both MaxBin (Wu et al. 2015) and MetaBat (Kang et al. 2015), then merges equivalent pairs of binned sequences with DAS Tool (Sieber et al. 2018), and evaluates for quality assembly with CheckM (Parks et al. 2015). Each merged bin represents an individual MAG.

To investigate the phylogenetic affiliation of the recovered good quality MAGs (i.e., completeness  $\geq$  90% and contamination  $<$  5%), we used the Insert Set of Genomes Into Species Tree 2.1.10 application of KBase (Arkin et al. 2018), which estimates an approximately maximum-likelihood phylogenetic tree with FastTree-2 (Price et al. 2010) considering a reference of 49 Clusters of Orthologs Groups from closely related genomes selected based on sequence identity. The phylogenetic analysis allowed the preliminary identification of good quality cyanobacterial MAGs unambiguously affiliated to the genus *Microcystis* (details provided in the Results section).

## Assembly and annotation of the genome of a microcystin-producing cyanobacteria from Washademoak Lake

To improve the assembly (scaffolding) of the *Microcystis* MAG, likely linked to microcystin production (e.g., 27 August 2018 sample) in Washademoak Lake, we first performed independent assemblies of the 27 August read collection with MEGAHIT v1.2.9 (*de novo* assembly) (Li et al. 2015), MetaCompass v2.0.0 (reference-guided assembly with the complete genome of *Microcystis aeruginosa* NIES-843 (accession number NC\_010296) as reference) (Cepeda et al. 2017), and SPAdes v3.12.0 (with the “trusted contigs” option, using a reference-guided assembly) (Bankevich et al. 2012). Then, we used Metassembler v1.5 (Wences and Schatz 2015) to merge the *Microcystis* MAGs recovered from our four alternative independent assembling tries, which included the initial metaSPAdes try plus the MEGAHIT, MetaCompass, and SPAdes trusted-contigs assemblies. After merging the four *Microcystis* MAGs, we used GenomeFinisher v1.4 (Guizelini et al. 2016) and the complete genome of *M. aeruginosa* NIES-843 as reference for a final scaffolding step.

We used the stand-alone software package of the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al. 2016) to annotate the resulting *Microcystis* scaffolds. Then, to identify homologous genes shared between our *Microcystis* scaffolds and the complete genomes of the closely related *M. aeruginosa* strains NIES-843 and NIES-2549 (Kaneko et al. 2007; Yamaguchi et al. 2015), we used the software GET\_HOMOLOGUES v3.3.2 (Contreras-Moreira and Vinuesa 2013) with the orthoMCL algorithm (75% minimum coverage in BLAST pairwise alignments and max E-value  $1e-05$ ) (Li et al. 2003). The sequence Average Nucleotide Identity (ANI) between our scaffolded *Microcystis* genome and data from different *Microcystis* species, including isolates from freshwater bodies in Canada (Pérez-Carrascal et al. 2019), was calculated with OrthoANI v1.2 with default parameters (Lee et al. 2016). Contig mapping to reference sequences was carried out with BWA-MEM (Li 2013) with default parameters.

## Prediction of biosynthetic gene clusters in the *Microcystis* genome

To investigate the genetic potential of the *Microcystis* MAG from Washademoak Lake to produce secondary metabolites, we used the DeepBGC v0.1.18 tool (Hannigan et al. 2019) with a 0.9 threshold score to predict the presence of biosynthetic gene clusters (BGCs) and antiSMASH online bacterial version (Blin et al. 2019) to get the most similar known BGC according to MIBiG 2.0 database (Kautsar et al. 2019). We also used DeepBGC to predict the “activity” and “product class” associated to each detected BGC.

## Results

### Field observations

Chalky blue-green clumps of cyanobacteria (~1 mm in diameter) were visible in Washademoak Lake from 30 July (the first day of sampling) through to 23 October 2018, with an intense bloom visible at site B on 30 July. *Dolichospermum* sp. was identified via microscopy as the dominant species in the 30 July sample. Similar clumps were also observed at Harvey Lake from 13 August to 25 September, though their abundance was very low relative to Washademoak Lake, and this water body was not considered to be blooming during our study period.

Surface water temperature was highest in Washademoak Lake on 30 July (31 °C) and on 13 August in Harvey Lake (28 °C) (Fig. S1). By 10 November, the temperature had decreased to 8 °C in Washademoak, and 6 °C in Harvey, with a brief warm period elevating the temperature/decreasing cooling around 8 October.

### Potential for cyanotoxin production: PCR detection of *mcyE* and *anaC* sequences

More than 70% (34/48) of the surface water samples from Harvey and Washademoak lakes were PCR positive for the presence of the *mcyE* gene, with positive samples obtained from the beginning of the sampling period in late July through late October. No *mcyE*-positive samples were detected in November. Interestingly, the *mcyE* assay was negative for Washademoak site B on 30 July, when an intense cyanobacterial bloom was observed. All *mcyE*-positive samples were also positive for the *Microcystis*-specific *mcyE* primers. Only one sample (Humphrey's Wharf (site B), Washademoak Lake) was positive for *mcyE* amplification using the *Planktothrix*-specific primers, and all evaluated samples were negative for the *Dolichospermum*-specific *mcyE* priming sequences (summary of PCR results in Table 1).

PCR screening for the presence of the *anaC* gene resulted in positives for 18 of the 48 surface water samples from Harvey and Washademoak Lakes (Table 1) collected throughout the summer and fall. No samples tested positive in November.

Samples that tested negative for the *mcyE* and *anaC* gene were assayed with a universal primer pair (27F1 and 1492R1; Table S2) that amplifies the bacterial 16S rRNA gene to determine if the negative results were due to PCR inhibition. All tested samples were positive for 16S rRNA amplicons.

### The bacterial community of Washademoak and Harvey lakes

The Illumina reads produced from the last sampling date (10 November 2018) from both lakes were discarded because of low quality. Taxonomic binning of the raw reads with MG-RAST indicates that the bacterial communities in samples collected in Washademoak and Harvey lakes were mainly composed of Proteobacteria, Cyanobacteria, and Actinobacteria, which together account for at least 71% of all recovered major taxonomic groups in any DNA sample (Fig. 2a).

Cyanobacteria dominated in Washademoak Lake on 30 July and 27 August, where their relative abundance was more than 88%. This coincides with peak surface water temperatures (29–31 °C), and an intense cyanobacterial bloom that was visible on 30 July at this location. Bacteroidetes, Firmicutes, Verrucomicrobia, Planctomycetes, Chloroflexi, and Acidobacteria were other bacterial lineages present, but their accumulated relative abundance was not higher than 19% and 26% in Washademoak and Harvey lakes, respectively (Fig. 2a).

**Table 1.** Presence/absence of cyanotoxin biosynthesis genes in samples collected from Harvey and Washademoak Lakes in 2018.

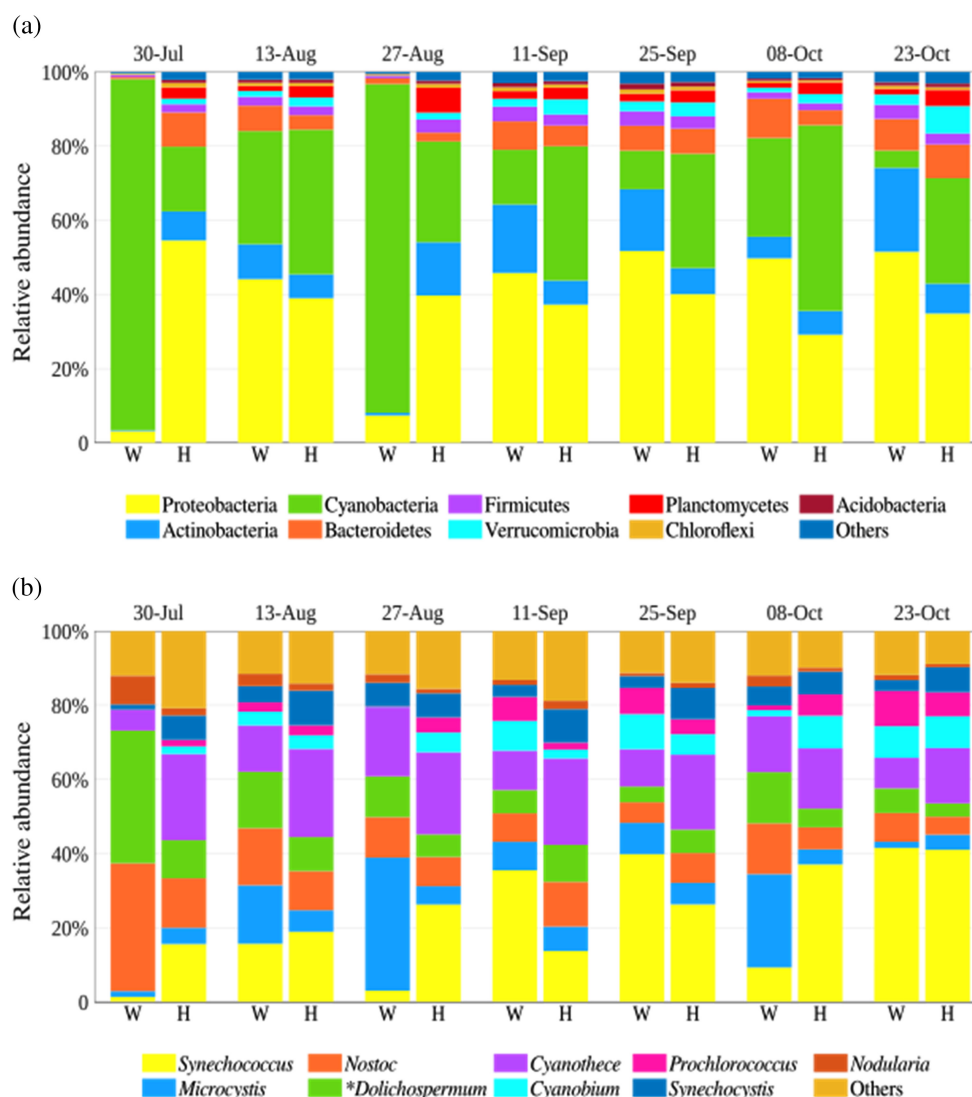
Site	PCR Assay	Date							
		30.07	13.08	27.08	11.09	25.09	08.10	23.10	10.11
Harvey Lake									
A	<i>mcyE</i>	+	–	–	+	+	+	+	–
	<i>Mic/Dol/Plank</i>	+/-/-			+/-/-	+/-/-	+/-/-	+/-/-	
	<i>anaC</i>	+	+	+	+	+	+	–	–
B	<i>mcyE</i>	+	+	–	+	+	+	+	–
	<i>Mic/Dol/Plank</i>	+/-/-	+/-/-		+/-/-	+/-/-	+/-/-	+/-/-	
	<i>anaC</i>	–	–	–	–	–	–	–	–
C	<i>mcyE</i>	+	+	+	+	+	+	+	–
	<i>Mic/Dol/Plank</i>	+/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/-/-	
	<i>anaC</i>	+	–	–	–	+	–	–	–
Washademoak Lake									
A	<i>mcyE</i>	+	+	+	+	–	+	+	–
	<i>Mic/Dol/Plank</i>	+/-/-	+/-/-	+/-/-	+/-/-		+/-/-	+/-/-	
	<i>anaC</i>	+	+	–	+	+	–	–	–
B	<i>mcyE</i>	–	+	+	+	+	+	–	–
	<i>Mic/Dol/Plank</i>		+/-/-	+/-/-	+/-/-	+/-/+	+/-/-		
	<i>anaC</i>	–	–	–	–	+	+	–	–
C	<i>mcyE</i>	+	–	–	+	+	+	+	–
	<i>Mic/Dol/Plank</i>	+/-/-			+/-/-	+/-/-	+/-/-	+/-/-	
	<i>anaC</i>	+	–	–	–	+	+	+	–

The MG-RAST analysis also revealed that members of the *Synechococcus*, *Cyanothece*, *Microcystis*, *Nostoc*, and *Dolichospermum* (planktonic *Anabaena* species have been transferred to the genus *Dolichospermum* (Wacklin et al. 2009)) genera were the more abundant cyanobacteria in both lakes (65%–79% in Washademoak and 65%–68% in Harvey) (Fig. 2b). Well-known bloom-forming genera (*Microcystis*, *Nostoc*, and *Dolichospermum*) were more prevalent in Washademoak with higher relative abundances (>45% in 4 samples) than in Harvey Lake (not higher than 29% in any sample) (Fig. 2b). In particular, the Washademoak Lake sample collected on 30 July was dominated by *Dolichospermum* and *Nostoc*, accounting for circa 70% of the total cyanobacterial abundance; yet, the PCR assays indicate there was no microcystin potential. *Microcystis* was relatively abundant in the same lake from August through October, peaking on 27 August (>35%) and 8 October (>25%), which coincides with the general and *Microcystis*-specific PCR assays indicating microcystin production potential from August through October.

The genome of *Microcystis aeruginosa* WS75, a toxin-producing cyanobacterium from Washademoak Lake

Our assembly and binning strategy recovered three good quality MAGs from Washademoak Lake, but it failed to identify assemblies of comparable quality in Harvey Lake samples. The three MAGs

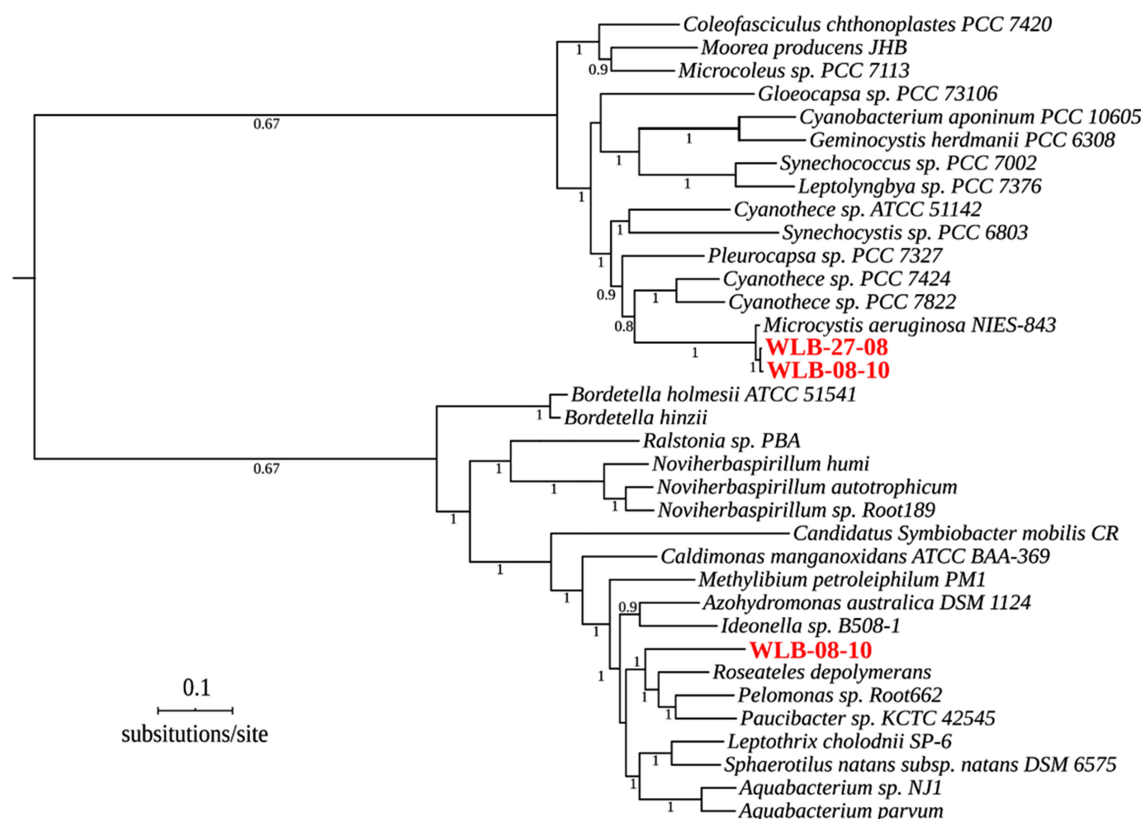




**Fig. 2.** Relative abundance and taxonomic affiliations of metagenomic sequences with (a) bacterial phyla (“Others” includes rare phyla with relative abundance < 1%) and (b) cyanobacterial genera (“Others” includes rare cyanobacterial genera with relative abundance < 5%) in Washademoak (W) and Harvey (H) lakes during 2018. \**Dolichospermum*, formerly *Anabaena*.

assembled from Washademoak Lake DNA samples (from 27 August and 8 October 2018) revealed a good degree of completeness (from 90% to 95%) and low levels of sequence contamination (from 0.5% to 4.5%). The phylogenetic analysis of a concatenated alignment of 49 core genes (Clusters of Orthologous Groups families preselected by KBase) shows that two of three Washademoak Lake MAGs belong to cyanobacteria of the genus *Microcystis*, whereas the third recovered genome is a  $\beta$ -proteobacteria of the Order Burkholderiales (Fig. 3).

The additional scaffolding steps allowed us to assemble a nearly complete cyanobacterial genome from the 27 August sample (hereafter referred as *Microcystis aeruginosa* WS75) (Table S3) highly similar (ANI > 97.4%) to *Microcystis aeruginosa* strains isolated from lakes Champlain and Caron and the reservoir Choinière in Quebec, Canada (Pérez-Carrascal et al. 2019) (Fig. S2), suggesting a



**Fig. 3.** Phylogenetic affiliation of the three good quality Metagenome Assembled Genomes (MAGs) from Washademoak Lake samples collected on 27 August (WLB-27-08) and 8 October (WLB-08-10). The phylogenetic tree was estimated with the approximately maximum-likelihood method implemented in FastTree2 (Price et al. 2010) via KBase (Arkin et al. 2018) considering a multiple alignment of 49 core genes defined by COG (Clusters of Orthologous Groups) gene families. The 49 COGs were identified and retrieved from the Washademoak MAGs with KBase. Branch length is proportional to the substitutions per site indicated by the scale bar. Values underneath branches indicate the split support evaluated with the Shimodaira-Hasegawa test.

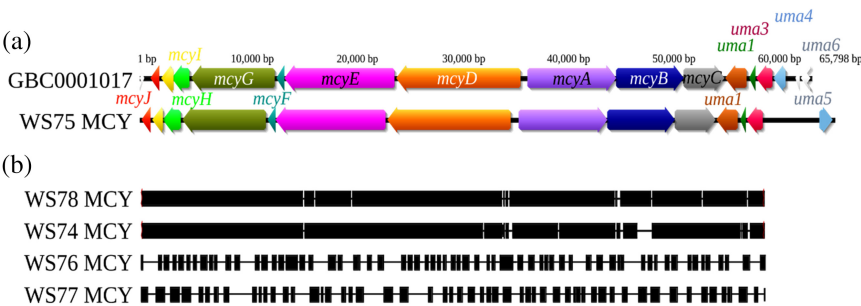
close phylogenetic relationship between these lineages from New Brunswick and Quebec (Lee et al. 2016; Jain et al. 2018). The sequence identity of *M. aeruginosa* WS75 to genomes of other *Microcystis* species (e.g., *M. flos-aquae* TF09 and *M. wesenbergii* TW10) is circa 95% ANI (Fig. S2).

Regardless of differences in size and total number of genes identified, the G+C content and number of CRISPR arrays detected in the *M. aeruginosa* WS75 assembly (circa 4.9 Mbp) are comparable to those from *M. aeruginosa* NIES-2549 and *M. aeruginosa* NIES-843 (Kaneko et al. 2007; Yamaguchi et al. 2015) (Table 2). The repertoire of RNA-coding genes (rRNAs and tRNAs) of *M. aeruginosa* WS75 is identical to the NIES-2549 strain. In contrast, only 65%–71% of the 4914 predicted genes in *M. aeruginosa* WS75 are shared with both NIES-843 (3504 shared) and NIES-2549 (3209 shared). The reciprocal ortholog detection also revealed that *M. aeruginosa* WS75 contains a set of 765 exclusive genes not shared with the other two investigated *M. aeruginosa* genomes.

The genome-mining survey detected 29 BGCs in *M. aeruginosa* WS75 accounting for more than 5% of the total assembled genomic sequences of this cyanobacterium (Table S4). According to DeepBGC, a “product activity” label was assigned to 20 of the clusters, whereas only 13 were assigned to a “product class” (Table S4). Although the antiSMASH survey indicates that most of these BGCs have no evident homologous counterparts in the MIBiG repository, 11 of them are similar to known

**Table 2.** Genomic data for *M. aeruginosa* WS75, *M. aeruginosa* NIES-2549 (Yamaguchi et al. 2015) and *M. aeruginosa* NIES-843 (Kaneko et al. 2007).

Features	<i>M. aeruginosa</i> WS75 (this study)	NIES-2549	NIES-843
Genome size (pb)	4,891,847	4,301,200	5,842,795
G+C content (%)	42.4	42.92	42.3
Total genes	4,914	4,046	5,680
RNA genes	51	51	52
CRISPR arrays	3	4	3



**Fig. 4.** The microcystin biosynthesis gene cluster of *M. aeruginosa* WS75. (a) Pairwise comparison of the gene clusters involved in biosynthesis of microcystin (MCY BGC) from *M. aeruginosa* PCC 7806 (BGC0001017) and *M. aeruginosa* WS75 (WS75 MCY). (b) The solid blocks represent mapped MCY BGC fragments from different Washademoak metagenome assemblies to the *M. aeruginosa* WS75 MCY BGC. WS78 (8 October 2018), WS74 (13 August 2018), WS76 (11 September 2018), and WS77 (25 September 2018).

clusters available in this database. These include complete, or near complete, BGCs involved in the biosynthesis of piricyclamides (5.7 Kbp, 91% of the BGC in *M. aeruginosa* PCC 7005; Fig. S3), the trypsin inhibitor aeruginosin 98- A,B,C (21.5 Kbp, 78% of the BGC in *M. aeruginosa* NIES-98; Fig. S4), micropeptin K139 (100% of the BGC in *M. aeruginosa* K-139)/cyanopeptolin (100% of the BGC in *Microcystis* sp. NIVA-CYA 172/5) (38 Kbp; Fig. S5) and, important to this work, the production of microcystin (60 Kbp; Fig. 4a). The BGC predicted with cytotoxic activity (hereafter WS75 MCY BGC) was very similar in order and orientation to the microcystin BGC of *M. aeruginosa* PCC 7806 (82% of the BGC) (Tillett et al. 2000) (Fig. 4a). We did not identify evidence of an anatoxin-a BGC in *M. aeruginosa* WS75.

### MCY biosynthetic gene clusters from incomplete genome assemblies

Our PCR analysis detected the *mcyE* gene in most DNA samples from both lakes, but besides the WS75 MCY BGC, no other contigs containing the complete MCY cluster were recovered from other samples. However, when contigs assembled with metaSPAdes were mapped against WS75 MCY cluster, fragments (contigs) of the MCY cluster were found in samples from 8 October (WS78), 13 August (WS74), 11 September (WS76), and 25 September (WS77), which matches with the PCR results (Fig. 4b). The contig mapping coverage against the WS75 MCY cluster varies from the almost complete reconstruction of the MCY BGC in samples WS78 and WS74 to the highly fragmented assemblies from WS76 and WS77 (Fig. 4b). Importantly, there is no evidence of the MCY BGC in

the Washademoak Lake samples collected on 30 July and 23 October, which is consistent with the negative *mcyE* PCR results for those same dates (Table 1). No contigs from Harvey Lake mapped to WS75 MCY BGC. We did not find evidence of additional gene clusters associated with production of other cyanotoxins in the metagenomic contigs from both lakes.

## Discussion

### PCR detection of toxin-producing cyanobacteria

The PCR screening indicates that during the 2018 sampling period, the potential for microcystin production, as inferred from the presence of the *mcyE* gene, was present throughout the summer and early fall of the year in both Washademoak and Harvey lakes. The use of different pairs of genus-specific *mcyE* primers allowed us to preliminarily identify *Microcystis* as the main contributor to the latent production of microcystin in these lakes. Despite the high relative abundance of *Dolichospermum* sequences in both lakes, which has been associated with microcystin production elsewhere (Huisman et al. 2018), no *Dolichospermum*-specific *mcyE* sequences were amplified by PCR. This result suggests that toxic genotypes of this taxa were either not present or below the limit of our PCR protocol during the period of study. The PCR survey also indicates that *Planktothrix* was a rare contributor to toxin production in certain locations. The primers used in our *anaC* PCR survey were designed to amplify sequences from diverse cyanobacterial genera (e.g., *Anabaena*, *Aphanizomenon*, *Oscillatoria*, *Dolichospermum*, and *Microcoleus*); therefore, we were unable to distinguish the particular cyanobacterial taxa associated with anatoxin-a potential. Recent reports have identified anatoxin-a, likely produced by *Microcoleus*, as a threat for human and animal health in Wolastoq/Saint John River in New Brunswick (McCarron et al. 2019), and our results indicate that this potent neurotoxin is also a concern for lakes in the same region.

The PCR approach permitted the detection of microcystin toxin potential in Harvey Lake, which has had blooms in the past but did not during this study period. This highlights the sensitivity of this approach and its ability to identify lakes at risk for toxic blooms, even under nonblooming conditions. As the frequency and severity of cyanobacterial blooms is anticipated to increase with climate change and cultural eutrophication (Huisman et al. 2018), the sensitivity of PCR provides an early-warning indicator for studying how lakes such as Harvey Lake, which have no or sporadic toxic blooms now, may transition to having more severe or frequent toxic blooms in the future.

### The microbial community composition of Washademoak and Harvey lakes

The microbial community in both lakes was dominated by Cyanobacteria, Proteobacteria, Actinobacteria, Bacteroidetes, and Verrucomicrobia, which is consistent with previous metagenomic surveys in other lacustrine environments (Eiler and Bertilsson 2004; Pope and Patel 2008; Steffen et al. 2012). Unlike Harvey Lake, typical bloom-forming cyanobacteria (*Microcystis*, *Dolichospermum*, and *Nostoc*) dominated in Washademoak Lake during 2018, and indeed, a surface cyanobacterial bloom was present during the July sampling period. Dominance by bloom-forming cyanobacteria is typically associated with specific environmental conditions (Zohary and Breen 1989; de J. Magalhães et al. 2019). For example, *Microcystis* presence is commonly correlated with high retention times in the water column, low water transparency, and more stable and prolonged periods of thermal stratification (Soares et al. 2009; Rangel et al. 2016; Guedes et al. 2018), while being more sensitive to mixing and turbulence (Reynolds 2006). Total cyanobacterial biomass is also positively correlated with water temperature and nutrient concentration (i.e., phosphorus) (Beaulieu et al. 2013; Smith 2019). Certainly, the surface water temperatures in Washademoak Lake reached a higher maximum than in Harvey Lake in 2018 (31 °C vs 28 °C) (Fig. S1), and Washademoak Lake is mesotrophic whereas

Harvey Lake is oligotrophic (New Brunswick Department of Environment and Local Government 2019).

## The genome of *Microcystis aeruginosa* WS75 from Washademoak Lake and its potential to produce bioactive metabolites

We assembled a near-complete genome of a toxigenic *Microcystis aeruginosa* strain inhabiting Washademoak Lake. The difference in genome size and presence of unique genes in *M. aeruginosa* WS75 relative to other strains of the same species (i.e., NIES-843 and NIES-2549) can be explained, in part, not only by disparities in the number of genes involved in DNA replication, recombination, and repair (Yamaguchi et al. 2018), but also by the high number of taxon-specific genes likely involved in the biosynthesis of toxins and other secondary metabolites, which is an important characteristic of this cyanobacterial lineage (Humbert et al. 2013; Huang and Zimba 2019). The high sequence identity at the genomic level between *M. aeruginosa* WS75 and *M. aeruginosa* strains characterized in fresh water bodies in Quebec strongly suggests these isolates are part of the same locally adapted lineage of the globally distributed *M. aeruginosa* (Pérez-Carrascal et al. 2019).

The recovery of MAGs from environmental samples has opened the possibility of screening for new genes and gene clusters, unlocking the previously underestimated metabolic potential of bacterial communities from lakes and other aquatic systems (McMahon 2015; Cuadrat et al. 2018). Metagenomics has been particularly useful for studying potentially toxic bloom-forming cyanobacteria, avoiding the challenges and limitations of culture-based studies (Alvarenga et al. 2017; Semedo-Aguiar et al. 2018; Pérez-Carrascal et al. 2019). Additionally, genome-scale investigations of bloom-forming and toxin-producing cyanobacteria have been key to appreciating their genetic diversity and capabilities (Pérez-Carrascal et al. 2019), genome plasticity (Meyer et al. 2017), and to improve our capacity to manage freshwater ecosystems during blooming events (Yamaguchi et al. 2020).

Our genome mining analysis revealed a high potential for *M. aeruginosa* WS75 to produce diverse secondary metabolites. That 5% of the *M. aeruginosa* WS75 genome involved in the production of secondary metabolites is consistent with previous observations that suggest cyanobacteria dedicate between 5% and 6% of their coding capacity to produce secondary metabolites, with some genomes encoding up to 23 BGCs (Shih et al. 2013; Calteau et al. 2014). Identifying the MYC BGC in the *M. aeruginosa* WS75 genome is not surprising given that members of this genus are recognized as producers of microcystins (Pineda-Mendoza et al. 2016), but contributes to understanding the genomic footprint of this toxic genus in the Atlantic region of Canada. The MYC BGC of *M. aeruginosa* WS75 lacks the *uma5* and *uma6* genes, but the *uma* coding regions (*uma1* to 6) have been reported in genomes of both toxic and nontoxic strains and are likely not involved in the biosynthesis of microcystins (Tillett et al. 2000). Consistent with the PCR survey, we found evidence of fragmented MYC BGCs in all PCR-positive samples from Washademoak Lake.

In addition to the MYC BGC, we also identified diverse gene sets associated with the production of other bioactive secondary metabolites that have not received the same attention as microcystins (Pearson et al. 2019). Cyanobacteria, and species of *Microcystis* in particular, are known to produce potentially bioactive peptides that are underexplored with respect to their relevance in human health and ecological effects (Janssen 2019). The *M. aeruginosa* WS75 biosynthetic repertoire includes the ability to produce aeruginosin, which is a potent inhibitor of serine proteases such as thrombin (involved in the human blood coagulation enzymatic cascade) and trypsin (part of the digestive enzymatic battery) (Ersmark et al. 2008; Pearson et al. 2019), with some variants being highly toxic to invertebrates (Scherer et al. 2016). We also identified BGCs involved in the production of cyanopeptolins and micropeptins (CTPs), which can act as inhibitors of the coagulation Factor Xla, human kallikrein, plasmin, thrombin, trypsin, and chymotrypsin. Due to their protease inhibition activity,



CTPs cause high mortality rates in freshwater water flea (*Daphnia* sp.) and fairy shrimp (*Thamnocephalus platyurus*) (Huang and Zimba 2019).

Secondary metabolites also play important roles in microbial community dynamics and are thought to be produced in response to abiotic and biotic stress, thus providing advantages to the holder over other organisms coexisting in the same environment (Kultschar and Llewellyn 2018). For example, microcystin produced during a *Microcystis* bloom has an allelopathic effect, inhibiting the growth of *Synechococcus elongatus* (Hu et al. 2004). In general, bioactive metabolites can modify the abiotic and biotic conditions of aquatic environments to favor dominance of bloom-forming cyanobacteria (Kurmayer et al. 2016).

Our metagenomic analysis failed to recover a MAG from Harvey Lake or genome fragments coding for the anatoxin-a BGC in either lake, despite the *anaC* gene being detected by PCR analysis. In contrast to PCR detection, shotgun sequencing of environmental DNA is typically biased towards the dominant organisms in each sample, with low probabilities of detecting rare members of the community (Bharagava et al. 2019). Accordingly, abundance of anatoxin-a-producing genotypes in both lakes is expected to be low, and microcystin producing cyanobacteria would not be dominant in Harvey Lake.

## From detection to comparative metagenomics of toxin production cyanobacteria in Washademoak and Harvey lakes

PCR-based surveys targeting specific genes involved in toxin biosynthesis are widely used, rapid, and a cost-effective method for qualitative (conventional PCR) and (or) quantitative (qPCR) identification of toxic cyanobacterial genotypes (Dittmann et al. 2017; Rantala-Ylinen et al. 2017). The sensitivity of PCR enables the detection of individual cells even before a cyanobacterial bloom occurs, providing time to initiate appropriate management actions such as public warnings and to employ more costly chemical analyses for identification and quantification of cyanotoxins (Kurmayer et al. 2017).

The sensitivity of PCR allowed us to identify, but with limited taxonomic precision, the genetic potential for microcystin and anatoxin-a production in two New Brunswick lakes that have experienced recurrent cyanobacterial blooms. This rapid and effective approach requires limited expertise to undertake, making it an accessible tool for other understudied regions where toxin potential is not well-characterized. Findings can then be used to advocate for more comprehensive monitoring programs and employing quantitative approaches, such as qPCR for gene abundance and chemical analyses for toxin concentration. In addition, the PCR results allowed us to direct our metagenomic survey to recover the almost complete genome of the microcystin-producing cyanobacteria inhabiting Washademoak Lake. Our analyses revealed that *M. aeruginosa* WS75 from Washademoak Lake belongs to a lineage with toxigenic potential widely distributed in other lakes across eastern Canada.

Detailed characterization of cyanobacteria inhabiting Atlantic Canada freshwater systems is important for a region that is relatively understudied and becoming increasingly impacted by cyanobacterial blooms. Although our PCR-based survey was targeted to genes involved in microcystin and anatoxin biosynthesis and the metagenomic approach did not recover BGCs for the production of other cyanotoxins, investigations into the cyanobacterial genetic potential to produce other, but less common, toxic secondary metabolites (e.g., cylindrospermopsins and saxitoxins) should be explored in freshwater systems of Atlantic Canada.

We hope that surveys combining PCR and metagenomic approaches, which also are becoming a relatively low-cost survey option, are regularly employed to explore the toxin and other bioactive secondary metabolite potential of cyanobacteria communities in other understudied regions.

## Author contributions

CV-C, AR-P, and JL conceived and designed the study. KH, RC, and JL performed the experiments/collected the data. CV-C, AR-P, and JL analyzed and interpreted the data. AR-P and JL contributed resources. CV-C, AR-P, and JL drafted or revised the manuscript.

## Competing interests

The authors have declared that no competing interests exist.

## Data availability statement

All relevant data are within the paper and in the Supplementary Material.

## Supplementary material

The following Supplementary Material is available with the article through the journal website at doi:[10.1139/facets-2021-0109](https://doi.org/10.1139/facets-2021-0109).

Supplementary Material 1

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