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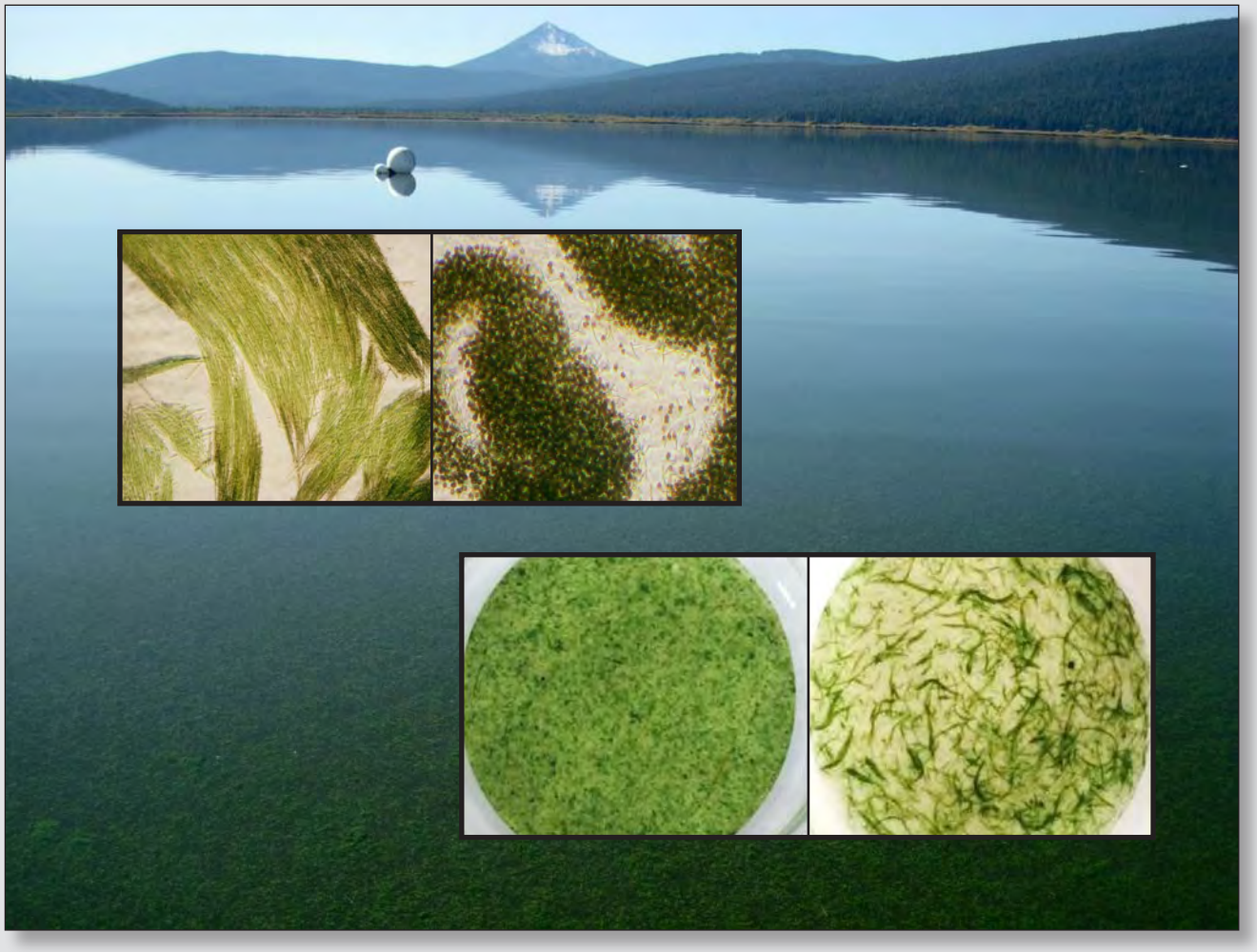
Using High-Throughput DNA Sequencing, Genetic Fingerprinting, and Quantitative PCR as Tools for Monitoring Bloom-Forming and Toxigenic Cyanobacteria in Upper Klamath Lake, Oregon, 2013 and 2014



Scientific Investigations Report 2017–5026

U.S. Department of the Interior
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Cover:
Background: Upper Klamath Lake during the late-season bloom, 2009.
Foreground top left: Phase-contrast microscope image of *Aphanizomenon* colony (magnification = 100×) in a water sample from Upper Klamath Lake, Oregon, September 2009.
Foreground top right: Phase-contrast microscope image of *Microcystis* colony (magnification = 100×) in a water sample from Upper Klamath Lake, Oregon, July 2009.
Foreground bottom right: Filtered water-column sample from Upper Klamath Lake, Oregon, showing *Aphanizomenon* dominance, June 2015.
Foreground bottom left: Filtered water-column sample from Upper Klamath Lake, Oregon, showing *Microcystis* dominance, August 2015.
(All photographs by Sara Eldridge, U.S. Geological Survey.)

Using High-Throughput DNA Sequencing, Genetic Fingerprinting, and Quantitative PCR as Tools for Monitoring Bloom-Forming and Toxigenic Cyanobacteria in Upper Klamath Lake, Oregon, 2013 and 2014

By Sara L. Caldwell Eldridge, Connor Driscoll, and Theo W. Dreher

Scientific Investigations Report 2017–5026

U.S. Department of the Interior
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U.S. Department of the Interior
RYAN K. ZINKE, Secretary

U.S. Geological Survey
William H. Werkheiser, Acting Director

U.S. Geological Survey, Reston, Virginia: 2017

Suggested citation:
Eldridge, S.L.C., Driscoll, Connor, and Dreher, T.W., 2017, Using high-throughput DNA sequencing, genetic fingerprinting, and quantitative PCR as tools for monitoring bloom-forming and toxigenic cyanobacteria in Upper Klamath Lake, Oregon, 2013 and 2014: U.S. Geological Survey Scientific Investigations Report 2017–5026, 50 p., https://doi.org/10.3133/sir20175026.

ISSN 2328-0328 (online)

Contents

Abstract....1
Introduction....1
Taxonomic Notes....3
Purpose and Scope....3
Background....5
Cyanobacteria and Microcystins in Upper Klamath Lake, Oregon....5
Genetic Tools for Monitoring Cyanobacteria Blooms....6
Study Area....6
Genetic Fingerprinting and High-Throughput DNA Sequencing to Characterize Community Structure....7
Genetic Fingerprinting: Cyanobacteria....7
High-Throughput DNA Sequencing: Total Bacteria....8
Methods: Genetic Fingerprinting and High-Throughput DNA Sequencing....9
Genetic Fingerprinting with T-RFLP....9
High-Throughput DNA Sequencing....9
Microscopic Analysis....11
Results: Genetic Fingerprinting and High-Throughput DNA Sequencing....11
Cyanobacterial Diversity Determined by T-RFLP, 2013....11
Bacterial Taxonomic Composition Based on 16S rRNA Sequencing, 2013 and 2014....12
Comparison of Phytoplankton Occurrence—DNA Sequencing Contrasted with Light Microscopy in 2013....18
Discussion: Genetic Fingerprinting and High-Throughput DNA Sequencing....21
Genetic Fingerprinting as a Tool for Taxonomic Comparisons....21
High-Throughput DNA Sequencing as a Tool for Describing Bacteria and Phytoplankton Community Structure....23
qPCR for Quantitative Analysis of Toxin-Producing and Bloom-Forming Cyanobacteria....24
Quantification of Potentially Toxigenic and Non-Toxigenic Strains of *Microcystis* using qPCR....25
Methods: Total Microcystins Analysis....25
Methods: qPCR Analysis of Total and Potentially Toxigenic *Microcystis*....26
Data Reporting and Analysis....27
Results: qPCR Analysis of Total and Potentially Toxigenic *Microcystis* and Comparisons with Microcystin Concentrations, 2013 and 2014....28
Comparison of Phytoplankton Occurrence Determined by qPCR and Light Microscopy, 2013....34
Quantification of *Aphanizomenon* and Total *Microcystis* using qPCR....34
Methods: qPCR Analysis of *Aphanizomenon* and *Microcystis*....35
qPCR Design to Target *Aphanizomenon* from Upper Klamath Lake, Oregon....35
qPCR of *Microcystis cpc*B....37
Results: qPCR Analysis of *Aphanizomenon cpc*A and Comparisons with Relative Copy Numbers of *Microcystis cpc*B, 2014....38
Discussion: qPCR as a Tool for Quantifying Populations of Bloom-Forming and Toxigenic Cyanobacteria....38

Contents—Continued

Quality Control for Bias and Variability in Sampling and Analysis 41
Field Sampling Variability 41
Analytical Variability....42
Summary....42
Acknowledgments....43
References Cited....43
Glossary....49

Figures

1. Map showing location of sampling site MDT, Upper Klamath Lake, Oregon, 2013 and 2014....4
2. Graph showing time series showing dominant terminal restriction fragments (T-RFs) detected and their log peak areas determined by T-RFLP analysis of cyanobacteria in water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013 12
3. Graphs showing proportion of the total reads classified as cyanobacteria and rarefaction curves of high-throughput DNA sequencing in water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013 and 2014 13
4. Graphs showing relative abundances of high-throughput DNA sequencing reads based on the 16S rRNA gene at phylum level of Bacteria obtained from water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013 and 2014....15
5. Graphs showing relative abundances of high-throughput DNA sequencing reads based on the 16S rRNA gene at order level of cyanobacteria obtained from water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013 and 2014....16
6. Graphs showing relative abundances of high-throughput DNA sequencing reads based on the 16S rRNA gene at genus level of cyanobacteria obtained from water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013 and 2014....17
7. Graph showing relative abundances of cyanobacteria determined by direct microscopic cell counts at the level of order in water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013 18
8. Graph showing relative abundances of cyanobacteria determined by direct microscopic cell counts at the level of genus in water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013....19
9. Comparison of cyanobacteria genera detected by microscopy contrasted with high-throughput DNA sequencing (HTS) by sample date at site MDT, Upper Klamath Lake, Oregon, 2013....20
10. Graphs showing changes in biovolume and counts of selected cyanobacteria genera measured by microscopy and high-throughput DNA sequencing (HTS) in water column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013....22
11. Graph showing total microcystins concentrations (as the sum of concentrations in the dissolved and particulate fractions) by date in water column samples collected at site MDT, Upper Klamath Lake, Oregon, 2009–2014 28
12. Graphs showing ratios of total *Microcystis* to total cyanobacteria and ratios of *mcy*E to total *Microcystis* at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014 31

Figures—Continued

13. Graphs showing total *Microcystis* and *mcy*E copy numbers determined by qPCR and microcystin concentrations in the particulate phase of water-column samples at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014 32
14. Graph showing log of *mcy*E copy numbers contrasted with *Microcystis* 16rRNA copy numbers as determined by qPCR and total microcystins concentrations in samples collected from site MDT in Upper Klamath Lake, Oregon, 2013 and 2014 33
15. Graph showing comparison of qPCR-estimated results to cell counts by microscopy, 2013.....35
16. Graph showing log concentrations of *Aphanizomenon cpc*A and *Microcystis cpc*B gene targets determined by qPCR in water-column samples collected from site MDT, Upper Klamath Lake, Oregon, 2014 38
17. Graphs showing ratios of *Microcystis cpc*B to *Aphanizomenon cpc*A, *mcy*+ *Microcystis* to total *Microcystis*, and *mcy*+ *Microcystis* to *Aphanizomenon cpc*A on different scales, 0 to less than 50 and 0.0–1.0, determined by qPCR in samples collected from site MDT, Upper Klamath Lake, Oregon 2014.....39

Tables

1. Collected water samples and analyses performed in this study, sampling site MDT (USGS site identification No. 422305121553800), Upper Klamath Lake, Oregon, 2013 and 2014.....5
2. Primers and probes used in analyses of bloom-forming and toxigenic cyanobacteria, Upper Klamath Lake, Oregon, 2013 and 2014 10
3. Percentage of relative abundances of cyanobacteria genera measured by microscopy (natural units per milliliter) and by high-throughput DNA sequencing (reads) in water-column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013 21
4. Standard curve characteristics for qPCR of total cyanobacteria, total *Microcystis*, and *Microcystis mcy*E in water column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014 26
5. Results of qPCR-based quantification of target genes and total microcystins concentrations in water-column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014.....27
6. Microcystin concentrations in the particulate phase of water-column samples as a percentage of qPCR-estimated 16S rRNA and *mcy*E gene targets specific to Microcystis in samples collected from site MDT, Upper Klamath Lake, Oregon, 2013 and 2014.....29
7. Relative abundances of total *Microcystis*, *mcy*+ *Microcystis*, and *Aphanizomenon* in water-column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014.....30
8. Spearman correlation coefficients, n, and p-values for correlations of total *Microcystis*, *mcy*+ *Microcystis*, and *Aphanizomenon* copy numbers with total microcystins concentrations in water column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014 34
9. Master mix constituents for qPCR of *cpc*A gene sequences in *Aphanizomenon*, Upper Klamath Lake, Oregon, 2014.....37
10. qPCR conditions to quantify *cpc*A gene sequences in *Aphanizomenon*, Upper Klamath Lake, Oregon, 2014.....37

Conversion Factors

International System of Units to U.S. customary units

Multiply	By	To obtain
	Length	
micrometer (μm)	3.937×10^{-5}	inch (in.)
nanometer (nm)	3.937×10^{-8}	inch (in.)
meter (m)	3.281	foot (ft)
meter (m)	1.094	yard (yd)
	Area	
square kilometer (km^2)	0.3861	square mile (mi^2)
	Volume	
liter (L)	33.814	ounce, fluid (fl. oz)
milliliter (mL)	0.03814	ounce, fluid (fl. oz)
microliter (μL)	3.3814×10^{-5}	ounce, fluid (fl. oz)
liter (L)	2.113	pint (pt)
liter (L)	1.057	quart (qt)
liter (L)	0.2642	gallon (gal)
liter (L)	61.02	cubic inch (in^3)
	Mass	
gram (g)	0.03527	ounce, avoirdupois (oz)
milligram (mg)	3.527×10^{-5}	ounce, avoirdupois (oz)
microgram (μg)	3.527×10^{-8}	ounce, avoirdupois (oz)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

$$°F = (1.8 \times °C) + 32.$$

Datums

Vertical coordinate information is referenced to the Upper Klamath Lake Vertical Datum (UKLVD), Bureau of Reclamation, which is 1.78 feet above National Geodetic Vertical Datum of 1929 (NGVD 29).

Horizontal coordinate information is referenced to the North American Datum of 1927 (NAD 27).

Elevation, as used in this report, refers to distance above the Upper Klamath Lake Vertical Datum (UKLVD), which is used by the Bureau of Reclamation for reporting the elevation of Upper Klamath Lake.

Using High-Throughput DNA Sequencing, Genetic Fingerprinting, and Quantitative PCR as Tools for Monitoring Bloom-Forming and Toxigenic Cyanobacteria in Upper Klamath Lake, Oregon, 2013 and 2014

By Sara L. Caldwell Eldridge[1], Connor Driscoll[2], and Theo W. Dreher[2]

Abstract

Monitoring the community structure and metabolic activities of cyanobacterial blooms in Upper Klamath Lake, Oregon, is critical to lake management because these blooms degrade water quality and produce toxic microcystins that are harmful to humans, domestic animals, and wildlife. Genetic tools, such as DNA fingerprinting by terminal restriction fragment length polymorphism (T-RFLP) analysis, high-throughput DNA sequencing (HTS), and real-time, quantitative polymerase chain reaction (qPCR), provide more sensitive and rapid assessments of bloom ecology than traditional techniques. The objectives of this study were (1) to characterize the microbial community at one site in Upper Klamath Lake and determine changes in the cyanobacterial community through time using T-RFLP and HTS in comparison with traditional light microscopy; (2) to determine relative abundances and changes in abundance over time of toxigenic *Microcystis* using qPCR; and (3) to determine relative abundances and changes in abundance over time of *Aphanizomenon*, *Microcystis*, and total cyanobacteria using qPCR. T-RFLP analysis of total cyanobacteria showed a dominance of only one or two distinct genotypes in samples from 2013, but results of HTS in 2013 and 2014 showed more variations in the bloom cycle that fit with the previous understanding of bloom dynamics in Upper Klamath Lake and indicated that potentially toxigenic *Microcystis* was more prevalent in 2014 than in years prior. The qPCR-estimated copy numbers of all target genes were higher in 2014 than in 2013, when microcystin concentrations also were higher. Total *Microcystis* density was shown with qPCR to be a better predictor of late-season increases in microcystin concentrations than the relative proportions of potentially toxigenic cells. In addition, qPCR targeting *Aphanizomenon* at one site in Upper Klamath Lake indicated a moderate bloom of this species (corresponding to chlorophyll *a* concentrations between approximately 75 and 200 micrograms per liter) from mid-June to mid-August, 2014. After August 18, the *Aphanizomenon* bloom was overtaken by *Microcystis* late in the season as microcystin concentrations peaked. Overall, results of this study showed how DNA-based, genetic methods may provide rapid and sensitive diagnoses for the presence of toxigenic cyanobacteria and that they are useful for general monitoring or ecological studies and identification of cyanobacterial community members in complex aquatic habitats. These same methods can also be used to simultaneously address spatial (horizontal and vertical) and temporal variation and under different conditions. Additionally, with some modifications, the same techniques can be applied to different sample types, including water, sediment, and tissue.

Introduction

Freshwater cyanobacterial blooms are of increasing global concern because they impair water quality and produce toxins (cyanotoxins) or taste-and-odor compounds. In Upper Klamath Lake, Oregon, dense blooms (characterized by chlorophyll *a* concentrations greater than 200 micrograms per liter [µg/L]; Wood and others, 2006; Lindenberg and others, 2009) occur annually from late spring to early summer and increase lake water pH (9.5 and higher; Kann and Smith, 1999) by their photosynthetic activity. These blooms usually decline by mid-summer, increasing concentrations of ammonia and other dissolved nutrients (Lindenberg and others, 2009). This seasonal cycle of cyanobacterial growth and decline also causes oxygen concentrations to fluctuate from super-saturation to near anoxia (dissolved oxygen concentrations less than 1 milligram per liter [mg/L]; Wood and others, 2006), depending on the magnitude of the bloom (Kann and Welch, 2005). Cyanotoxins are of particular interest because they are widespread in temperate and tropical aquatic systems and have been implicated in the illnesses or deaths of humans, mammals (wild and domestic), birds,

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and fish in over 50 countries worldwide (Falconer, 1999; Sivonen and Jones, 1999; Carmichael and others, 2001). Hepatotoxic microcystins, first isolated from *Microcystis aeruginosa* (Krishnamurthy and others, 1986), are produced by several genera of cyanobacteria and are the most abundant and frequently occurring of these compounds (Sivonen and Jones, 1999). Considering the anticipated climate change and increased eutrophication expected to occur globally in the future, there also is concern that cyanobacterial blooms will become more prevalent and severe and that more toxigenic populations will be favored over those that are nontoxigenic (Paerl and Otten, 2013; Wells and others, 2015).

Combinations of biological and environmental factors that promote toxigenic cyanobacterial blooms complicate the accurate prediction of bloom occurrences and the degree to which a bloom will be toxic. The use of genetic tools has led to major advances in understanding bloom dynamics, and these tools have proved to be an integral part of ongoing and developing research (Ouellette and Wilhelm, 2003; Dorigo and others, 2005; Martins and Vasconcelos, 2011). Genetic approaches can help to identify toxin-producing strains, how toxin-producers supplant non-toxin producers, and the role of bacteria and viruses in bloom stability and persistence. Genetic tools also have the potential to be used to improve the early detection of toxigenic blooms and to enable rapid interventions by resource management. In Upper Klamath Lake, genetic tools may be used to quantify the relations between water-quality conditions, cyanobacterial community dynamics, and toxin occurrence; highlighting the need for a multi-faceted monitoring program.

The information provided by different genetic tools can vary substantially depending on the DNA or RNA regions, or genes, targeted and the techniques used to reveal variations within these regions. Such genetic variability can be used to infer taxonomic or physiological differences between populations or within microbial communities. With respect to water quality monitoring, and monitoring cyanobacterial blooms in particular, genetic tools may be used routinely for detecting and quantifying the presence of key groups or species and for determining the expression of genes involved in biological processes that contribute to poor water quality, such as cyanotoxin production. In designing a data-collection program using genetic tools, choice of gene target(s) and analytical technique(s) depend on the biological question(s) asked or the nature of the required monitoring data, or both. Most environmental microbiology studies use genetic tools to address the following fundamental questions: (1) what type of microorganisms are present? (2) what are these microorganisms doing? (3) how do the activities of these microorganisms relate to environmental processes or ecosystem function?

Broadly, genetic techniques used in microbial ecology may be categorized as targeted or untargeted. Targeted approaches usually include polymerase chain reaction (PCR)-based methods where DNA or RNA extracted from an environmental sample is used as a template to characterize the microorganisms in the sample. From this basic starting point, samples may be further analyzed to assess diversity and biological function by using gene fingerprinting methods, analyzing the DNA sequences of the targeted genes, and quantifying the number of genes in the sample. Terminal restriction fragment length polymorphism, or T-RFLP, is a qualitative gene fingerprinting technique that generates a characteristic profile of a microbial community to demonstrate a natural or artificial effect on the community or differences between different communities without providing direct taxonomic identities and quantitative relations between populations. Quantitative PCR (qPCR; also known as real-time PCR) is used to simultaneously amplify, detect, and quantify specific, known gene targets in a sample. Quantification is achieved by comparisons with a standard curve constructed from known concentrations of properly characterized standards, so this technique provides a quantitative estimate of targeted gene sequences, which can be related to cell abundances in the original sample.

Untargeted approaches offer a more comprehensive view of genetic diversity compared to PCR-based genetic approaches that target only a single or few genes. These techniques attempt to analyze regions or all of the genetic information present in total DNA or messenger RNA (mRNA) extracted from an environmental sample. Specifically, large-scale DNA sequencing technologies, also called "next-generation" or "high-throughput" sequencing, allow investigations of microbial communities at much higher resolutions than more traditional sequencing methods (such as the Sanger method) and at relatively low cost per base pair (bp) of information generated. These large-scale sequencing techniques are used in many applications such as whole-genome sequencing, metagenomics, metatranscriptomics, and proteogenomics.

In this study, we used a variety of genetic tools to identify and enumerate key taxa known to create poor water quality conditions and produce microcystins in water-column samples from Upper Klamath Lake and to track changes in the presence and abundances of these organisms over the 2013 and 2014 bloom seasons. The specific objectives were (1) to characterize the microbial community at one site in Upper Klamath Lake and determine changes in the community through time using T-RFLP genetic fingerprinting of total cyanobacteria and high-throughput DNA sequencing of the total bacterial community based on a single gene target in comparison with traditional light microscopy; (2) to determine the abundances of potentially toxigenic and non-toxigenic strains of *Microcystis* and the changes through time of these abundances using qPCR; and (3) to determine the abundances of *Aphanizomenon*, *Microcystis*, and total cyanobacteria and the changes through time of these abundances using qPCR. Results of this study can be easily expanded and incorporated into future monitoring of Upper Klamath Lake or used in short-term studies where knowledge of cyanobacterial or general bacterial community dynamics is required.

Taxonomic Notes

Taxonomic ranks are referred to as a means of grouping organisms, or taxa, on the basis of shared characteristics. The seven primary taxonomic ranks used here (listed in order from more general categories to groups more closely related) are Domain (or Kingdom), Phylum, Class, Order, Family, Genus, and Species.

Previous microscopic analyses of cyanobacteria based primarily on morphology identified *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* as the key microcystin-producing and most abundant bloom-forming species, respectively, in Upper Klamath Lake. However, given that the genetic tools discussed in this report detected multiple species of *Microcystis* and *Aphanizomenon*, and because the species designation for *M. aeruginosa* may soon be revised to include all groups into a single-species complex (based on the genetic definition of a bacterial "species"), these genera are identified hereinafter as *Microcystis* spp. and *Aphanizomenon* spp. when referring to these groups at the species level. Additionally, a recent genetic evaluation of the genus *Anabaena* determined this group to be genetically heterogeneous and called for the taxonomic separation of planktonic *Anabaena*-morphotypes possessing gas vesicles from the benthic, mat-forming species (Wacklin and others, 2009). The planktonic species were reclassified into a new genus, *Dolichospermum*, whereas the benthic species remain in the genus *Anabaena*. Because this distinction is based primarily on habitat characteristics, it is not carried over into the microscopic analysis. Therefore, *Anabaena* and *Dolichospermum* appeared as distinct genera in the high-throughput DNA sequencing (hereinafter referred to as "HTS") dataset, but they were combined as the single genus *Anabaena* in the microscopy data. Furthermore, the planktonic *Dolichospermum* was not distinguished from *Aphanizomenon* in the HTS dataset because phylogenetic analysis targeting the 16S ribosomal RNA (rRNA), internal transcribed spacer 1 (ITS1), and RubisCO (*rbcLX)* gene regions (Gugger and others, 2002; Rajaniemi and others, 2005) and recent analyses of metagenomes obtained from site MDT in Upper Klamath Lake (Connor Driscoll, Theo Dreher, and others, Oregon State University, written commun., 2016) showed that these genera are indistinguishable although they differ morphologically. Therefore, in the HTS dataset, *Dolichospermum* and *Aphanizomenon* were classified into the same operational taxonomic unit (OTU; a user-defined definition of a species based solely on DNA sequence data), even though they were split into the morphotypes *Anabaena* and *Aphanizomenon* in the microscopic analysis.

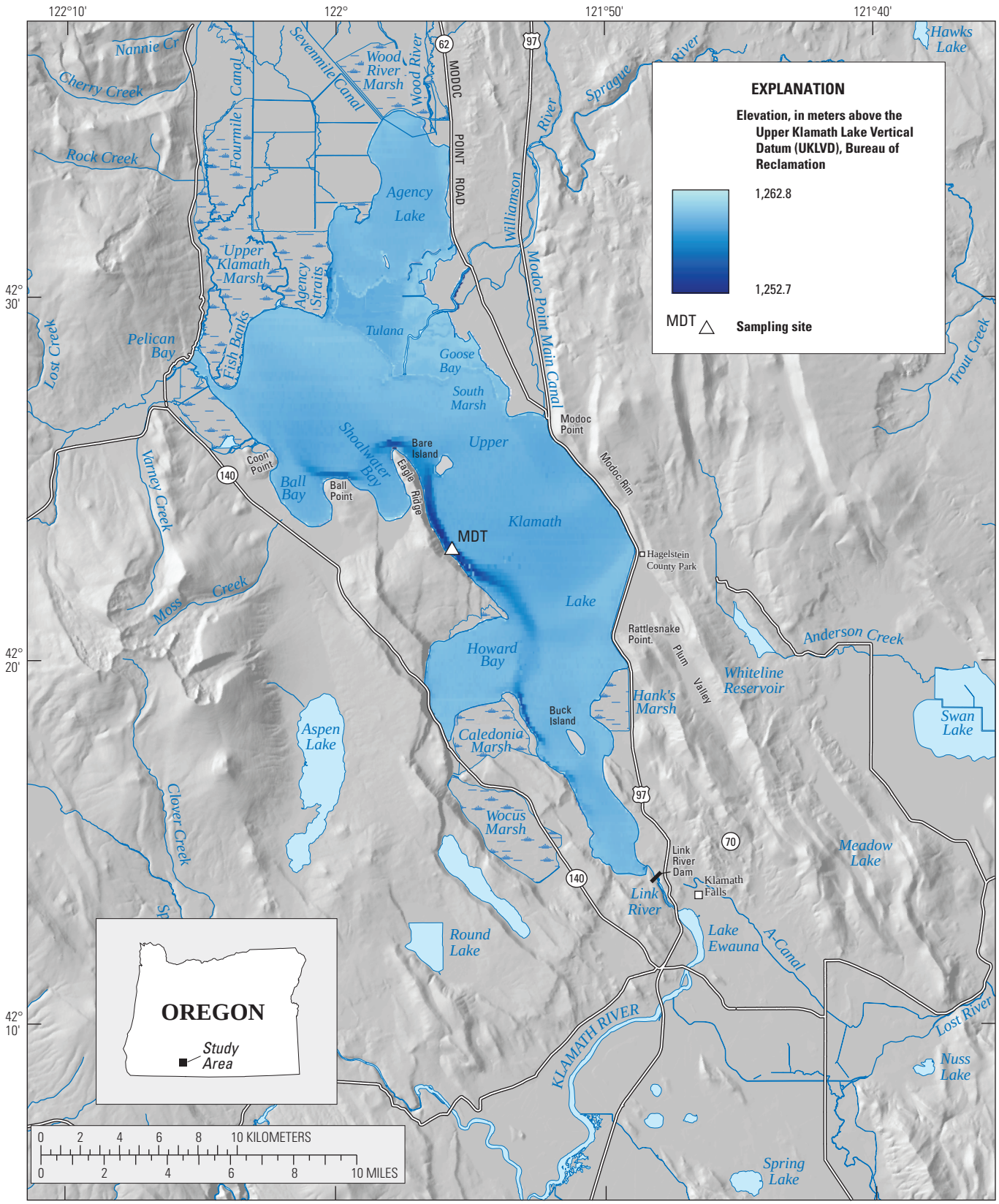
Purpose and Scope

This report presents results of genetic fingerprinting, DNA sequencing, qPCR, and microcystins analyses as outlined in the study objectives. This study was designed to support and be implemented in conjunction with the U.S. Geological Survey (USGS) Upper Klamath Lake long-term water quality monitoring program (Eldridge and others, 2014). Samples were collected from one site (site MDT, USGS site identification No. 422305121553800) on the lake between June and September in 2013 and 2014 (table 1; fig. 1). Results are discussed here in detail, but more general discussions are also included of how the tools implemented in this project can be used for other water-quality monitoring programs. Additional analytical methods are mentioned briefly to illustrate different ways of using genetic-based data in ecological or water-quality studies.

High-throughput DNA sequencing to target all bacteria (16S ribosomal RNA gene) and qPCR analyses of the gene targets specific to toxigenic *Microcystis* (microcystin synthetase E gene [*mcy*E]) and total cyanobacteria (16S ribosomal RNA gene) were performed in both study years along with direct measurements of microcystin concentrations using the enzyme-linked immunosorbent assay (ELISA). Microcystins were measured separately in the dissolved and particulate (> 63 micrometers [µm]) phases of water-column samples in accordance with the ongoing water-quality monitoring program, and the results were compared with the qPCR-determined abundances of *Microcystis* to investigate the relations between microcystin concentrations, *Microcystis* growth dynamics, and relative proportions of *Microcystis* cells containing a gene for microcystin production (*mcy*E). T-RFLP fingerprinting of the cyanobacterial community was done only in 2013, and qPCR analysis of the alpha subunit of c-phycocyanin photopigment gene (*cpc*A) from *Aphanizomenon* was done only in 2014. The design and methods of *Aphanizomenon cpc*A qPCR are provided here in more detail because these methods were not reported previously. The beta subunit of the c-phycocyanin gene (*cpc*B) from *Microcystis* also was analyzed in 2014 only so that changes in the abundance of this genus could be compared with the abundance of *Aphanizomenon* using similar, single copy gene targets and the same samples (other comparisons were made between split samples; see section, "Quality Control for Bias and Variability in Sampling and Analysis").

In 2013 only, samples were also collected and analyzed for phytoplankton identification and enumeration by microscopy. At least 400 units per sample were counted following filtration. Results of microscopically determined biovolume estimates and direct cell counts were compared with results of qPCR and high-throughput DNA sequencing to show the resolution and sensitivity more advanced technologies afford in detecting bloom-forming and toxin-producing cyanobacteria.

In addition, statistical and time-series comparisons were made between the genetic taxonomic data or microcystin concentration results presented in this report and concurrently collected water-quality and environmental data to evaluate potential triggers for increased lake toxicity and to understand the changes in phytoplankton community composition observed between 2013 and 2014. For simplicity, these results will be presented and discussed in a separate, follow-up report; this current report will focus primarily on the methods used and how they can be incorporated into a routine water-quality monitoring program.



Base modified from USGS and other digital sources, various scales.
Coordinate system is UTM, Zone 10N; North American Datum of 1927.

Figure 1. Location of sampling site MDT (USGS site identification No. 422305121553800), Upper Klamath Lake, Oregon, 2013 and 2014.

Table 1. Collected water samples and analyses performed in this study, sampling site MDT (USGS site identification No. 422305121553800), Upper Klamath Lake, Oregon, 2013 and 2014.

[**Analysis:** T-RFLP, terminal restriction fragment length polymorphism; HTS, high-throughput DNA sequencing; qPCR, quantitative polymerase chain reaction; 16S rRNA, 16S ribosomal RNA gene; *cpc*A, alpha subunit of the c-phycocyanin photopigment gene; *cpc*B, beta subunit of the c-phycocyanin gene; *mcy*E, microcystin synthetase E gene; ELISA, enzyme-linked immunosorbent assay. All samples were collected by depth integration from site MDT (USGS site identification No. 422305121553800) between June and September. Number of samples does not include samples collected for quality assurance]

Analysis	Number of samples		Purpose
	2013	2014	
T-RFLP	10	0	Fingerprint cyanobacteria for diversity
HTS	10	14	Identify bacteria present
qPCR,16S rRNA	10	14	Quantify total cyanobacteria
qPCR,16S rRNA	10	14	Quantify total *Microcystis*
qPCR, *mcy*E	10	14	Quantify *mcy*+ *Microcystis*
qPCR, *Aphanizomenon cpc*A	0	16	Quantify *Aphanizomenon*
qPCR, *Microcystis cpc*B	0	9	Quantify total *Microcystis*
ELISA	10	14	Quantify microcystin concentrations
Microscopy	6	0	Identify and enumerate phytoplankton

Background

Phytoplankton are photosynthetic microorganisms that form the base of the aquatic food chain in almost all freshwater and marine ecosystems. Excessive growth, or "blooms," of phytoplankton may substantially threaten biodiversity and ecosystem function, and they are often used as indicators of aquatic ecosystem status or health (Eiler and others, 2013). Therefore, it is important to evaluate the genetic diversity, composition, and growth dynamics of phytoplankton communities to better understand how they interact with their ecosystem and respond in composition and biomass to shifting environmental conditions, such as climate change, nutrient concentrations, and changes in land use or flow regimes (Paerl and Huisman, 2008; Posch and others, 2012). To effectively describe or predict bloom patterns, collection of taxonomic phytoplankton data requires representative, detailed, and precise methods.

Cyanobacteria and Microcystins in Upper Klamath Lake, Oregon

In Upper Klamath Lake, seasonal phytoplankton blooms are dominated by cyanobacteria, and previous studies using microscopy and direct observation have shown that the cyanobacterium *Aphanizomenon flos-aquae* comprises more than 90 percent of the phytoplankton community (by biovolume) during dense bloom periods (when rapid accumulation occurs and is recognized by a change in water coloration to green or blue-green; Kann and Smith, 1999; Carmichael and others, 2000). In most years, phytoplankton blooms in the lake begin in March, when they primarily consist of the diatom *Fragilaria* (Carmichael and others, 2000). The *Aphanizomenon*-dominated bloom typically begins to develop in late May or early June (Kann, 1997; Carmichael and others, 2000) and often co-occurs with small blooms of *Dolichospermum flos-aquae* (formerly of the genus *Anabaena*), which constitutes less than 1 percent of the total biomass during that time. In July of most years, *Microcystis* spp., *Woronichinia* sp., and *Gloeotrichia* sp. appear in the water column, and along with *Aphanizomenon* spp., these groups often persist in the lake until late autumn. During the summer or autumn in some years, *Microcystis* accumulates at high concentrations near the lake surface, particularly along the shoreline, and often appears as surface scum. However, *Microcystis* has historically represented a minor portion of the total biomass, usually less than 1 percent (Kann, 1997). Although the overall pattern in the occurrence of specific phytoplankton species between April and October each year in Upper Klamath Lake is generally understood, there is considerable variation in the timing and duration of the occurrence of specific groups, and huge variations in the concentrations of cells for a given species (*Microcystis* spp., in particular) are often observed throughout each season and from year to year. These variations may be indicative of environmental conditions (Eldridge and others, 2012, 2013), but more work is needed to better constrain those relations.

Members of the genus *Aphanizomenon* may produce hepatotoxic cylindrospermopsins and other cyanotoxins, anatoxin-a and saxitoxin (Preussel and others, 2006), but only *Microcystis* has been directly linked to microcystin occurrence in Upper Klamath Lake (Saker and others, 2007). The USGS

recently reported results of a study which characterized and compared microcystin concentrations in water samples collected from five sites in Upper Klamath Lake during the 2007–09 field seasons (Eldridge and others, 2012). Comparisons of time-series trends and statistical variations were made between daily median microcystin concentrations and other environmental parameters, including chlorophyll *a* concentrations; total, dissolved, and particulate nutrient (nitrogen and phosphorus) concentrations; and nutrient ratios. These comparisons showed microcystin concentrations generally increased following the decline of the first of two *Aphanizomenon*-dominated blooms of each season in response to an increase in bioavailable nitrogen and phosphorus. Nitrogen (N_2) fixation by *Aphanizomenon* early in the sample season (May through June) appeared to stimulate growth of toxigenic *Microcystis* later in the season (late-July through August), and these groups co-existed until late September.

Phytoplankton samples are collected routinely by the Klamath Tribes (Chiloquin, Oregon) and USGS as a component of their water-quality monitoring programs in Upper Klamath Lake (Kann and others, 2014). Samples are currently analyzed by microscopic counts of visibly recognizable groups, as microscopy is one of the primary techniques used in most quantitative studies (Soares and others, 2011). However, the high diversity of phytoplankton shapes and sizes can complicate quantification, particularly for large, irregular-shaped colonies and for cyanobacteria that are not easily observed, such as the picoplankton, *Synechococcus* spp. Furthermore, *Microcystis* spp. populations can contain both toxigenic and nontoxigenic strains, and the ability of a cell to produce microcystins cannot be determined by microscopy since there is no corresponding morphological trait for toxin production. Since the 1970s, biochemical, or molecular, techniques have been used to identify phytoplankton species based on such markers as carbohydrates, proteins, and DNA or RNA (Rohrlack and others, 2008; Gjølme and others, 2009; Ebenezer and others, 2012). Such methods have multiple advantages over using microcopy alone and, therefore, are increasingly popular for use in ecosystem studies.

Genetic Tools for Monitoring Cyanobacteria Blooms

Genes and gene expression is a subject of molecular biology. Molecular analyses allow for the use of informational molecules to characterize populations, infer microbial biomass, or locate and quantify specific organisms of interest. Ribosomal RNA (rRNA) is the most commonly used of these information molecules for assessments of community structure and for categorization of Bacteria because this molecule is highly conserved (has a very slow mutation rate) among genera and species. The ribosome is the highly complex subcellular structure on which protein synthesis occurs (Lake, 1985). Bacterial ribosomes contain large conserved RNAs (16S and 23S), of which 16S rRNA is the most extensively used for sequence comparisons. RNA is transcribed (copied) from chromosomal DNA, and the portion of the DNA from which rRNA is transcribed is the rRNA gene. Analysis of 16S rRNA sequences, and analogous 18S rRNA sequences in eukaryotes, led to the conclusion that all organisms are related and that DNA (or corresponding RNA) sequence comparisons could be used to infer a universal phylogeny, or a "tree of life" (Woese, 1987). Thus, specific gene sequence information provides information on the phylogenetic context of an organism. Once a phylogeny is established by genetic evaluations of cultured organisms or by sequencing and assembly of genomes from environmental samples, comparisons of DNA sequences obtained from organisms in the environment can be used to place these new sequences into the working phylogeny without isolation and culturing required. Parts of the ribosome evolve very slowly, so they are nearly identical in all organisms. Such regions are considered to be universally conserved. Other areas of the 16S rRNA gene are less well conserved, so they are common only to certain groups or taxa. These more variable regions can be used as targets in PCR-based techniques to identify specific phylogenetic groups of interest. Besides RNA, many other genes are routinely used for genetic studies to identify an organism or a potential metabolic characteristic of an organism. Genes used to target cyanobacteria include 16S rRNA, the internal transcribed spacer (ITS) targeting the 16S-23S boundary of bacterial ribosomal RNA genes, and the phycocyanin intergenic spacer (PC-IGS), among others. Although choices of gene target may vary, the importance of identifying specific genes or gene regions to target only the taxa of interest remains, and this is often the key consideration in using genetic tools.

DNA-based detection methods are often preferred over traditional methods because of their potential specificity, sensitivity, and speed. Such methods provide rapid detection of toxigenic cyanobacteria and are useful for general ecological studies. Genetic methods have been extensively used to characterize the cyanobacterial community in complex aquatic and sediment habitats (Kolmonen and others, 2004; Rinta-Kanto and others, 2005; Zwart and others, 2005; Ouellette and others, 2006; Becker and others, 2007; Junier and others, 2007; Latour and others, 2007; Bozarth and others, 2010; Ye and others, 2011). These same methods also have been used to investigate spatial and temporal variability in the cyanobacterial community (Ye and others, 2011; Otten and others, 2012; Eldridge and others, 2013).

Study Area

Upper Klamath Lake is within the Klamath Graben structural valley at the base of the Cascade Mountains (eastern slope) in south-central Oregon. It is a naturally formed, large and shallow hypereutrophic lake with a surface area of 305 square kilometers (km^2), an average depth of 2.6 meters

(m), and a drainage basin of 9,415 km^2 (fig. 1). More than 90 percent of the lake is less than 4 m deep, but along the western shoreline between Eagle Ridge and Buck Island, the depth may reach 15 m. The Williamson River enters the lake from the north and contributes approximately 46 percent, on average, of the annual total inflowing water (including groundwater, precipitation, and surface-water runoff) to the lake (Johnson, 1985).

Since the mid-1800s, major land-use changes in the watershed, including the clear-cutting of forest, the grazing of cattle in upstream flood plains, the degradation of riparian corridors, the draining of neighboring wetlands, and the subsequent degradation of peat soils, have coincided with higher sediment accumulation rates, higher nutrient concentrations, and a decrease in the total nitrogen to total phosphorus ratio (TN:TP; Eilers and others, 2004). Although the lake is natural, it is artificially controlled. Upper Klamath Lake has been the primary water source for the Klamath Project, which has supplied water to agricultural areas within the Upper Klamath Basin since the completion of the Link River Dam at the southern outlet of the lake in 1921 (Stene, 1994). This has enabled regulation of lake water levels to produce more extreme annual fluctuations in surface water elevation than occurred under pre-dam conditions.

Upper Klamath Lake is at the head of the Klamath River, where blooms of *Microcystis* and *Aphanizomenon* occur infrequently in the small JC Boyle Reservoir but frequently and at high densities in the large Copco and Iron Gate Reservoirs. Large *Aphanizomenon* blooms also can occur in the small Keno Reservoir just downstream of Upper Klamath Lake, but the short residence time here suggests these "blooms" may only be from inflow from Upper Klamath Lake. These blooms are thought to be sustained by diffuse nutrient inputs and by microbial cycling of organic matter and nutrients that originate in Upper Klamath Lake (Kann and Asarian, 2007; Moisander and others, 2009; Otten and others, 2015). The presence of *Microcystis* in Upper Klamath Lake indicates that the lake may be a source of *Microcystis* cells to the Copco and Iron Gate Reservoirs (Otten and others, 2015), so results of monitoring or research in Upper Klamath Lake has implications for downstream processes.

Genetic Fingerprinting and High-Throughput DNA Sequencing to Characterize Community Structure

Previous studies of the Upper Klamath Lake phytoplankton community and the relations between phytoplankton density and nutrient dynamics have focused primarily on *Aphanizomenon* spp. because of the apparent dominance of this species, based on microscopic cell counts. Likewise, *Microcystis* spp. is most often targeted for microcystins monitoring in the lake because of previous work that showed microcystin production by this species. However, despite the direct significance of these groups to water quality, the phytoplankton community in Upper Klamath Lake has been shown to exhibit far more diversity and temporal variation than is often revealed by studies targeting one or two key species (Kann and others, 2014). To that end, the first objective of this study was to use genetic fingerprinting and high-throughput DNA sequencing as tools to describe the microbial community in water samples collected from a single site over 2 years. Although understanding patterns of cyanobacterial growth and decline (increasing and decreasing cell densities and effects of their metabolic activity) has been the primary concern related to water quality in Upper Klamath Lake, total bacteria, including cyanobacteria, were targeted for HTS in this study to provide a more comprehensive view of the microbial community in water-column samples and to show the overall contribution of cyanobacteria within that community.

Genetic Fingerprinting: Cyanobacteria

T-RFLP analysis was used to create genetic fingerprints of cyanobacteria in water-column samples from one site in Upper Klamath Lake. A genetic fingerprint is a unique pattern of DNA fragments (terminal restriction fragments [T-RFs]) of different sizes (expressed as DNA base pairs) created from cleavage by specific restriction enzymes that may be used to distinguish between genetically distinct organisms based on where the enzymes cleave the DNA. As with other genetic fingerprinting techniques, T-RFLP allows the products of PCR-amplified variants of a gene target to be separated, resulting in distinctive patterns, or fingerprints (Liu and others, 1997). T-RFLP analysis is a sensitive, rapid, and highly reproducible method for assessing the diversity of complex bacterial communities and for rapidly comparing the community structure and diversity between samples, substrates, or ecosystems. Reviews by Marsh (1999) and Kitts (2001) provide examples of T-RFLP applications to soils, bioreactors, aquifer sand, and groundwater.

In this method, T-RF patterns represent different genetic variants and are generated by amplifying DNA fragments from a common gene of a mixed community in an environmental sample using PCR with one or two fluorescently labelled primers. The PCR products are then digested with restriction enzymes that cleave DNA strands at or near specific sequence motifs. The resulting patterns, which are visualized as a graph, or electropherogram, of fluorescence intensity as a function of the number of base pairs can then be used to assess the diversity of microbial communities in the samples (Zhang and others, 2008). A comparison of electropherograms among samples collected at different times or from different sites can be used to assess relative temporal and spatial variability in the microbial community. An advantage of the T-RFLP method over other fingerprinting methods is that the data can be compared with available databases, such as the

Ribosomal Database Project (RDP; http://rdp.cme.msu.edu) and Phylogenetic Assignment Tool (PAT; Kent and others, 2003) to assess potential identities of the bacteria contributing to each fragment. T-RFLP is a powerful and effective tool for comparisons in taxonomic diversity between samples, but caution should be used in interpreting T-RFLP profiles because, as with other PCR-based methods, this technique has some inherent biases and may underestimate bacterial diversity (Liu and others, 1997).

High-Throughput DNA Sequencing: Total Bacteria

Genetic fingerprinting allows for rapid screening of environmental samples for genetic diversity, but as a "next step" in detecting or identifying specific species or taxa present in a sample, DNA sequencing methods are routinely used. Over the past decade, next-generation HTS technologies have emerged that provide unprecedented throughput, scalability, and speed far exceeding the capacity of traditional Sanger DNA sequencing. Sequence-based analyses have provided unique insights into microbial diversity and have allowed for more detailed explorations of microbial communities. HTS can be applied to small, targeted regions of DNA or to the entire genome through a variety of methods, offering advantages in throughput and scale not previously achieved. Large stretches of DNA can be sequenced in a massively parallel fashion with HTS. After assembly and analysis, such massive sequence datasets can, for example, help ecologists identify rare or niche organisms present in an ecosystem that would have been missed with the traditional Sanger sequencing technique. The most comprehensive method is the sequencing of whole genomes or communities of genomes (shotgun metagenomes). See Ju and Zhang (2015) for a review of metagenomics applied to environmental science and biotechnology. As an alternative to shotgun metagenomic sequencing, targeted sequencing of PCR-amplified gene regions (amplicon sequencing) can be used to sequence a subset of the genome or regions of a gene, allowing researchers to focus time, expenses, and analysis on specific areas of interest. This generates a smaller, more manageable dataset and reduces sequencing costs, turnaround time, and data analysis burdens. This also enables "deep" sequencing (where the total number of sequenced reads is many times larger than the length of the sequence under study) to detect more rare genetic variants.

Compared to microscopy, HTS provides multiple advantages for analysis of phytoplankton communities, including the automation of sample handling and preparation which may lower analytical costs and provide results more quickly (Eiler and others, 2013). Automated processing also permits increased sampling frequency and large-scale comparisons of samples from various locations, time points, and substrates. HTS can also be standardized between laboratories and provides more reproducible results than can be achieved with microscopy, which relies heavily on the skills and experience of the taxonomist doing the analysis. This is of particular concern in long-term studies, in that more than one operator or laboratory may be employed for analysis over the duration of the study, which is likely to vary the results considerably (Eiler and others, 2013). Genetic methods also have the advantage of detecting nano- and picophytoplankton or functional types that cannot be discriminated by morphology. Furthermore, HTS provides greater resolution in detecting rare taxa, discriminates between distinct taxa with similar or identical morphologies, and does not discriminate between life stages (Eiler and others, 2013).

The popularity of HTS-based methods may prompt the use of this tool as a way to directly infer species abundances in the sampled environment, because the HTS data generated from complex communities reveals the composition and relative abundances of specific taxonomic groups. However, the relation between environmental species abundance and the abundances of sequence reads in an HTS dataset has not been sufficiently tested in natural communities, especially in aquatic ecosystems (Sun and others, 2015). In addition, differences in DNA (or RNA) extraction techniques or efficiencies (not standardized) can produce variable outcomes, and no distinction can be made between living or dead cells using genetic methods. Therefore, HTS should be regarded as relative or proportional, and interpretations of HTS data as quantitative inference regarding environmental communities should be taken with caution. It is also important to note that the resolution in taxonomic identification obtained by HTS relies on comparisons with existing databases which may lack cyanobacterial or other sequences. In such cases, database gaps can lead to results that mischaracterize the community being studied.

HTS technology has been used successfully to study microbial diversity at much greater resolution than is possible with microscopy alone. However, it is important to note a primary factor in designing a HTS-based study or data-collection program is to select the most appropriate gene target or taxonomic indicator. Most phytoplankton communities are composed of a collection of both Bacteria (cyanobacteria, for example) and Eukarya (diatoms and green algae, for example). But there is currently no universal primer that targets both domains; the 16S rRNA gene is typically used for bacteria, and in eukarya the target is typically the 18S rRNA gene. Simultaneous detection may be achieved by shotgun metagenomics or by focusing on the 16S rRNA gene because it is universal in bacteria and is also present in the chloroplasts of photosynthetic eukaryotes (Eiler and others, 2013). But considerable differences are likely between cyanobacteria and eukaryotic algae in the priming sites to target the 16S rRNA gene, which may result in an underestimation of eukaryote abundances using the 16S rRNA gene target rather than the 18S rRNA gene (Timothy G. Otten, Bend Genetics, written commun., 2016).

Methods: Genetic Fingerprinting and High-Throughput DNA Sequencing

Genetic Fingerprinting with T-RFLP

Depth-integrated water-column samples were collected weekly from July 9, 2013, to September 10, 2013, from site MDT in Upper Klamath Lake (fig. 1) as outlined in Eldridge and others (2012), and shipped overnight to the USGS Ohio Water Microbiology Laboratory (OWML; Columbus, Ohio) on ice. Samples (20–100 milliliters [mL], depending on bloom density) were filtered in duplicate at OWML onto nucleopore polycarbonate filters (Whatman™/GE Healthcare®, Piscataway, New Jersey) with 0.4-micrometer (µm) pore size. Filters were placed into 2-mL vials containing 0.3 gram (g) of acid-washed glass beads (Sigma-Aldrich®, St. Louis, Missouri) and held at -70 °C until further analysis. One set of the duplicate filters remained at OWML for qPCR analysis, and the other set was sent to the USGS Michigan Bacteriological Research Laboratory (MI-BaRL; Lansing, Michigan).
At MI-BaRL, DNA was extracted from filters using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California) following the manufacturer's instructions; DNA extracts ranged in concentration between 3.0 and 8.5 ng/µL. PCR was performed using cyanobacterial specific 16S rRNA gene primer sequences (forward and reverse primers CYA359F, CYA718R(a) and CYA718R(b); table 2) and PCR conditions as described in Nübel and others (1997). A 2-microliter (µL) aliquot of template DNA was used per 25 µL reaction. PCR conditions were as follows: 95 °C for 10 minutes then 25 cycles of 95 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1 minute with a final extension at 72 °C for 7 minutes. The PCR master mix consisted of $MgCl_2$ (1.8 millimolar [mM]), a mixture of four deoxyribonucleotides (dNTPs; 0.2 mM), bovine serum albumin (BSA; 0.1 µL), AmpliTaq Gold® DNA polymerase with buffer II (0.2 units/µL and 1.5×, respectively; Life Technologies™, Grand Island, New York). The labeled PCR reactions used for T-RFLP included dual high performance liquid chromatography-purified, fluorescein amidite (FAM)-labeled forward primer and the reverse primers. The final concentrations of all primers were 200 nanomolar (nM). For restriction digests, PCR reactions were scaled up to 3 × 100 µL reactions for each sample. After amplification, triplicate reactions were pooled and the approximately 420 base-pair PCR product was visualized using a FlashGel® system for DNA (LONZA Inc., Walkersville, Maryland). PCR products were purified using a PCR purification kit (Qiagen, Valencia, California) and quantified using a NanoDrop® 2000 (Thermo Scientific, Williamton, Delaware). Approximately 200 nanograms (ng) of DNA was digested with 30 units of MspI and RsaI restriction endonuclease (New England Biolabs®, Ipswich, Massachusetts) for 3 hours at 37 °C following the manufacturer's instructions. The enzyme digests were separated into two technical replicates and sent to Genewiz, Inc. (Plainfield, New Jersey) for T-RFLP analysis. T-RFLP fragments were run on an ABI® 3730 DNA analyzer, and the results were evaluated at MI-BaRL using Peak Scanner® software (Applied Biosystems, Grand Island, New York). Changes in the cyanobacterial community through time were estimated from the electropherogram peak areas in each sample, which represented the relative abundances of each T-RF, or genetic variant.

High-Throughput DNA Sequencing

A portion of the DNA extracted prior to T-RFLP at MI-BaRL was sent for next generation sequencing using the Illumina MiSeq® system (Illumina, Inc., San Diego, California) at the Research Technology Support Facility (RTSF Genomics Core) at Michigan State University (East Lansing, Michigan). Community DNA from each sample was submitted for sequencing using 16S rRNA universal primers specific to the V3/V4 region of the 16S rRNA gene. These primers were designed to target the total bacterial community of the sample. The Illumina MiSeq system uses clonal amplification and sequencing by synthesis chemistry. The process simultaneously identifies DNA bases while incorporating them into a nucleic acid chain. Each base emits a unique fluorescent signal as it is added to the growing strand which is used to determine the order of the DNA sequence (http://www.illumina.com/systems/miseq.html). The V3/V4 region of the 16S rRNA gene was amplified using Illumina fusion primers as described by Caporaso and others (2012). Individual PCR products were normalized using a SequalPrep® DNA normalization plate (Invitrogen®, Carlsbad, California), and the normalized products were pooled. After quantitation, the pool was loaded on a standard Illumina MiSeq flow cell and sequenced in a 2 × 250 bp paired-end format using a MiSeq v2 500 cycle reagent kit. Base calling was performed by the Illumina Real-Time Analysis (RTA) software, version 1.18.54, and the output of the RTA analysis was demultiplexed and converted to FASTQ (text-based) format with the Illumina Bcl2FastQ software, version 1.8.4. After sequencing, the FASTQ files were processed using the Mothur program (Schloss and others, 2009; http://www.mothur.org) at the USGS Klamath Falls Field Station. Each sample was analyzed separately.

Output from the sequencer as FASTQ format files, together with a list of the samples and associated file names (forward and reverse reads separately), were first used to generate contiguous, overlapping sequence reads (contigs) in the Mothur program. Ambiguous sequences and poorly generated reads (as indicated by their unexpected lengths) were then removed from the data, and duplicates were removed to simplify further computations in the program.

Table 2. Primers and probes used in analyses of bloom-forming and toxigenic cyanobacteria, Upper Klamath Lake, Oregon, 2013 and 2014.

[**Purpose:** T-RFLP, terminal restriction fragment length polymorphism; HTS, high-throughput DNA sequencing; qPCR, quantitative polymerase chain reaction. **Primer or probe:** Forward and reverse designations refer to primer orientation in relation to the rRNA gene. F, forward; R, reverse. **Specificity:** *cpc*A, alpha subunit of the c-phycocyanin photopigment gene; *cpc*B, beta subunit of the c-phycocyanin gene; *mcy*E, microcystin synthetase E gene. **Sequence (5′→3′):** Y indicates a C/T nucleotide degeneracy]

Purpose	Primer or probe	Specificity	DNA sequence (5′→3′)	Reference
T-RFLP	CYA359F	Cyanobacteria 16S rRNA	GGGGAATYTTCCGCAATGGG	Nübel and others, 1997
T-RFLP	CYA718R(a)	Cyanobacteria 16S rRNA	GACTACTGGGGTATCTAATCCCATT	Nübel and others, 1997
T-RFLP	CYA718R(b)	Cyanobacteria 16S rRNA	GACTACAGGGGTATCTAATCCCTTT	Nübel and others, 1997
HTS	357F	Bacteria 16S rRNA	CTCCTACGGGAGGCAGCA	Caporaso and others, 2012
HTS	806R	Bacteria 16S rRNA	GGACTACCAGGGTATCTAATCCTGTT	Caporaso and others, 2012
qPCR	CYAN 108F	Cyanobacteria 16S rRNA	ACGGGTGAGTAACRCGTRA	Rinta-Kanto and others, 2005
qPCR	CYAN 377R	Cyanobacteria 16S rRNA	CCATGGCGGAAAATTCCCC	Rinta-Kanto and others, 2005
qPCR	TaqMan probe	Cyanobacteria 16S rRNA	CTCAGTCCCAGTGTGGCTGNTC	Rinta-Kanto and others, 2005
qPCR	MICR 184F	Total *Microcystis* 16S rRNA	GCCGCRAGGTGAAAMCTAA	Rinta-Kanto and others, 2005
qPCR	MICR 431R	Total *Microcystis* 16S rRNA	AATCCAAARACCTTCCTCCC	Rinta-Kanto and others, 2005
qPCR	TaqMan probe	Total *Microcystis* 16S rRNA	AAGAGCTTGCGTCTGATTAGCTAGT	Rinta-Kanto and others, 2005
qPCR	127F	*Microcystis mcy*E	AAGCAAACTGCTCCCGGTATC	Sipari and others, 2010
qPCR	247R	*Microcystis mcy*E	CAATGGGAGCATAACGAGTCAA	Sipari and others, 2010
qPCR	TaqMan probe, 186P	*Microcystis mcy*E	CAATGGTTATCGAATTGACCCCGGAGAAAT	Sipari and others, 2010
qPCR	AFA_cpcA_F	*Aphanizomenon cpc*A	TTAACCGCTAAAGCTCAACA	This study
qPCR	AFA_cpcA_R	*Aphanizomenon cpc*A	CATCGGATGCGTATTGATTA	This study
qPCR	Fluorigenic probe	*Aphanizomenon cpc*A	[6-FAM]-TTACAGCAAATTCCCTTACACCACATCT-[BHQ1a-Q]	This study
qPCR	cpcBF	*Microcystis cpc*B	CATCTKGCGMTATGTTACCTAC	Kurmayer and Kutzenberger, 2003
qPCR	cpcBR	*Microcystis cpc*B	CGMAGMCCATTTAAGCAACGAT	Kurmayer and Kutzenberger, 2003
qPCR	TaqMan probe	*Microcystis cpc*B	[6-FAM]-TACGCTACCTTCKCTGGCGA-[BHQ1]	Kurmayer and Kutzenberger, 2003

The resulting sequences were then aligned to the SILVA small subunit rRNA gene database, release 119 (http://www.arb-silva.de/; Quast and others, 2013). Aligned sequences were filtered to remove overhangs (misaligned sequences) and gaps, and the sequences were pre-clustered to further reduce noise in the data. Chimeras were removed using the UCHIME algorithm (Edgar and others, 2011) called within the Mothur program. Finally, sample sequences were assigned to OTUs by splitting sequences into bins and clustering within each bin with a 97 percent identity cutoff. Sequences were split at the order level and classified to the genus level using the Greengenes reference taxonomy (DeSantis and others, 2006). Final OTU results were compiled for all samples, and the relative percentages of the more abundant groups (arbitrarily determined, generally more than about 20 reads per sample) were used for further analysis and interpretation. Because of their large abundance in the resulting dataset, unclassified bacteria were excluded from figures and interpretations of sequencing data at the genus level.

Microscopic Analysis

Subsamples (500 mL) split from the same depth-integrated water-column samples collected at site MDT (fig. 1) for T-RFLP and DNA sequencing following USGS water sampling protocols (Eldridge and others, 2012) were preserved with glutaraldehyde to produce a final concentration of 0.25–0.50 percent in the sample. Microscopy samples were collected approximately every week between July 23 and September 10, 2013, and shipped to PhycoTech®, Inc. (St. Joseph, Michigan) for analysis. Phytoplankton were enumerated with an Olympus® BX51 compound microscope and proprietary database-driven software that allows up to 400 taxa to be enumerated per sample. All calculations, including concentrations, biovolumes, biomasses, and diversity indices were completed within this program. Multiple magnifications between 400 and 1,000× were used for analysis and varied with the size of the dominant taxa and the size and number of particulates in each sample. Biovolume was measured by determining the maximum greatest axial length dimension and the length, width, and depth of different aspects of colonies or cells. Cell and colony shapes were approximated to a geometric figure. Phytoplankton composition was determined to the finest level possible, usually species. Between 10 and 30 natural units were measured for each taxon depending on variability and number encountered. The number of fields counted was spread evenly over 3 replicate slides for each sample (30 total fields, 10 fields per slide). Counting was completed when the standard error of the mean of the total number of natural units per field was less than 10 percent. Recounts were done for quality control on approximately every 10th sample.

Results: Genetic Fingerprinting and High-Throughput DNA Sequencing

Cyanobacterial Diversity Determined by T-RFLP, 2013

Electropherograms based on MspI digestion showed a dominant T-RF at 172 bp in all samples (fig. 2). This indicated the presence of a dominant genetic cyanobacterial variant, although variations in peak area of up to 10-fold were observed in some samples. Results of the RsaI digestion (data not shown in fig. 2) showed two T-RFs located at 101.5 bp and at 102.5 bp across all samples analyzed. An additional, smaller T-RF was also identified at 60 bp in the sample collected on September 3, 2013. In reference to a draft genome of an *Aphanizomenon flos-aquae* culture established from Upper Klamath Lake in 2013 (GeneBank accession LJOY00000000; Connor Driscoll, Theo Dreher, and others, Oregon State University, written commun., 2016), the expected TR-F sizes for MspI and RsaI digestions are 175 and 103 nucleotides, respectively. Therefore, these data are consistent with *Aphanizomenon* being the dominant cyanobacterium detected.

In evaluating relative diversity, different T-RFs in a sample may be considered to represent distinct genotypes, and the peak areas of these T-RFs indicate the relative abundances of these genotypes in the sample. Assuming the observed variations in the number of T-RF peaks present could be attributed to the presence of multiple genotypes; samples collected July 30, August 6, August 13, and August 20, 2013, from site MDT exhibited more than one cyanobacterial genotype and, therefore, may be described as having genetic diversity; samples collected from July 9 to 23 and from August 27 to September 10 were dominated by a single cyanobacterial type, as indicated by the presence of a single peak on their electropherograms. Changes in T-RF peak areas along with the number of unique T-RFs present in the samples (fig. 2) indicated higher overall diversity among cyanobacteria in early and late July through August relative to later in the autumn.

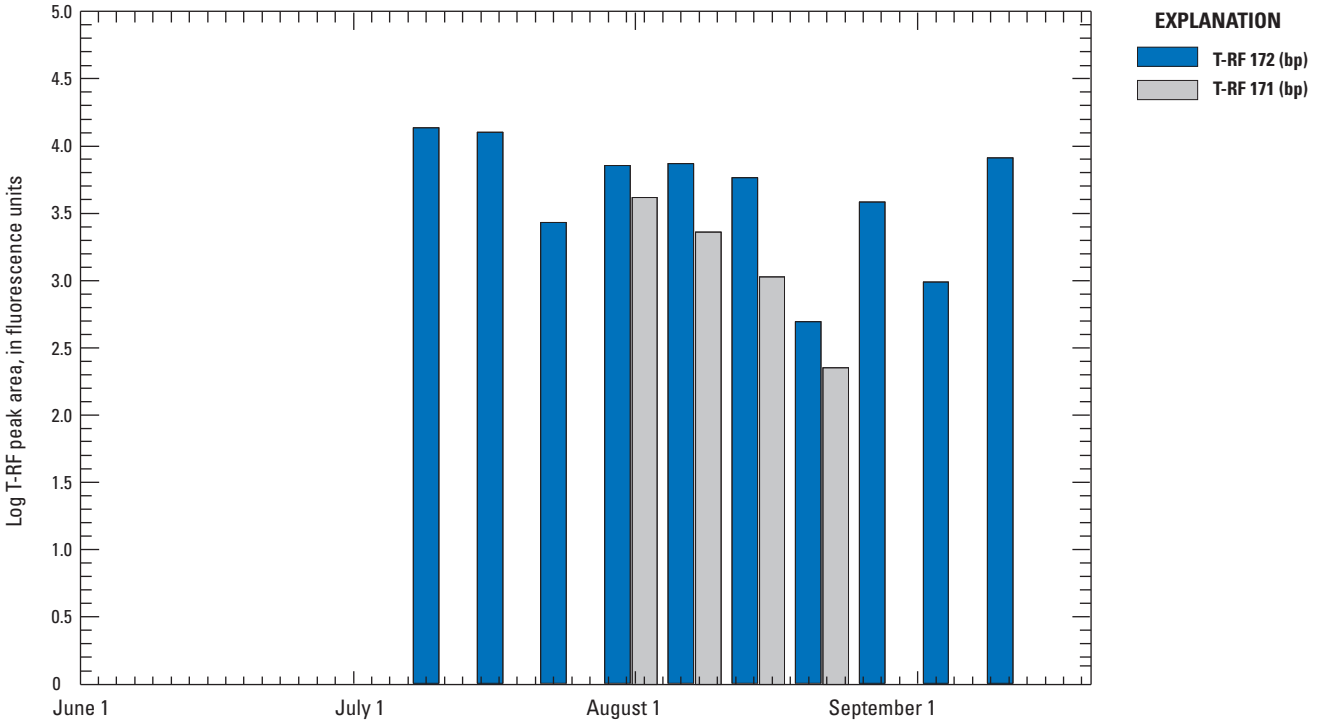


Figure 2. Time series showing dominant terminal restriction fragments (T-RFs) detected and their log peak areas determined by T-RFLP analysis (MspI digestion) of cyanobacteria in water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013.

Bacterial Taxonomic Composition Based on 16S rRNA Sequencing, 2013 and 2014

Results of high-throughput DNA sequences are shown in Eldridge and others (2017). After sequence processing, 1,074,845 reads were generated from 10 primary samples collected from site MDT in 2013, and 3,782,360 reads resulted from sequencing 14 MDT samples in 2014. These sequences were binned into 79,342 16S rRNA OTUs in 2013 and 437,638 OTUs in 2014. OTUs were defined as sequences being greater than 97 percent similar. Of the total number of sequences generated in 2013 and 2014, 2 percent were classified as cyanobacteria. The per-sample sequencing effort ranged from 98,173 to 124,874 sequences in 2013 and from 136,253 to 387,803 sequences per sample in 2014. All other bacteria (non-cyanobacterial groups), including unclassified bacteria, occurred in much higher proportion in the sample data than cyanobacteria (fig. 3*A*). The largest proportion of cyanobacteria in the 2014 dataset occurred at an average of 16 percent, and in the 2013 dataset, the average proportion of cyanobacteria was 6 percent.

Rarefaction analysis is a technique most commonly used to determine whether sufficient observations have been made to reasonably estimate the number of different species present in an environment or ecosystem. Rarefaction curves plot the measured number of species as a function of the number of observations made. There are different ways to define the quantity of species, such as richness or alpha diversity, but in the current study, species were quantified as the number of OTUs, and the number of sequence reads per sample was used as the number of observations. In general, rarefaction curves are created by randomly re-sampling the pool of observations multiple times and plotting the average number of species found as a function of the number of observations. These curves generally grow exponentially to begin with and then plateau as the more rare species remain un-sampled. Although the potential for deep sequencing is higher for Illumina sequence technology, rarefaction curves (fig. 3*B*) did not plateau in 2013 or 2014, indicating that the diversity in the samples was probably underestimated.

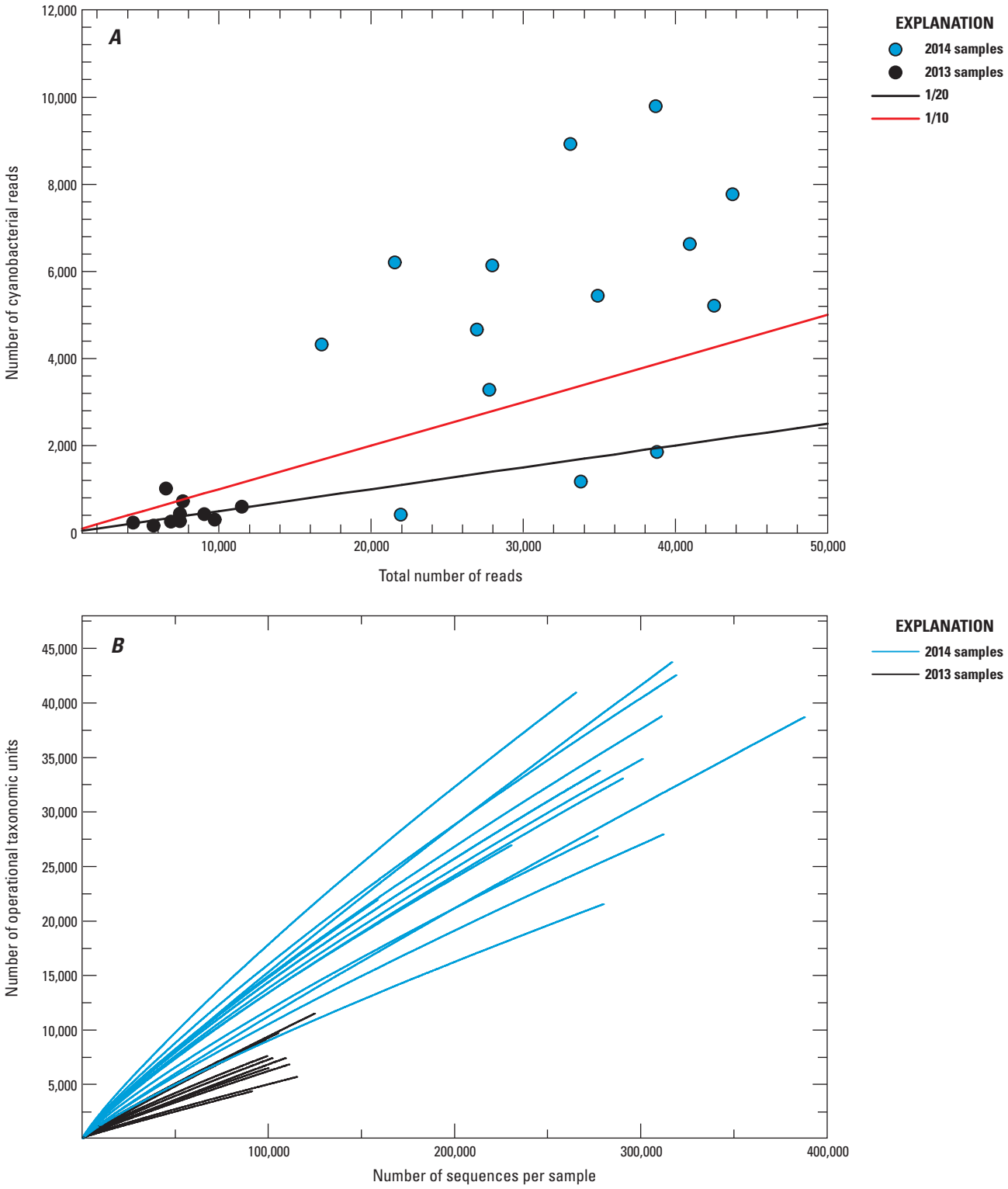


Figure 3. Proportion of the total reads classified as cyanobacteria (*A*) and rarefaction curves of high-throughput DNA sequencing (V3 and V4 regions, 16S rRNA gene) (*B*) in water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013 and 2014. The 1/20 and 1/10 lines in (*A*) depict ratios between cyanobacterial reads to the total number of reads in the datasets.

Among the more abundant phyla present in the HTS data, the *Actinobacteria* phylum was more dominant in 2013 than in 2014, composing up to 70 percent of the bacterial sequences by late August (fig. 4). Other phyla, including the *Bacteroidetes*, *Cyanobacteria*, *Proteobacteria*, and *Verrucomicrobia* also were relatively abundant in the dataset (occurring more than a few times). The distribution of phyla in 2014 was more diversified, in that more significant contributions were made that year among the *Bacteroidetes* (20 percent on average) and *Cyanobacteria* (16 percent on average), in addition to the *Actinobacteria* (18 percent on average); *Proteobacteria* (27 percent on average) abundances were similar in 2013 and 2014. In 2013, cyanobacteria were at peak abundances of 17 percent of the bacterial sequences on September 9 and 9.5 percent of bacteria on July 16 (fig. 4). In 2014, the abundance of cyanobacteria in the HTS dataset generally followed a two-bloom pattern often seen in Upper Klamath Lake (Lindenberg and others, 2009): values were greater than 20 percent in late-June to early-July (when chlorophyll *a* concentrations ranged from 109 to 153 µg/L), decreased to 2 percent on July 21 (98 µg/L chlorophyll *a*), and subsequently increased to 28 percent in early August (greater than 100 µg/L chlorophyll *a*).

HTS results at the level of order among cyanobacteria showed the *Nostocales*, which includes the genera *Anabaena*, *Dolichospermum*, *Aphanizomenon*, *Cylindrospermopsis*, and *Gloeotrichia*, to be most abundant overall, at 58 percent, on average, in all sequence data obtained in 2013 and at 59 percent in the 2014 sequence data (fig. 5). However, distinct differences were observed in the overall distribution of sequences between 2013 and 2014. *Synechococcales* were much more abundant in 2013 sample data from late July to mid-September, while *Chroococcales*, which includes the genera *Microcystis*, *Coelosphaerium*, *Merismopedia*, and *Woronichinia*, were more abundant in 2014 sample data (average 5 percent in 2013; average 22 percent in 2014). *Chroococcales* was more abundant in the HTS data collected later in both years, but in 2014, this group increased in the dataset abruptly after August 4, composing more than one-half the sample sequences from August 18 through September 8 (fig. 5). HTS results at the genus level (fig. 6) showed that the *Chroococcales* was composed almost entirely of *Microcystis* in 2014 and on August 27 and September 10 in 2013.

The USGS water-quality monitoring program in Upper Klamath Lake since 2005 has focused primarily on environmental parameters that affect the health and survival of endemic fish populations (Burdick and Hewitt, 2012; Hewitt and others, 2014; Hereford and others, 2016). The key variables are dissolved oxygen, pH, ammonia, and microcystins, which are largely regulated by oxygenic photosynthesis and microcystin production of the more abundant and metabolically active phytoplankton. Therefore, water-quality studies in Upper Klamath Lake have focused on the ecology of species thought to contribute most to changes in these water-quality parameters, namely *Aphanizomenon* spp., *Dolichospermum* spp., and *Microcystis* spp. Within that scope, results of HTS of water samples in 2014 showed trends consistent with patterns in chlorophyll *a* (a surrogate for biomass of the more abundant *Aphanizomenon/Dolichospermum*) and microcystin concentrations (as a surrogate for *Microcystis*) measured by the USGS since 2007 (Lindenberg and others, 2009; Eldridge and others, 2012) that define the cycle of cyanobacteria bloom growth and decline observed most years. The successional patterns in blooms of *Aphanizomenon*, *Dolichospermum*, *Microcystis*, *Gloeotrichia*, and *Woronichinia* reflected in the HTS dataset (fig. 6) also agreed generally with the pattern of occurrence described by Carmichael and others (2000). However, as with the HTS results at the levels of phylum and order, the 2014 sequence data appeared quite different from 2013 in the temporal distribution of the genera present (fig. 6), which likely reflected differences in environmental conditions between 2013 and 2014. Overall, the bloom-forming *Aphanizomenon/Anabaena/Dolichospermum* group composed the largest proportion of HTS reads in 2013 (average relative abundance in all sample sequences of more than 35 percent, excluding unclassified genera) and in 2014 (average abundance over all sample sequences of more than 44 percent). Note that *Aphanizomenon* and *Anabaena/Dolichospermum* are phylogenetically intermixed (Gugger and others, 2002; Rajaniemi and others, 2005) and can not be separated based on 16S rRNA gene sequences. On average, the relative abundance of *Microcystis* across all sample sequences was about 20 percent in 2013 and almost 47 percent in 2014 (fig. 6), indicating a potential predominance of *Microcystis* in 2014 based on the relative number of cells (a measure distinct from biomass) in collected samples. Assuming this predominance corresponded qualitatively with a dense *Microcystis* bloom at MDT in 2014, these results showed a rapid increase of *Microcystis* at a single site, which was not previously observed in Upper Klamath Lake. A convention based on microscopic data since at least the early 1990s is that *A. flos-aquae* makes up more than 99 percent of the biomass during bloom periods (Kann, 1997; Carmichael and others, 2000) as a consequence of their larger cell and colony sizes. It is also noteworthy that the abundance of *Synechococcus* (a unicellular cyanobacterium, found within the smaller picoplankton) was more than 34 percent, on average, in the 2013 sample sequences (excluding unclassified groups), but in 2014, *Synechococcus* made up only 1 percent of the genera-level HTS dataset. In contrast, the abundance of *Gloeotrichia* was more than 4 percent of the HTS reads in 2014, but was not found at all in samples collected in 2013 (fig. 6).

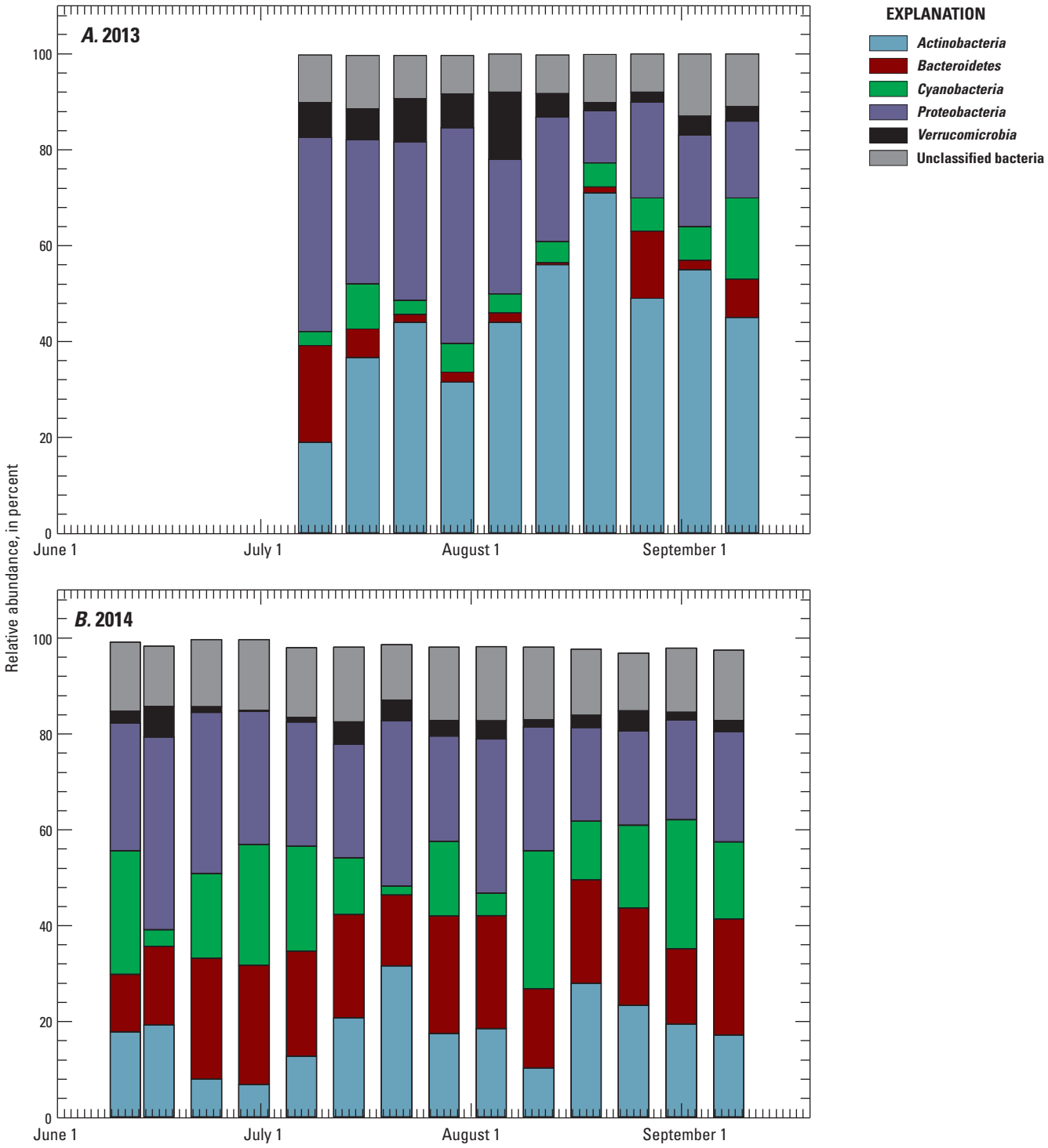


Figure 4. Relative abundances of high-throughput DNA sequencing reads based on the 16S rRNA gene at phylum level of Bacteria obtained from water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013 and 2014. Relative abundances were calculated from the number of reads of each taxon as a percentage of the total number of phylum-level reads in the dataset, including unclassified bacteria. Phyla that were represented in less than 2 percent of the reads are not shown in the figure.

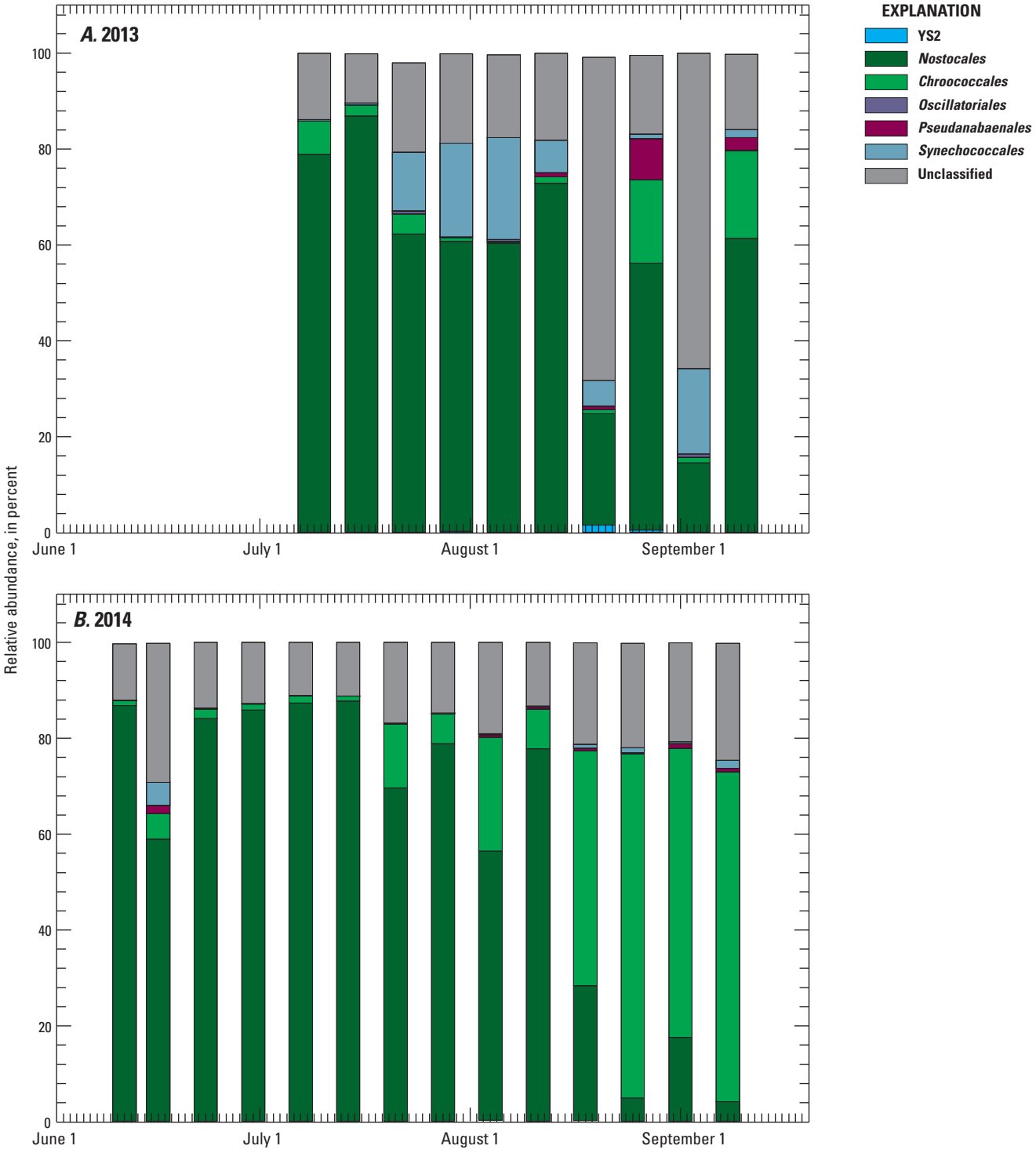


Figure 5. Relative abundances of high-throughput DNA sequencing reads based on the 16S rRNA gene at order level of cyanobacteria obtained from water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013 and 2014. Relative abundances were calculated from the number of reads of each taxon as a percentage of the total number of order-level reads in the dataset, including unclassified bacteria. Orders with less than 2 percent relative abundance are not shown in the figure.

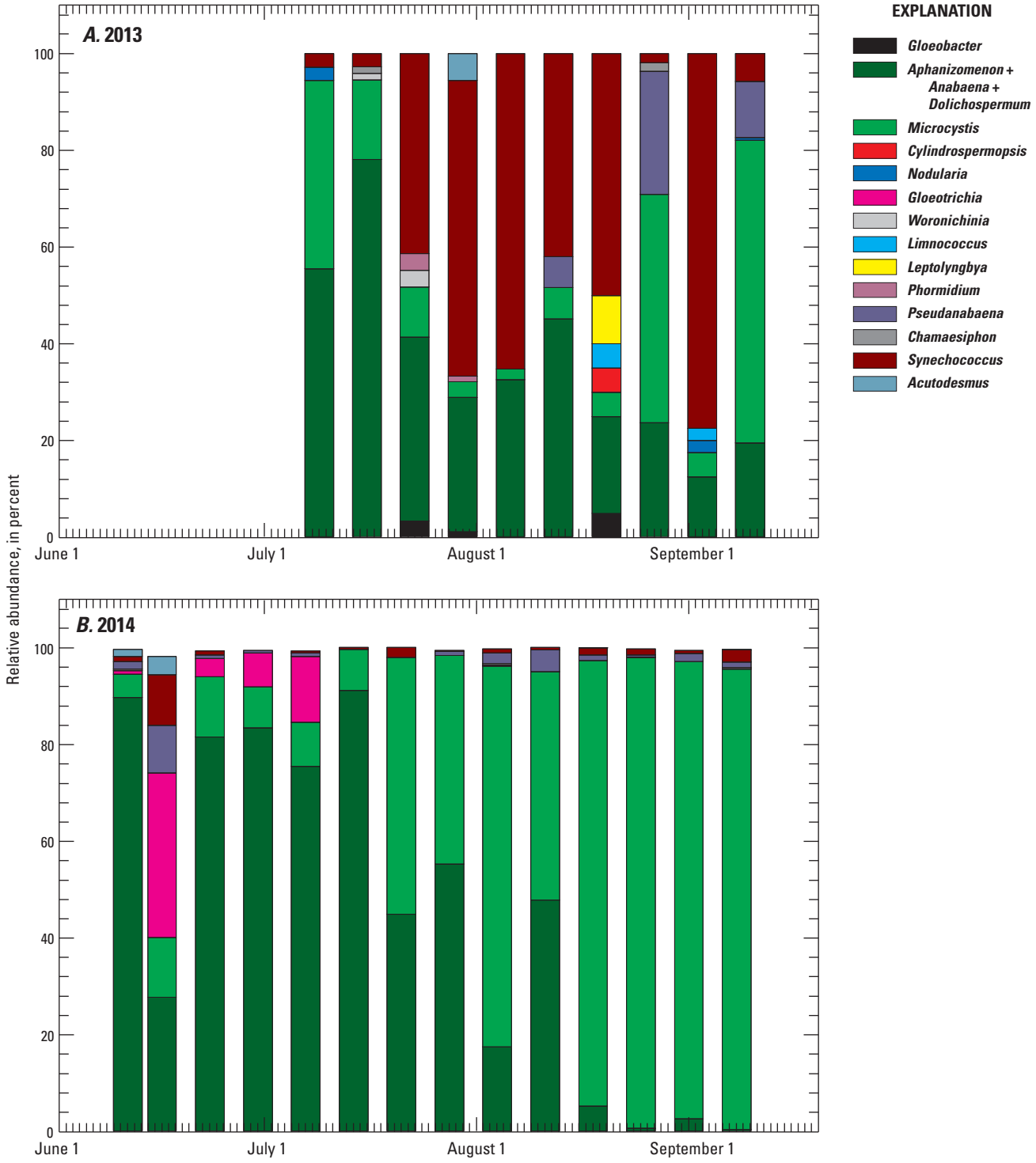


Figure 6. Relative abundances of high-throughput DNA sequencing reads based on the 16S rRNA gene at genus level of cyanobacteria obtained from water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013 and 2014. Relative abundances were calculated from the number of reads of each taxon as a percentage of the total number of genus-level reads in the dataset; unclassified groups were excluded. Genera, not including unclassified reads, which were represented in less than 2 percent of the reads are not shown in the figure.

Over time, the *Aphanizomenon/Anabaena/Dolichospermum* bloom occurred throughout July and August in both years, with higher relative abundances early in 2014 (fig. 6; mean chlorophyll *a* concentrations were 228 µg/L in 2013 and 154 µg/L in 2014 during this time). In 2013, the corresponding bloom appeared to diminish by mid-July and did not increase again to the level seen in early-season samples. However, in 2014, an apparent "crash" in this bloom was evident by August 4, and by August 25, this group was almost completely replaced by *Microcystis*. This trend also was observed directly by field and lab personnel. *Microcystis* was present in all samples collected in the study and occurred in relatively high abundance in both years, but the striking contrast between 2013 and 2014 was in how *Microcystis* appeared to dominate the community in 2014 in a way not seen in previously reported data. This has clear implications for water quality in terms of the potential for high microcystin concentrations and of the underlying environmental conditions that may have prompted this shift in phytoplankton community dynamics between 2013 and 2014.

Comparison of Phytoplankton Occurrence—DNA Sequencing Contrasted with Light Microscopy in 2013

Results of microscopic and HTS analyses are provided in Eldridge and others (2017). The composition, relative quantities, and temporal trends in cyanobacteria occurrence detected by HTS and light microscopy were compared at the levels of order (figs. 5 and 7) and genus (figs. 6 and 8) on six dates when samples were collected for both analyses. Microscopic counts were reported as natural units per milliliter (NU/mL), and the proportions of each taxon were determined as the relative percentages of all groups detected at the corresponding taxonomic level; these values were compared with the relative percentages of reads in the HTS dataset. Microscopic counts were reported as natural units because it is a more statistically valid counting unit for estimates of precision. Natural units may be single cells, filaments, or colonies (Ehrlich, 2010), but in the current study, natural units were determined primarily from colony counts. Therefore, absolute abundances determined by microscopy were not interchangeable with HTS-determined abundances, so comparisons were based on presence/absence detections, relative proportions of detected groups, and time-series trends.

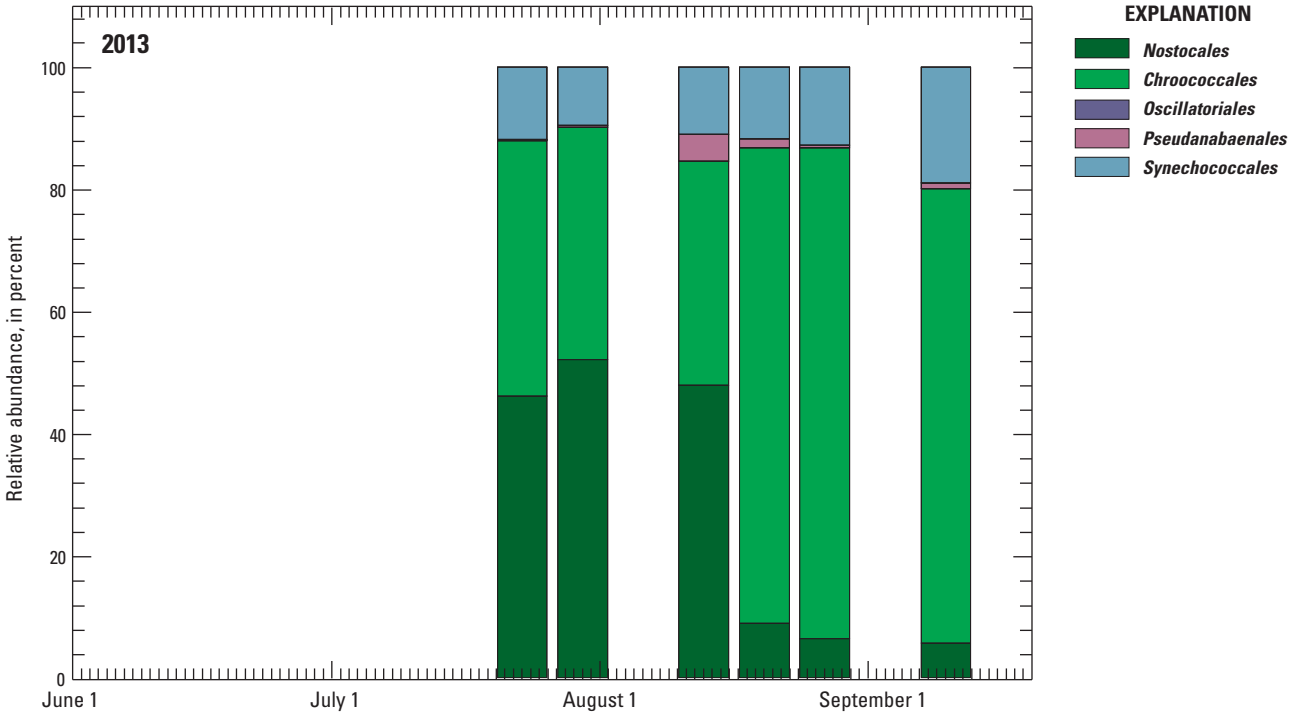


Figure 7. Relative abundances of cyanobacteria determined by direct microscopic cell counts (natural units per milliliter) at the level of order in water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013. Percentages were based on the relative percentage of all counts in the dataset.

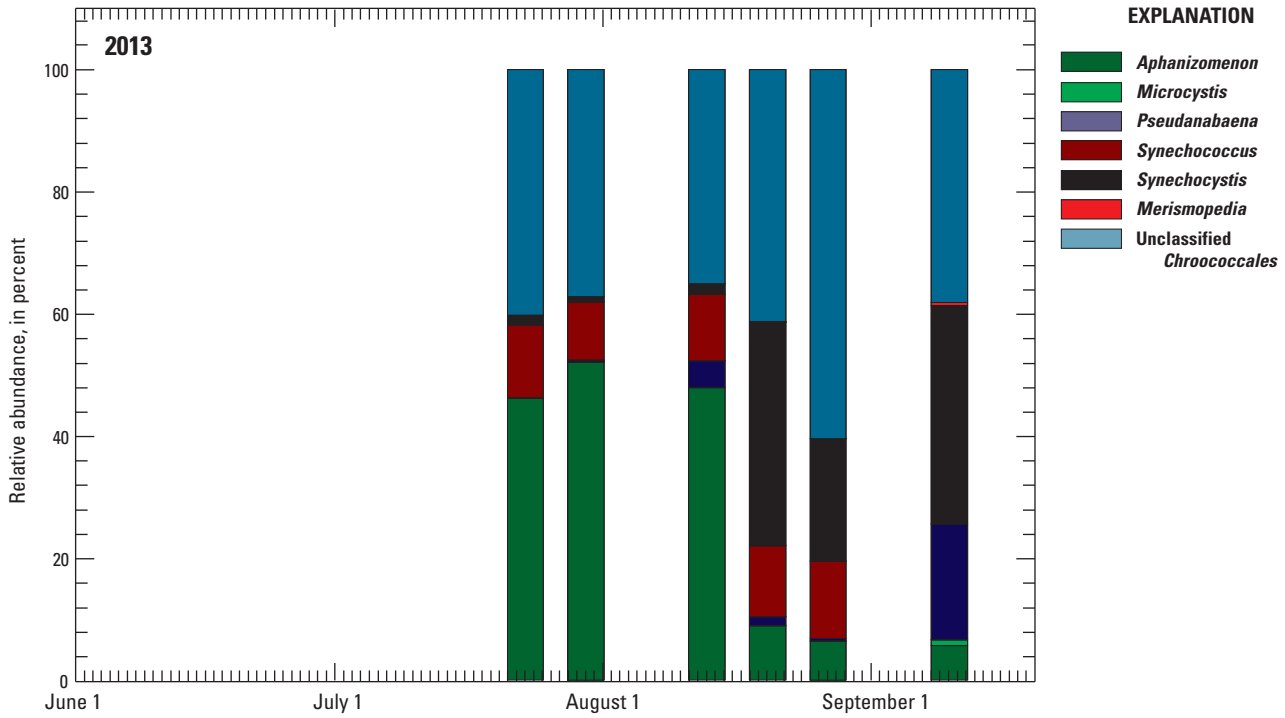


Figure 8. Relative abundances of cyanobacteria determined by direct microscopic cell counts (natural units per milliliter) at the level of genus in water-column samples from site MDT, Upper Klamath Lake, Oregon, 2013. Percentages were based on the relative percentage of all counts in the dataset.

In general, the HTS approach for cyanobacteria identification exhibited a higher sensitivity for detection of specific groups than light microscopy. Microscopy detected 5 orders (fig. 7) and 6 genera (fig. 8) of cyanobacteria in 2013. In contrast, HTS revealed 9 orders (*Euglenozoa*, *Streptophyta*, and *Gloeobacterales* are not shown in fig. 5) and 15 genera of cyanobacteria (*Dolichospermum* was combined with *Aphanizomenon/Anabaena* in fig. 6). Among cyanobacteria, microscopy did not detect any species of the genera *Gloeobacter*, *Cylindrospermopsis*, *Dolichospermum* (*Anabaena*), *Limnococcus*, *Nodularia*, *Woronichinia*, *Chamaesiphon*, *Phormidium*, *Leptolyngbya*, or *Chamaesiphon* (figs. 8 and 9), but HTS results indicated that these groups all occurred in samples at relatively low abundance. However, *Synechocystis* and *Merismopedia* were detected in microscopy samples but not in samples analyzed by HTS (*Merismopedia* was detected in only one sample, August 20). There are 17 unique *Synechocystis* sp. sequences identified in the Greengenes database as "unclassified" and 9 unique *Synechocystis* sp. sequences classified under Cyanobacteria (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). So, it is likely that *Synechocystis* sp. was present in the samples, but they were not detected by HTS because the strains present were more closely related to these unclassified strains, and therefore, denoted in the dataset as "unclassified." For most genera, detection by either HTS or microscopy varied by sample date (fig. 9). There were few dates between July 23 and September 10 when any single genus was detected by both methods, although there was more agreement between methods regarding the absence of genera (not found by either method). *Microcystis* was detected by both methods on a single day, *Pseudanabaena* was detected by both on 3 days (one-half the dates), and *Synechococcus* was detected by both on 5 days (fig. 9). Only *Aphanizomenon*/*Anabaena* was identified in all samples by HTS and microscopy.

For the two genera of interest to water-quality studies in Upper Klamath Lake, *Aphanizomenon*/*Anabaena* and *Microcystis*, the results varied widely between methods in terms of presence or absence over time and in the relative quantities detected. The average relative abundance of *Aphanizomenon* in microscopy samples, based on natural units and including unclassified *Chroococcales*, was 28 percent; in the HTS dataset, 19 percent of reads (relative abundance, on average) were classified as *Aphanizomenon*/*Anabaena* or *Dolichospermum* in 2013. *Microcystis* was determined by microscopy to occur at an average relative abundance of 0.16 percent (based on natural units), but when analyzed by HTS, the average relative abundance of *Microcystis* (based on number of reads) was 20 percent.

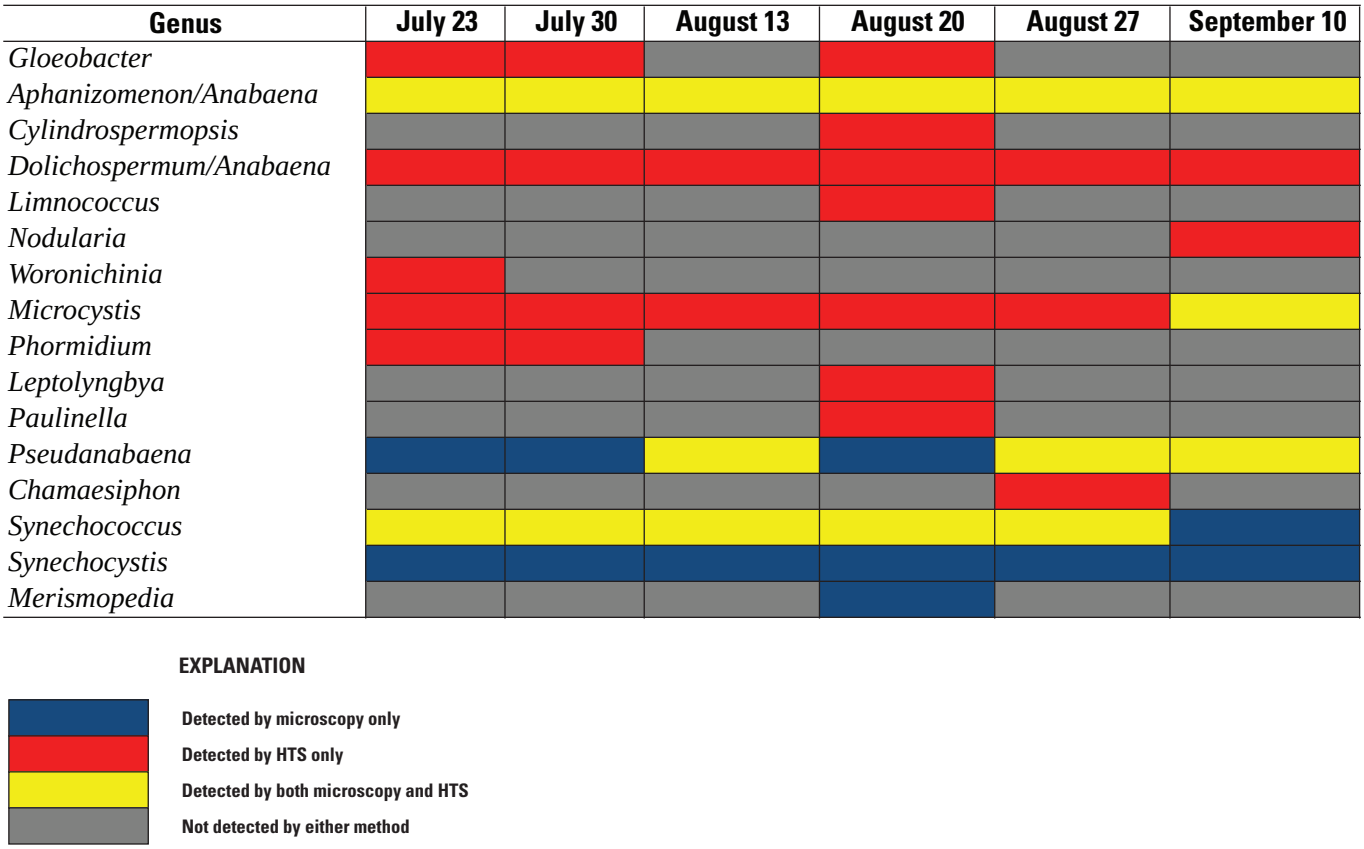


Figure 9. Comparison of cyanobacteria genera detected by microscopy contrasted with high-throughput DNA sequencing (HTS) by sample date at site MDT, Upper Klamath Lake, Oregon, 2013. *Aphanizomenon*/*Anabaena* detected by microscopy were all identified as "*Aphanizomenon,*" because detections by microscopy were based on morphology. *Aphanizomenon* was not distinguished from *Anabaena* by DNA sequencing, so these genera are combined in the figure. HTS detections, shown here as "*Dolichospermum*/*Anabaena,*" were all classified in the HTS dataset as "*Dolichospermum.*" Microcopy did not detect any cells or colonies that could be identified morphologically as *Anabaena*.

Some of this difference between methods may be attributed to the inherent variabilities introduced during sampling and analysis, particularly given the differences in the analytical approaches. However, analysis of quality-control samples (see section, "Quality Control for Bias and Variability in Sampling and Analysis") in this study showed high reproducibility among replicate and split samples. So, it is unlikely that the discrepancies observed in detecting *Microcystis* cells in water samples contributed much bias or error.

Considering only the four more abundant cyanobacterial genera detected by both methods in this study, *Aphanizomenon*/*Anabaena/Dolichospermum*, *Microcystis*, *Pseudanabaena*, and *Synechococcus*, the *Aphanizomenon*/*Anabaena/Dolichospermum* group appeared to be overrepresented in the 2013 microscopy counts, relative to HTS data, when this population was most abundant in July and August (table 3). There was general agreement between the methods on September 10, but the relative abundance of this group was greater when quantified by microscopy in all prior samples. *Microcystis* was not detected by microscopy until the last sample date, September 10, but HTS detected *Microcystis* on all dates sampled. The relative abundance of *Microcystis* varied in the HTS dataset from 3.61 percent (July 30) to 66.9 percent (September 10; table 3). This shows a striking underrepresentation of *Microcystis* in all microscopic count data when compared to results from HTS.

Time-series trends in the microscopy-determined biovolume estimates (as relative percentage) showed less correspondence with HTS data than microscopy counts based on natural units (fig. 10). The relative percent biovolume for *Aphanizomenon* was nearly 100 percent in all 2013 samples, and for *Microcystis*, *Pseudanabaena*, and *Synechococcus*, the relative percent biovolume was less than 5 percent throughout the season. Compared with results of HTS analysis, the relative percent biovolumes of the genera shown in figure 10 did not fluctuate over the sample season and suggested that most of the biomass in the collected samples was composed of the much larger *Aphanizomenon*, despite the higher relative abundances of smaller-sized cells like *Synechococcus* (higher abundances earlier) and *Microcystis* (higher abundances later)

Table 3. Percentage of relative abundances of cyanobacteria genera measured by microscopy (natural units per milliliter) and by high-throughput DNA sequencing (reads) in water-column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013.

[**Abbreviations:** HTS, high-throughput DNA sequencing; ND, not detected]

Date	*Aphanizomenon/ Anabaena/ Dolichospermum*		*Microcystis*		*Pseudanabaena*		*Synechococcus*	
	HTS	Microscopy	HTS	Microscopy	HTS	Microscopy	HTS	Microscopy
	(percent)		(percent)		(percent)		(percent)	
07-23-13	42.3	79.4	11.5	ND	ND	0.34	46.2	20.3
07-30-13	30.1	89.3	3.61	ND	ND	3.07	66.3	7.62
08-13-13	45.2	75.7	6.45	ND	6.45	6.97	41.9	17.3
08-20-13	28.6	40.8	7.14	ND	ND	6.56	64.3	52.7
08-27-13	24.1	33.2	48.1	ND	25.9	2.14	1.85	64.6
09-10-13	20.8	22.0	66.9	2.02	12.4	3.61	ND	72.3

in the HTS dataset (fig. 10). Overall, these results showed the differences in results obtained from various quantification techniques and highlighted the importance of choosing the appropriate method given the parameters needed for study.

Discussion: Genetic Fingerprinting and High-Throughput DNA Sequencing

Genetic Fingerprinting as a Tool for Taxonomic Comparisons

T-RF profiles are often used as indicators of microbial taxonomic richness (Denaro and others, 2005), and although the results of T-RFLP analysis likely vary by the study design (primarily, the choice of restriction enzyme used; Zhang and others, 2008), the T-RFLP fingerprinting method is a rapid, high-throughput means for comparing complex bacterial community structures. In this current study, T-RFLP analysis suggested that the cyanobacteria community was composed primarily of a few genetically distinct types (genotypes). Time-series trends in the 2013 T-RF profiles showed a possible succession of genotypes that coincided with changes in bloom-associated biomass, which, as previous data have shown, are related to the seasonal fluctuations in environmental factors (Wood and others, 2006; Lindenberg and others, 2009). As a water-quality monitoring tool, particularly in cases where the phytoplankton or bacterial community is not well known, variations in diversity along temporal or spatial gradients provide clues to environmental changes that may indicate physico-chemical gradients that can be further evaluated as controls of biomass or diversity. The low cost and high sensitivity of genetic fingerprinting also make these techniques well-suited as screening tools for selecting samples for further study, as a first step in evaluating less known sites or systems, or as stand-alone monitoring tools. If needed, T-RF profiles can be further analyzed by characterizing the community structure using multidimensional scaling ordination (Zhang and others, 2008) or by assigning phylogenetic identity using a public database (http://mica.ibest.uidaho.edu/). Yet a major disadvantage of using T-RFLP analysis is that it can fail to distinguish closely related genotypes that might be readily distinguished by other means, such as qPCR.

As an example of using genetic fingerprinting for water-quality monitoring, Loza and others (2013) used temperature gradient gel electrophoresis (TGGE) to examine benthic cyanobacterial communities associated with eutrophication from Guadarrama River (Spain) biofilms and compared the TGGE profiles with results of microscopic analyses of samples and cultured isolates from the river. Results of this study identified direct relations between TGGE band profiles and water quality (principally dissolved inorganic nitrogen and soluble reactive phosphorus) and validated the use of genetic fingerprinting as a monitoring tool for rivers.

Results of T-RFLP analysis in this current study also highlighted the need for careful consideration in the selection of gene targets for a water-quality or ecological study. Here, gene sequences specific to total cyanobacteria were targeted, so the T-RF profiles could only be resolved at that taxonomic level; no conclusions could be made regarding the diversity of other bacterial groups or of specific cyanobacterial species. To further explore community or population dynamics at different taxonomic scales, other genes or sequences may be targeted and(or) different tools may be used, based on the study objective or data needs. In the USGS Upper Klamath Lake water-quality monitoring program, identification and enumeration of groups or species affecting water quality (or groups affected by water quality) is desired. Therefore, high-throughput DNA sequencing of the 16S rRNA gene was used in this study following T-RFLP fingerprinting to further describe the bacterial community in lake water samples.

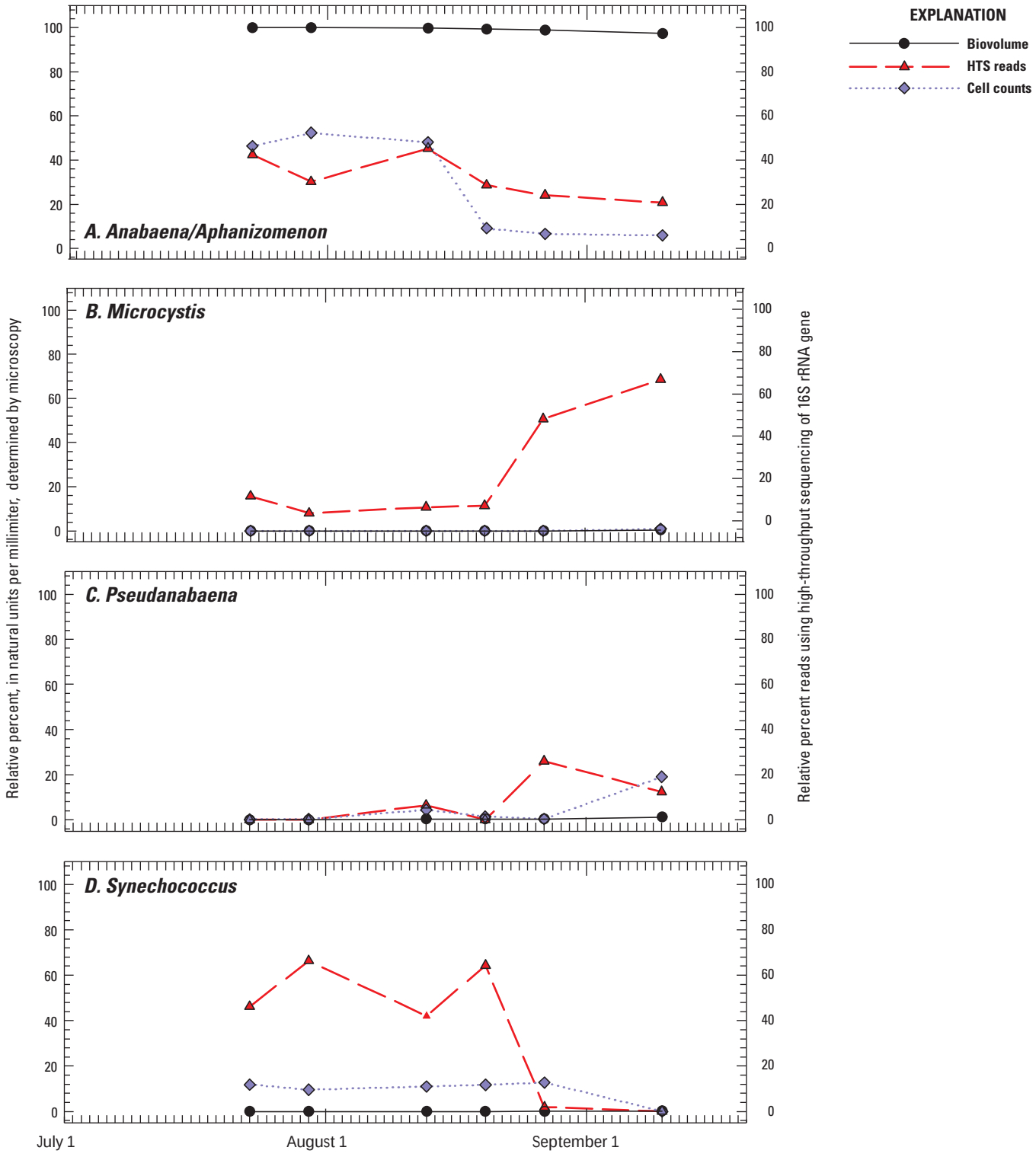


Figure 10. Changes in biovolume and counts of selected cyanobacteria genera measured by microscopy and high-throughput DNA sequencing (HTS) in water column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013.

High-Throughput DNA Sequencing as a Tool for Describing Bacteria and Phytoplankton Community Structure

As primary producers, phytoplankton directly influence nutrient cycling in surface aquatic systems. Therefore, they are sensitive to, and in turn signify changes in, the environment, particularly eutrophication and water quality. The phytoplankton community in Upper Klamath Lake has been studied for more than two decades (Kann and others, 2014), but this current study was the first to describe phytoplankton in this lake using next-generation, high-throughput DNA sequencing technology, and it illustrated the value of this tool for water-quality studies and for monitoring of this system. In this study, we used HTS to qualitatively describe changes in the phytoplankton community over two field seasons, 2013 and 2014, at a single site with a lower level of detection for less abundant species than by microscopic analysis. Relative abundances of cyanobacteria in the HTS dataset showed seasonal and interannual variations in the bloom cycle that fit with the general understanding, based on previous microscopy and biomass (chlorophyll *a*) data, of bloom growth dynamics in Upper Klamath Lake (Eldridge and others, 2012, 2013, 2014). HTS results over time also tracked the presence of *Microcystis* during the spring and summer and showed this group to be more prevalent in water samples than previously understood.

At the order and genus levels, more distinct taxa were detected by HTS in this study than by traditional microscopy. Xiao and others (2014) obtained similar results in a comparison of microscopy contrasted with HTS analyses in 300 freshwater samples collected weekly over 20 years. In the Xiao study, HTS detected more rare species and picoplankton than light microscopy and, therefore, gave a better assessment of phytoplankton diversity above the species level. Likewise, Eiler and others (2013) described the distribution patterns of cyanobacteria and chloroplasts across 49 lakes using HTS in direct comparison to microscopic identifications based on morphology. HTS captured the seasonality of phytoplankton succession as observed through microscopy and detected cyanobacteria at generally greater abundance and at a higher resolution of classification than microscopy.

In Eiler and others (2013), detailed HTS and microscopy taxonomic lists showed low correspondence, which the authors suggested was due to methodological constraints and discrepancies in the taxonomic frameworks of the different methods. Comparisons of microscopic (morphology-based) and genetic methods are often impeded by fundamental differences between these methods and by the inherent contradictions in the classification systems that the methods use. For example, sample filtration and DNA extraction, the first steps in HTS analysis, have been shown to affect species detection (Medinger and others, 2010). Furthermore, DNA sequence alignment and classification of HTS reads into distinct OTUs is based on comparison of the sequences to a database of known sequences. So, detection of taxa may be limited by the quality or completeness of the database used, and using databases that underrepresent taxa from the studied system can result in more unclassified results. Additionally, results of microscopic analysis may vary widely with the expertise of the taxonomist. Microscopic counts also are usually done on much smaller sample volumes than the samples filtered for HTS. This may result in the underestimation of less abundant groups by microscopy. Light microscopy also may fail to detect picoplankton or very small phytoplankton, as well as most heterotrophic bacteria, which are more likely to be detected with HTS. This was illustrated in the current study by the far greater abundances of *Synechococcus*, one of the most thoroughly studied picoplankton species, in more samples analyzed by HTS than by microscopy. Causes for the underrepresentation of taxa also may include taxon-specific loss of cells during preservation or handling prior to microscopic evaluation and errors in microscopic identification if cells are missing characters (akinetes, for example) or if key traits are hidden from view, making it impossible to discriminate morphological traits.

Results of HTS in this study also showed an abundance of heterotrophic bacteria in all collected samples, revealing a significant component of the pelagic microbial community that would have been missed if only phytoplankton were targeted for sequencing. It is well known that bacteria and phytoplankton dynamics are closely linked in both freshwater and marine systems (Cole, 1982; Bratbak and Thingstad, 1985; Rooney-Varga and others, 2005; Danger and others, 2007). Together, these groups control energy flow and nutrient cycling in aquatic ecosystems through primary production (phytoplankton) and decomposition (heterotrophic bacteria; Loreau, 2001). In brief, photosynthetic algae and cyanobacteria fix carbon dioxide into organic matter that is then metabolized by heterotrophic bacteria. Nutrients are remineralized by heterotrophs, which subsequently enables phototrophs to grow. The species that comprise these ecological groups are numerous and genetically diverse (Rooney-Varga and others, 2005); together they exhibit a vast array of physiological and chemical responses to the environment and to each other. Interactions between bacteria and phytoplankton range from obligate to facultative and from mutualistic to parasitic, and these interactions can be mediated by cell-to-cell attachment or through communication between cells facilitated by the release of chemicals. Many studies have shown interactions between phytoplankton and bacterial communities through the measurement of bulk community parameters, such as production, respiration, and nutrient fluxes (Jensen, 1983; Bratbak and Thingstad, 1985; Cole and others, 1988; Obernosterer and Herndl, 1995). In addition, studies of harmful algal blooms in marine systems have

shown that bacteria are capable of stimulating or inhibiting phytoplankton growth (Yoshinaga and others, 1997; Ferrier and others, 2002), algaecide or allelopathy (Imai and others, 1993; Yoshinaga and others, 1997; Lovejoy and others, 1998), or altering phytoplankton physiology (that is, production of algal toxins; Gallacher and others, 1997). Researchers have also reported evidence for species-specific interactions between bacteria and phytoplankton, which has led to the conclusion that bacteria can play a major role in controlling phytoplankton dynamics (Imai and others, 1993; Fukami and others, 1996; Yoshinaga and others, 1997). Although it is beyond the scope of this report to provide a detailed analysis of the bacteria-phytoplankton interactions in Upper Klamath Lake, results of this study highlighted the presence and quantitative significance of heterotrophic bacteria in the water column. Most heterotrophs cannot be detected by light microscopy because, unlike many phytoplankton species, they do not form large, visible colonies and do not have uniquely identifiable, morphological traits. Furthermore, by targeting all bacteria with the HTS method, this study illustrated how future water-quality studies may be designed to obtain a more comprehensive understanding of energy flow and nutrient dynamics by including both phytoplankton and heterotrophic bacteria in microbial community assessments.

The consensus among studies based on next-generation, HTS is that this tool has broadened our understanding of microbial diversity in the environment. Results of this study suggested that HTS has many advantages over traditional microscopy as a tool for phytoplankton monitoring, in that it allows the simultaneous detection of all taxa affecting water quality in a rapid, reproducible, high-throughput, and cost-effective manner. Overall, HTS-based techniques can provide a more comprehensive view of phytoplankton diversity and metabolic function, independent of cell or colony size, than using microscopy alone. As an alternative to direct cell counts, HTS analysis can reveal the presence and relative abundances of less-abundant taxa, or taxa that cannot be distinguished microscopically, and can be used to target genes that inform on metabolic processes of interest. Direct observations or cell counts by microscopy do permit observations of morphology which, for some groups, are more diagnostic than genotyping for identification. Depending on the data needed, microscopic observation also can be a more immediate, cost-effective means of detection (presence/absence) if it is done in-house or in the field. Furthermore, when biomass estimates are needed, microscopy-based analyses are favored over HTS-estimated abundances, given the limitations of HTS as a quantitative tool (described in section, "High-Throughput DNA Sequencing: Total Bacteria") and the ability microscopy affords to directly measure sample-specific variations in cell or colony sizes and shapes.

qPCR for Quantitative Analysis of Toxin-Producing and Bloom-Forming Cyanobacteria

One goal of the water-quality monitoring program in Upper Klamath Lake is to collect data that can be used to describe the community structure and dynamics of phytoplankton in the lake with an emphasis on the groups thought to have the greatest effect on water quality: *Aphanizomenon* and *Microcystis*. *Aphanizomenon* is the most abundant bloom-forming genus, by biomass, during dense bloom periods in the lake (characterized by chlorophyll *a* concentrations greater than 200 µg/L), and *Microcystis* has been directly linked with elevated microcystin concentrations in some years (Gilroy and others, 2000; Lawrence and others, 2001; Saker and others, 2007). In previous ecological or water-quality studies of Upper Klamath Lake, detection of toxin-producing and bloom-forming cyanobacteria was based on microscopic techniques combined with the chemical detection of microcystins and the pigments chlorophyll *a* and phycocyanin in water samples. This can be an adequate means of monitoring biomass and coarse population dynamics. But these are ineffective tools for monitoring the toxin-producing potential of species such as *Microcystis* spp. because it is not possible to determine whether a specific strain is toxigenic by observing the cellular or colonial morphology (Baker and others, 2002; Ouellette and Wilhelm, 2003) or by measuring pigments. The large, combined non-ribosomal peptide synthetase (NRPS)/polyketide synthetase (PKS) gene cluster involved in microcystin production (*mcy* A-J) was identified, sequenced, and confirmed to be required for microcystin synthesis more than 16 years ago (Tillett and others, 2000), and it was shown to be expressed constitutively in *Microcystis* cells possessing the operon (Tillett and others, 2000; Baker and others, 2002). The resolution at which mixed populations of toxigenic (capable of producing toxins) and nontoxigenic (not capable of producing toxins) cells are analyzed can be improved greatly by targeting regions of the *mcy* gene cluster in qPCR (Rinta-Kanto and others, 2005), a technique that has been increasingly used for monitoring potentially toxigenic cyanobacteria in diverse aquatic ecosystems globally (Pearson and Neilan, 2008; Kurmayer and Christiansen, 2009; Ostermaier and Kurmayer, 2010). As a water-quality monitoring tool, qPCR enables detecting and quantifying the total abundance of *Microcystis*, potentially toxigenic *Microcystis*, and total cyanobacteria in water samples. With some modification, it can also be used to study the relationships within microbial communities that influence the presence and(or) proliferation of specific species,

such as *Microcystis* spp. or *Aphanizomenon* spp. Comparing cyanobacterial communities from a single location over time, for example, may reveal a temporal fluctuation of one target species that correlates with changes in another. A further advantage of qPCR is that sample analysis may be expedited by reducing the number of steps required by microscopy and chemical assays.

Use of qPCR for the study of hepatotoxic cyanobacteria population dynamics was reviewed in Martins and Vasconcelos (2011). Techniques based on PCR allow for the detection of specific DNA sequences by targeted amplification (making many copies) of those specific DNA sequences. This facilitates identification of unique genotypes based on the presence or absence of target genes in the samples (Ouellette and Wilhelm, 2003), depending on the design of PCR primers, which are used to specify the DNA target. qPCR involves the same basic techniques of conventional PCR, but instead of providing an endpoint analysis of the final replication products (amplicons), qPCR quantifies the DNA products generated after each replication cycle using fluorescent markers that are incorporated into the growing PCR product. The increase in fluorescence is directly proportional to the number of amplicons generated in the exponential phase of the reaction (Heid and others, 1996), hence, the technique monitors the amplification of the DNA target in real-time, which can be used to determine absolute or relative amounts of a known DNA sequence in a sample.

Detection of the microcystin-producing gene is not direct evidence for microcystin production. Detection of the gene indicates that the organism is genetically capable of producing the toxin (that is, potentially toxigenic), but by itself, PCR or qPCR does not measure gene expression. Toxigenic cells must actively express ("turn on") the corresponding gene(s) by transcribing the DNA into mRNA to initiate biosynthesis of the toxin. RNA-based analytical techniques are necessary for determining the activity of cells or the expression of genes at the time the samples are collected. As an example of recent RNA-based analyses, Stelzer and others (2013) and Francy and others (2015) used the quantitative reverse-transcription PCR (qRT-PCR) to simultaneously identify toxin-producing genera and measure the level of toxin gene expression in Ohio recreational waters.

In this current study, qPCR was used to detect and quantify the key cyanobacteria species thought to have the greatest effect on water quality in Upper Klamath Lake and to describe the temporal changes in the relative proportions of these species within the total cyanobacterial community in lake samples. To do this, a multi-level approach was used to determine the abundances of potentially toxigenic and non-toxigenic strains of *Microcystis*, total cyanobacteria, and *Aphanizomenon*. The RNA-based approach was not used in this study for determination of gene expression. Instead, active expression was assumed based on concurrent detection of the *mcy* gene and direct, chemical measurement of microcystin concentrations in split water samples.

Quantification of Potentially Toxigenic and Non-Toxigenic Strains of *Microcystis* using qPCR

Methods: Total Microcystins Analysis

Water-column samples were collected by depth integration from June 11 to September 10, 2013, and June 11 to September 22, 2014, following the protocol outlined for water quality data collection in Eldridge and others (2012). Site MDT is approximately 14 m deep in early June, and water from the top 10 m was collected, composited in a churn splitter, and subsampled for microcystins and qPCR analyses. Total microcystins (congener-independent) concentrations were determined in water samples at the Klamath Falls Field Station (Klamath Falls, Oregon) using methods modified from Eldridge and others (2012). Water samples were filtered onto pre-weighed ProWeigh® glass fiber filters (Environmental Express®, Mt. Pleasant, South Carolina) immediately after returning from the field to independently measure extracellular (dissolved-phase of water samples) and intracellular (particulate-phase of water samples) microcystins. Dissolved-phase microcystins were analyzed directly without further concentrating. For particulate-phase microcystins analysis, filters were freeze-dried, weighed, and extracted three times with 4 mL of 80 percent aqueous methanol containing 0.1 percent trifluoroacetic acid for at least 30 minutes. Extracts were sonicated for 5 minutes and then centrifuged at 10,000 rpm for 15 minutes. The supernatant was filtered through at 0.45-µm UniPrep® syringeless glass microfiber filter (Whatman Inc., Piscataway, New Jersey). Samples were dried under nitrogen gas to remove methanol and resuspended in 10 mL of deionized water. Diluted extracts (1/10–1/1,000) were analyzed using the enzyme-linked immunosorbent assay (ELISA) to determine microcystin concentrations following the manufacturer's instructions (kit 520011; Abraxis, LLC, Warminster, Pennsylvania). Absorbances of the samples at 450 nm were determined within 10 minutes following addition of the final reagent; samples and standards were analyzed in duplicate. Concentrations were determined from a linear regression of the mean absorbances of calibration standards analyzed along with the environmental samples. The detection limit for the microcystins assay was 0.10 µg/L. However, the concentrations in some samples were reported at values less than 0.10 µg/L because the aqueous concentrations were calculated from the total extracted biomass and sample volumes.

Methods: qPCR Analysis of Total and Potentially Toxigenic *Microcystis*

Samples were collected in 500-mL polypropylene bottles for qPCR analysis from the same composite (depth-integrated) water-column samples collected for microcystins analysis from June 9 to September 11, 2013, and June 11 to September 25, 2014. All samples for qPCR analysis were shipped on ice overnight to the OWML. Once received, 20–100 mL (depending on the amount of particulates present) of each sample were filtered in duplicate onto 0.4-µm nucleopore polycarbonate filters (Whatman/GE Healthcare®, Piscataway, New Jersey), stored in screw-cap vials with 0.3 g of acid-washed glass beads (Sigma-Aldrich®, St. Louis, Missouri), and held at -70 ºC until further analysis. DNA was extracted from the filters using the GeneRite DNA-EZ extraction kit (GeneRite, North Brunswick, New Jersey) according to the manufacturer's instructions, except that no pre-filter was used and that the final elution volume was 100 µL. Extracted genomic DNA (gDNA) was stored at 4 °C. Prior to qPCR analysis, total measured DNA concentration (in nanograms per microliter) was measured for each sample using a Qubit® fluorometer and a dsDNA (double-stranded DNA) high sensitivity assay kit (Life Technologies®, Carlsbad, California). For total cyanobacteria and total *Microcystis*, 5 µL of gDNA was analyzed by qPCR in duplicate using the primers, probes, and conditions described in Rinta-Kanto and others (2005) and the *Microcystis*-specific *mcy*E gene assay as described in Sipari and others (2010; table 2). Run conditions of each PCR and additional reagents used are described in Stelzer and others (2013). All assays were run on either an Applied Biosystems® 7500 or a StepOne Plus™ (Foster City, California) thermal cycler (individual assays run on a single machine) and using a TaqMan® universal PCR master mix (Applied Biosystems, Foster City, California). Sample inhibition determination and creation of plasmid standards were performed as discussed in Stelzer and others (2013). Each qPCR run included a six-point standard curve (made by serial dilutions of known quantities of target DNA) analyzed in duplicate. The conversion of the qPCR output (cycle threshold [Ct] value) to microbial-source tracking marker concentrations was done by interpolating from these standard curves. Standard curve characteristics are listed in table 4. An amplification efficiency of 100 percent indicates the DNA target sequence was doubled during each cycle. The dynamic range (given in copies per reaction), is the range of standards used, and the limit of quantification is the minimum quantifiable concentration, or the minimum given in the dynamic range. The detection limits for all qPCR assays were determined by using the 95th percentile of detections in the blank samples. A limit of quantification was also established for each assay using the average Ct value of the corresponding limit of detection and subtracting two times the standard deviation of the Ct values of the lowest standard; values between the limit of quantification and the limit of detection were considered estimated values. Limits of quantification were not determined in 2013. The regression coefficient, R^2, was used to evaluate the standard curve as described in Stelzer and others (2013).

Table 4. Standard curve characteristics for qPCR of total cyanobacteria, total *Microcystis*, and *Microcystis mcy*E in water column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014.

[**Assay:** *mcy*E, microcystin synthetase E gene; *cpc*A, c-phycocyanin photopigment gene. **Limit of quantification:** ND, not determined]

Assay	Dynamic range (copies/reaction)	Amplification efficiency (percent)	Regression coefficient (R^2)	Limit of detection (copies/reaction)	Limit of quantification (copies/reaction)
		2013			
Total cyanobacteria	16.2–1.62×10^7	94	0.999	85	ND
Total *Microcystis* 16S rRNA	16.8–1.68×10^7	89	0.998	0.4	ND
*mcy*E	24.3–2.43×10^6	98	0.999	2.1	ND
		2014			
Total cyanobacteria	24–2.4×10^6	92	0.999	26	72
Total *Microcystis* 16S rRNA	26–2.6×10^6	89	0.998	27	55
*mcy*E	24–2.4×10^6	94	0.997	13	39
*Aphanizomenon cpc*A	10–1.0×10^6	[1]94–104	0.997	10	ND

[1]Efficiencies were determined separately for triplicate assays, and the range among triplicates was given.

Data Reporting and Analysis

The absolute quantities of individual target genes determined by qPCR were used in all analyses and reported as copies per milliliter (copies/mL; Stelzer and others, 2013), which is also referred to hereinafter as copy number or concentration. Cell equivalents were not determined in this study, although *Microcystis* and *Aphanizomenon* have multiple copies of the 16S rRNA gene per genome (see section, "Discussion: qPCR as a Tool for Quantifying Populations of Bloom-Forming and Toxigenic Cyanobacteria"). Correlations between the total and relative copy numbers of genes targeted in qPCR and microcystin concentrations were tested using Spearman's rank correlation analysis in RStudio, version 0.99.441 (pspearman package; R Development Core Team, 2009). Correlations with p-values less than 0.05 were considered significant. In (quantitative) comparisons between qPCR-estimated copy numbers and microscopic counts, microscopic counts as cells per milliliter (cells/mL) were used, although natural units/mL was used in comparisons with results of HTS analysis (qualitative). All qPCR results for this study are given in Eldridge and others (2017).

Results: qPCR Analysis of Total and Potentially Toxigenic *Microcystis* and Comparisons with Microcystin Concentrations, 2013 and 2014

Microcystins were detected higher than the method reporting limit in all samples collected after July 9, 2013, and after July 14, 2014 (table 5). Concentrations were primarily in the particulate-phase of water-column samples

Table 5. Results of qPCR-based quantification of target genes and total microcystins concentrations in water-column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014.

[Gray shading in 2014 indicates no sample for the corresponding date at MDT in 2013. The method detection limit for microcystins was 0.1 ppm. **Total *Microcystis*:** The symbol E denotes a value that was estimated because it was higher than the upper range observed for blank samples, but lower than the quantification limit, and, therefore, may be unreliable. **qPCR assays:** *cpc*A, alpha subunit of the c-phycocyanin photopigment gene; *cpc*B, beta subunit of the c-phycocyanin gene; *mcy*E, microcystin synthetase E gene; mL, milliliter; qPCR, quantitative polymerase chain reaction. **Total microcystins concentration:** Total microcystins shown as the sum of concentrations in the dissolved and particulate phases of water-column samples. ND, data not collected; µg/L, micrograms per liter. **Symbol:** <, less than]

Sample date	qPCR assays					Total microcystins concentration (µg/L)
	Total cyanobacteria (copies/mL)	Total *Microcystis* (copies/mL)	*Microcystis mcy*E (copies/mL)	*Aphanizomenon cpc*A (copies/mL)	*Microcystis cpc*B (copies/mL)	
			2013			
07-09-13	60,100	E 5.1	<1.4	ND	ND	< 0.1
07-16-13	24,700	40.8	3.50	ND	ND	0.08
07-23-13	28,300	119	4.54	ND	ND	0.01
07-30-13	20,200	68.5	3.64	ND	ND	0.18
08-06-13	19,900	410	31.3	ND	ND	0.35
08-13-13	15,100	330	10.0	ND	ND	0.19
08-20-13	4,480	1,010	16.9	ND	ND	0.22
08-27-13	2,530	842	24.8	ND	ND	0.85
09-03-13	4,120	1,020	29.1	ND	ND	0.65
09-10-13	19,200	2,160	379	ND	ND	0.91
			2014			
06-16-14	140,000	430	20.0	12,626	ND	< 0.1
06-23-14	14,000,000	110 E	<13	13,748	ND	< 0.1
06-30-14	5,200,000	<18	<8.7	28,182	ND	0.03
07-07-14	7,000,000	54.0	<8.7	28,361	ND	0.06
07-14-14	4,400,000	61.0	<10	13,273	ND	< 0.1
07-21-14	610,000	40.0	<10	10,923	1,697	0.13
07-28-14	350,000	1,100	140	22,392	7,841	0.08
08-04-14	490,000	7,200	1,500	13,462	1,774	0.15
08-11-14	12,000,000	7,600	580	58,002	19,271	0.27
08-18-14	990,000	19,000	4,400	2,584	4,144	0.83
08-25-14	300,000	46,000	5,700	906	8,554	6.60
09-02-14	810,000	300,000	47,000	5,539	101,788	53.7
09-08-14	280,000	73,000	13,000	560	30,250	5.64
09-15-14	460,000	92,000	10,000	798	8,530	4.75

(data not shown). In 2013, detectable concentrations were low, remaining less than 1 µg/L all season, and ranging from less than 0.01 to 0.91 µg/L. Additional samples not shown in table 5 were collected from June 11 to July 9, 2013, and contained concentrations near to or lower than the detection limit. Concentrations were much higher in 2014, ranging from less than 0.1 to 53.7 µg/L. Concentrations were higher in August and September than in June or July during 2013 and 2014, but in both years, concentrations in most samples were lower than the limit of 10 µg/L set by the Oregon Health Authority (OHA) for issuing a public health advisory for recreational water. The OHA threshold was exceeded by one sample collected September 2, 2014, when the microcystin concentration peaked at greater than 53 µg/L. This was higher than any concentration previously reported in water samples collected by the USGS since 2009 (fig. 11; Eldridge and others, 2012).

As with microcystin concentrations, copy numbers of cyanobacterial 16S rRNA, *Microcystis* 16S rRNA, and *Microcystis mcy*E target genes were higher in 2014 than in 2013 (table 5). Total cyanobacteria concentrations ranged from 2,530 to 60,100 copies/mL in 2013 and from 280,000 to 12,000,000 copies/mL in 2014. Total *Microcystis* copy numbers were more variable, ranging from about 5.1 (estimated value) to 2,160 copies/mL in 2013, and in 2014, ranged from less than 18 to 300,000 copies/mL. In both years, a subpopulation of *Microcystis* cells were potentially toxigenic because the cells possessed sequences of the *mcy*E gene that codes for microcystin production (*mcy*+, hereinafter). In 2014, concentrations of the *mcy*+ population ranged from less than 8.7 to 47,000 copies/mL, and in 2013, the range was from less than 1.4 to 379 copies/mL (table 5).

To determine per-cell microcystin quotas relevant to water-quality monitoring and public health, microcystin concentrations in the particulate phase of water-column samples (intracellular concentrations) only were used to calculate the percentages of microcystin concentrations per *Microcystis* cell estimated by qPCR quantification of the 16S rRNA and *mcy*E gene targets (table 6). Overall, results showed higher per-cell microcystin quotas (0.14–4.89 percent) in *mcy*+ cells detected in 2013. Given the much higher cell densities relative to microcystin concentrations in 2014, each *mcy*+ cell that year accounted for less than 0.1 percent of the particulate-phase microcystins.

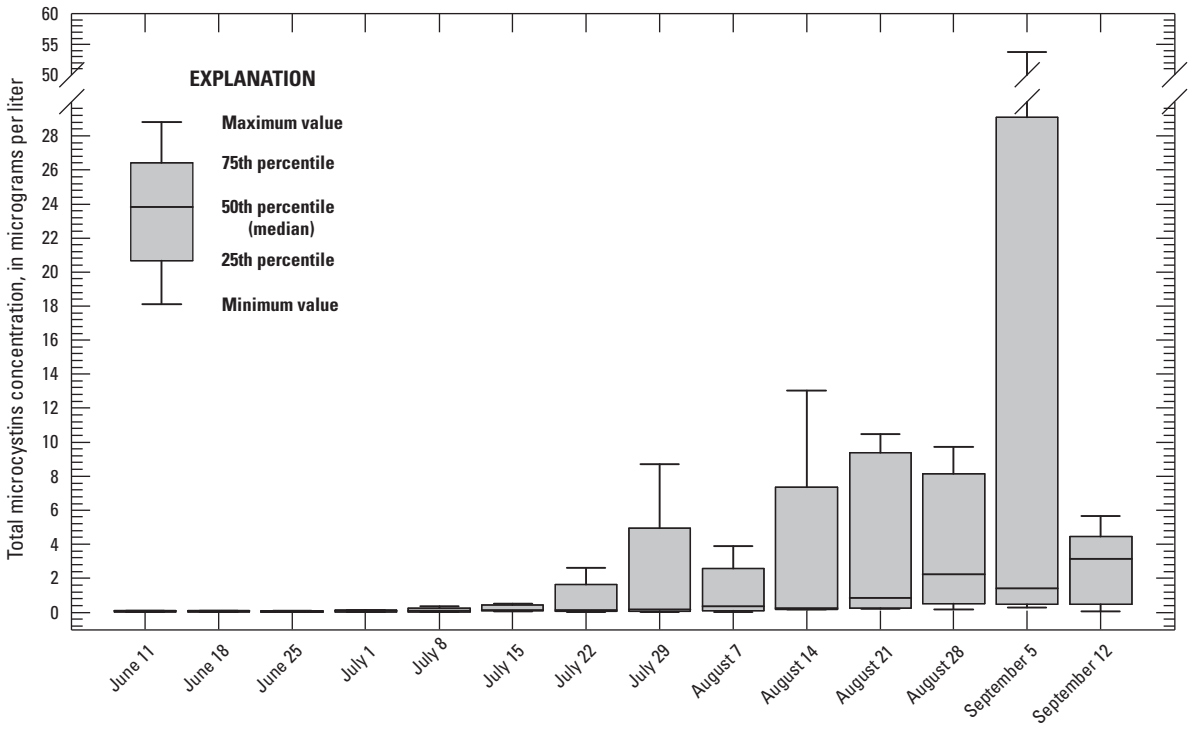


Figure 11. Total microcystins concentrations (as the sum of concentrations in the dissolved and particulate fractions) by date in water column samples collected at site MDT, Upper Klamath Lake, Oregon, 2009–2014. Sample dates are not actual. The first consensus date was chosen, and approximately 7 days were added to each subsequent date to represent weekly sampling across years.

Table 6. Microcystin concentrations in the particulate phase of water-column samples as a percentage of qPCR-estimated 16S rRNA and *mcy*E gene targets specific to *Microcystis* in samples collected from site MDT, Upper Klamath Lake, Oregon, 2013 and 2014.

[Method detection limit for microcystins was 0.1 ppm. **Total *Microcystis:*** The symbol E denotes a value that was estimated because it was higher than the upper range observed for blank samples, but lower than the quantification limit, and, therefore, may be unreliable. **Abbreviations:** *mcy*E, microcystin synthetase E gene; mL, milliliter; ND, not determined; qPCR, quantitative polymerase chain reaction; µg/L, micrograms per liter; <, less than]

Sample date	Intracellular microcystins concentration (µg/L)	Total *Microcystis* (copies/mL)	Microcystin concentration per total *Microcystis* cell (percent)	*Microcystis mcy*E (copies/mL)	Microcystin concentration per *mcy*E cell (percent)
			2013		
07-09-13	<0.1	E 5.1	ND	<1.4	ND
07-16-13	0.08	40.8	0.19	3.50	2.17
07-23-13	0.01	119	0.01	4.54	0.16
07-30-13	0.18	68.5	0.26	3.64	4.89
08-06-13	0.18	410	0.04	31.3	0.57
08-13-13	0.19	330	0.06	10.0	1.89
08-20-13	0.22	1,010	0.02	16.9	1.27
08-27-13	0.61	842	0.07	24.8	2.48
09-03-13	0.55	1,020	0.05	29.1	1.88
09-10-13	0.54	2,160	0.02	379	0.14
			2014		
06-23-14	<0.1	110 E	ND	<13	ND
06-30-14	0.03	<18	ND	<8.7	ND
07-07-14	0.06	54.0	0.11	<8.7	ND
07-14-14	<0.1	61.0	ND	<10	ND
07-21-14	0.13	40.0	0.32	<10	ND
07-28-14	0.08	1,100	0.007	140	0.06
08-04-14	0.15	7,200	0.002	1,500	0.01
08-11-14	0.10	7,600	0.001	580	0.02
08-18-14	0.30	19,000	0.002	4,400	0.01
08-25-14	4.13	46,000	0.009	5,700	0.07
09-02-14	51.9	300,000	0.02	47,000	0.11
09-08-14	5.21	73,000	0.007	13,000	0.04
09-15-14	3.52	92,000	0.004	10,000	0.04
09-22-14	1.24	92,471	0.001	10,061	0.01

To quantify changes in the relative abundances of species of interest to water quality in Upper Klamath Lake, quantities of qPCR-estimated gene targets were used to calculate the following ratios as relative percentages (based on copy numbers): (1) the percentage of total cyanobacteria that were *Microcystis* (*mcy*+ and *mcy*-), (2) the percentage of total cyanobacteria that were *mcy*+, and (3) the percentage of total *Microcystis* that were *mcy*+ (table 7). Overall, comparisons of the relative percentages calculated over the date range sampled in both years indicated that, although the cyanobacteria bloom was similarly comprised of *Microcystis* in 2014 (average 8.22 percent) and 2013 (average 9.70 percent), *mcy*+ cells made up a larger portion of the total *Microcystis* population (average 16.8 percent in 2014 and 5.93 percent in 2013) in 2014, and a larger portion of the total cyanobacteria community (average 2.19 percent in 2014 and average 0.43 percent in 2013).

Temporal changes in microcystin concentrations and the relative copy numbers of *mcy*+ *Microcystis*, total *Microcystis*, and cyanobacteria in 2013 and 2014 showed a correspondence between microcystin concentrations in Upper Klamath Lake and the total *Microcystis* population size as a percentage of the total cyanobacterial community (fig. 12) or as the absolute copy number determined by qPCR (fig. 13).

Table 7. Relative abundances of total *Microcystis, mcy*+ *Microcystis*, and *Aphanizomenon* in water-column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014.

[Relative abundances based on copy numbers. **Abbreviations:** *cpc*A, alpha subunit of the c-phycocyanin photopigment gene; *mcy*E, microcystin synthetase E gene; ND, data not collected, <, less than]

Sample date	Total *Microcystis* in total cyanobacteria (percent)	*Microcystis mcyE* in total cyanobacteria (percent)	*Microcystis mcy*E in total *Microcystis* (percent)	Total *Microcystis* in *Aphanizomenon cpc*A (percent)	Microcystis *mcy*E in *Aphanizomenon cpc*A (percent)
			2013		
07-09-13	0.01	0.01	<27.5	ND	ND
07-16-13	0.17	0.01	8.58	ND	ND
07-23-13	0.42	0.02	3.82	ND	ND
07-30-13	0.34	0.02	5.31	ND	ND
08-06-13	2.06	0.16	7.63	ND	ND
08-13-13	2.19	0.07	3.03	ND	ND
08-20-13	22.5	0.38	1.67	ND	ND
08-27-13	33.3	0.98	2.95	ND	ND
09-03-13	24.8	0.71	2.85	ND	ND
09-10-13	11.3	1.97	17.5	ND	ND
			2014		
06-16-14	0.31	0.01	4.65	ND	0.16
06-23-14	<0.01	<0.01	11.8	ND	0.09
06-30-14	<0.01	<0.01	48.3	ND	0.03
07-07-14	<0.01	<0.01	16.1	ND	0.03
07-14-14	<0.01	<0.01	16.4	ND	0.08
07-21-14	0.01	<0.01	25.0	15.5	0.09
07-28-14	0.31	0.04	12.7	35.0	0.63
08-04-14	1.47	0.31	20.8	13.2	11.1
08-11-14	0.06	<0.01	7.63	33.2	1.00
08-18-14	1.92	0.44	23.2	160	170
08-25-14	15.3	1.90	12.4	944	629
09-02-14	37.0	5.80	15.7	1,838	849
09-08-14	26.1	4.64	17.8	5,405	2,323
09-15-14	20.0	2.17	10.9	1,069	1,254

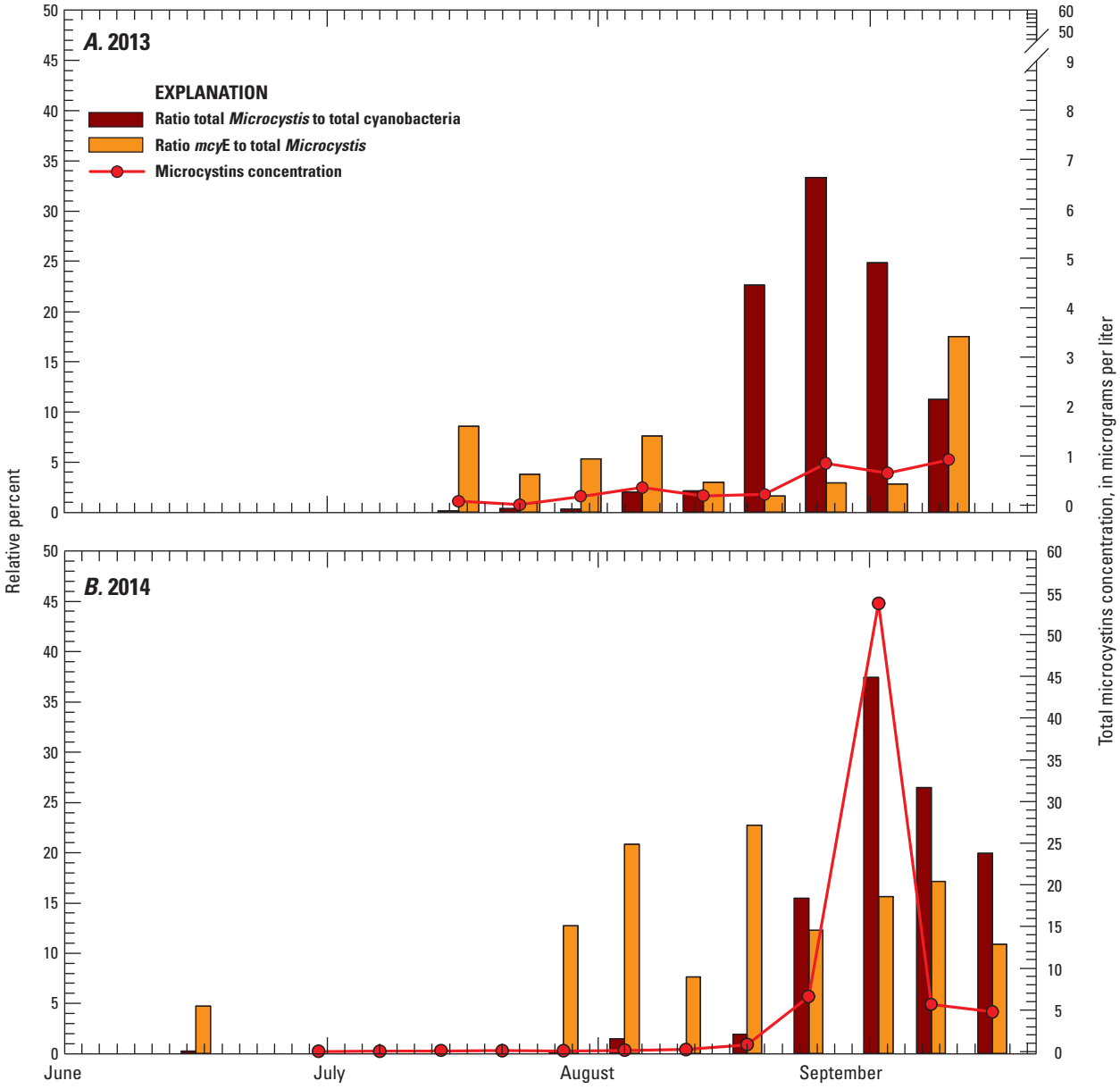


Figure 12. Ratios of total *Microcystis* to total cyanobacteria (copies per milliliter) and ratios of *mcy*E to total *Microcystis* (copies per milliliter) at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014. *mcy*E copies/mL were lower than the detection limit on July 9, 2013, and from June 23 to July 21, 2014.

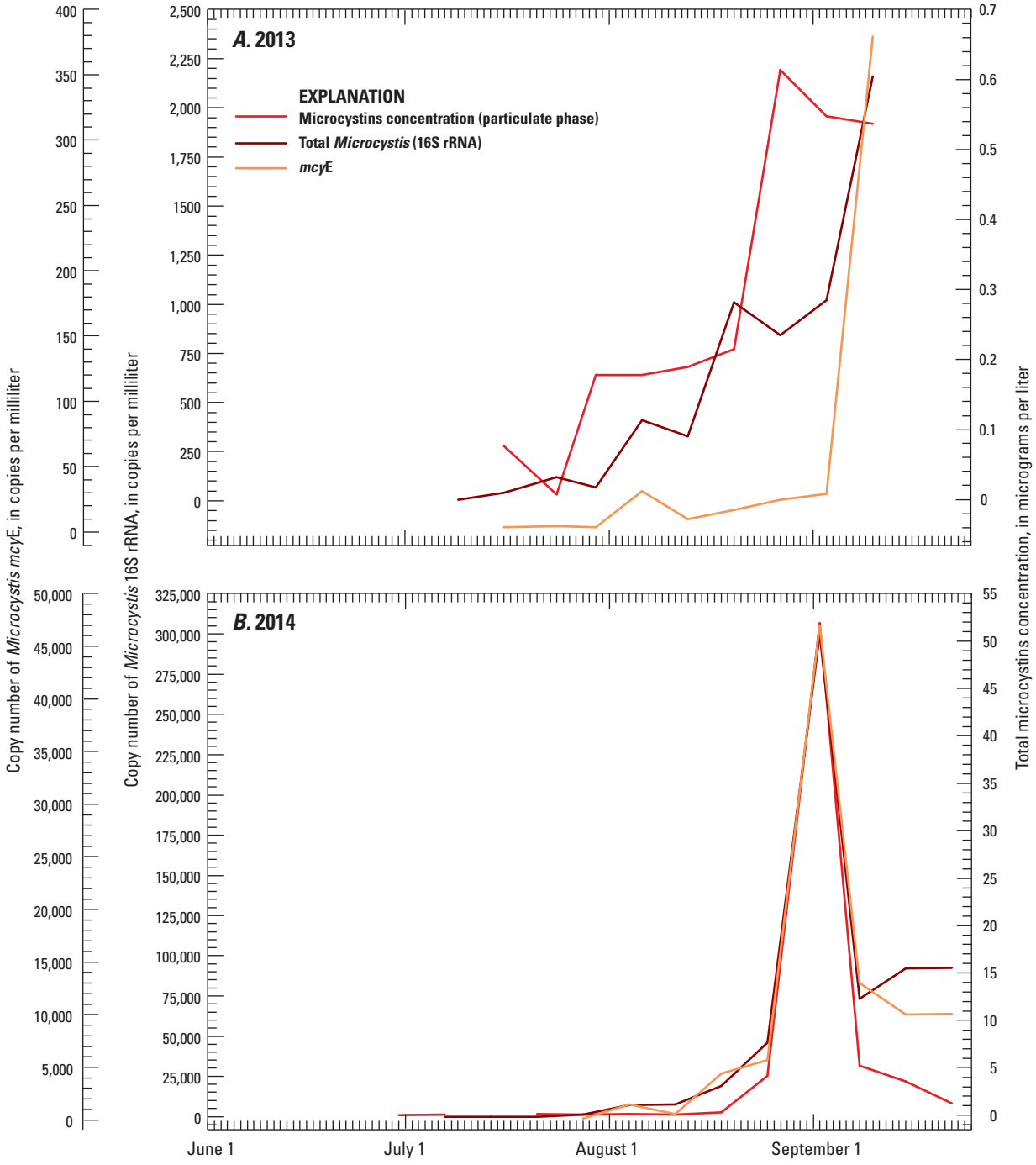


Figure 13. Total *Microcystis* and *mcy*E copy numbers determined by qPCR and microcystin concentrations in the particulate phase of water-column samples at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014.

In 2014, peaks in the proportions of *mcy*+ cells in the *Microcystis* population occurred when microcystin concentrations and the total *Microcystis* population size was low (fig. 12). So, over each sample season, microcystin concentrations showed less overlap with the trend in the *mcy*+/total *Microcystis* ratio. However, considering only the late-season periods of high microcystin concentrations, there was more agreement between changes in microcystin concentrations and proportions of *mcy*+ *Microcystis* to total *Microcystis*; this was also seen when absolute quantities were compared (fig. 13). Microcystin concentrations and ratios of *Microcystis* to total cyanobacteria showed corresponding peaks on September 2, when the microcystin concentration was 53.7 µg/L and the ratio of *Microcystis* 16S rRNA copies to that of total cyanobacteria was 37 percent. The relative copy number of *Microcystis* cells that were *mcy*+ remained relatively constant during the same time period, varying mostly between 10 and 25 percent and showing no corresponding peak on September 2. In 2013, when microcystin concentrations were much lower, there was not a clear correspondence between the microcystin concentrations and either total *Microcystis* or the proportion of *mcy*+ cells (fig. 12). Comparison of all log-transformed *Microcystis* and *mcy*E copy numbers and total microcystins concentrations for both years combined (fig. 14) showed positive correlations between *mcy*E copy numbers and total *Microcystis* copy numbers and between *mcy*E copy numbers and microcystin concentrations when microcystin concentrations were greater than 1.0 µg/L. Between 0.1 and 1.0 µg/L, closer to the detection limit of the microcystins assay and where greater error may be expected, the correlations between *mcy*+ cell copy number and microcystin concentration were not linear.

Results of Spearman's analysis showed that total *Microcystis*, *Microcystis mcy*E, and *Aphanizomenon* copy numbers were significantly correlated with microcystin concentrations in 2013 and 2014 (*Aphanizomenon* was quantified in 2014 only; table 8). Microcystin concentrations also were significantly correlated with the percentage of *Microcystis* in total cyanobacteria and with the percentage of total cyanobacteria that were *mcy*+ *Microcystis*.

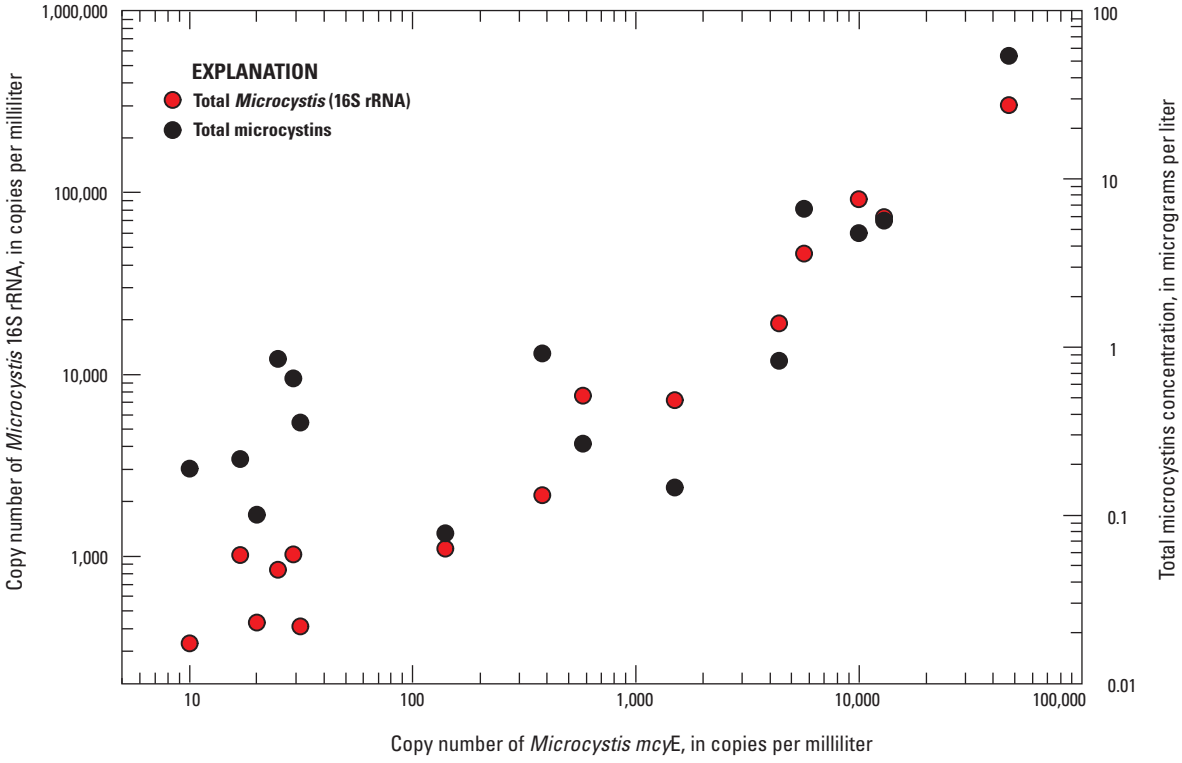


Figure 14. Log of *mcy*E copy numbers contrasted with *Microcystis* 16rRNA copy numbers as determined by qPCR and total microcystins concentrations in samples collected from site MDT in Upper Klamath Lake, Oregon, 2013 and 2014. Copy numbers lower than the detection limit were assigned a value of 1.0 copies per milliliter for plotting, and microcystin concentrations lower than the detection limit were considered equal to the detection limit (0.1 microgram per liter) in the figure.

Table 8. Spearman correlation coefficients, *n*, and p-values for correlations of total *Microcystis*, *mcy*+ *Microcystis*, and *Aphanizomenon* copy numbers with total microcystins concentrations in water column samples collected at site MDT, Upper Klamath Lake, Oregon, 2013 and 2014.

[Correlations were performed with 2014 samples collected on corresponding dates in 2013. Correlations were considered significant at $p < 0.05$. Significant correlations are indicated by **bold** p-values. **Gene target quantity:** *cpc*A, alpha subunit of the c-phycocyanin photopigment gene; *cpc*B, beta subunit of the c-phycocyanin gene; *mcy*E, microcystin synthetase E gene; mL, milliliter. **Abbreviations:** ND, data not collected; <, less than]

Gene target quantity (copies/mL)	2013			2014		
	Rho (ρ)	*n*	p-value	Rho (ρ)	*n*	p-value
*Aphanizomenon cpc*A	ND	ND	ND	-0.70	10	0.022
Microcystis 16S rRNA	0.86	10	**<0.001**	0.90	10	**<0.001**
*Microcystis mcy*E	0.87	10	**<0.001**	0.93	10	**<0.001**
*Microcystis cpc*B	ND	ND	ND	0.71	8	0.037
Percentage of total *Microcystis* in total cyanobacteria	0.794	10	**0.004**	0.833	10	**<0.001**
Percentage of *Microcystis mcy*E in total cyanobacteria	0.939	10	**<0.001**	0.819	10	**0.002**
Percentage of *Microcystis mcy*E in total *Microcystis*	-0.236	10	0.490	-0.127	10	0.707
Percentage of total *Microcystis* in *Aphanizomenon cpc*A	ND	ND	ND	0.762	8	**0.021**
Percentage of *Microcystis mcy*E in *Aphanizomenon cpc*A	ND	ND	ND	0.915	10	**<0.001**

However, correlations were not significant between microcystin concentrations and the percentage of *Microcystis* cells that were *mcy*+. In 2014, microcystin concentrations were negatively correlated with *Aphanizomenon* copy numbers, which follows from a negative correlation between *Aphanizomenon* and *Microcystis* copy numbers ($\rho = -0.59$, $p = 0.07$; not shown in table 8). Significant correlations between the ratio of total or *mcy*+ *Microcystis* to *Aphanizomenon* copy numbers and microcystin concentrations in 2014 indicated further that microcystin levels increased along with *Microcystis* copy numbers as *Aphanizomenon* copy numbers declined (table 8).

Comparison of Phytoplankton Occurrence Determined by qPCR and Light Microscopy, 2013

In 2013, counts determined by microscopy for cyanobacteria and *Microcystis* did not agree with qPCR-estimated copy numbers. In the six samples analyzed by both methods, qPCR copy numbers were lower for cyanobacteria 16S rRNA than the cell counts determined by microscopy (fig. 15). Cell equivalents were not determined in this study, so the qPCR results were not translated into natural cell densities. Both *Microcystis* and *Aphanizomenon* have multiple copies of the 16S rRNA gene per genome (see section, "Discussion: qPCR as a Tool for Quantifying Populations of Bloom-Forming and Toxigenic Cyanobacteria"), so it is likely that the qPCR estimates of gene copy number and relative abundances determined by HTS were greater than environmental cell concentrations for these species. The consistently high microscopy counts for cyanobacteria relative to qPCR agreed with comparisons of HTS and microscopy, because HTS showed that most of the cyanobacteria were composed of *Aphanizomenon/Anabaena* in the collected samples (fig. 6), and the corresponding microscopy analysis estimated *Aphanizomenon* at densities from two to nearly five times higher, indicating an overrepresentation of this group by microscopy. In contrast, *Microcystis* was not detected microscopically in five of the six samples analyzed by both methods (fig. 15). *Microcystis* was detected by both methods on September 10 only but at a 4-fold lower concentration in the microscopic count than by qPCR.

Quantification of *Aphanizomenon* and Total *Microcystis* using qPCR

Although qPCR has been used widely to detect and quantify toxigenic cyanobacteria, there have been very few studies that have used qPCR to monitor *Aphanizomenon*. In Upper Klamath Lake, *Aphanizomenon* is non-toxigenic and historically has been the most abundant bloom-forming species, and a recent USGS study of microcystin concentrations and associated nutrient concentrations and ratios indicated that microcystin occurrence in the lake may be associated with the decline of the first seasonal bloom of non-toxin-producing *Aphanizomenon* (Eldridge and others, 2012). This study highlighted the importance of understanding the ecological interactions between toxigenic and non-toxigenic species for predicting periods of elevated toxin concentrations. Therefore, to compare changes in the relative abundances of *Microcystis* and *Aphanizomenon* in lake water samples and to relate the abundances of these species to changes in the total cyanobacterial community, a protocol for using qPCR to quantify *Aphanizomenon* specific to Upper Klamath Lake was developed at Oregon State University and applied to samples collected from site MDT in 2014.

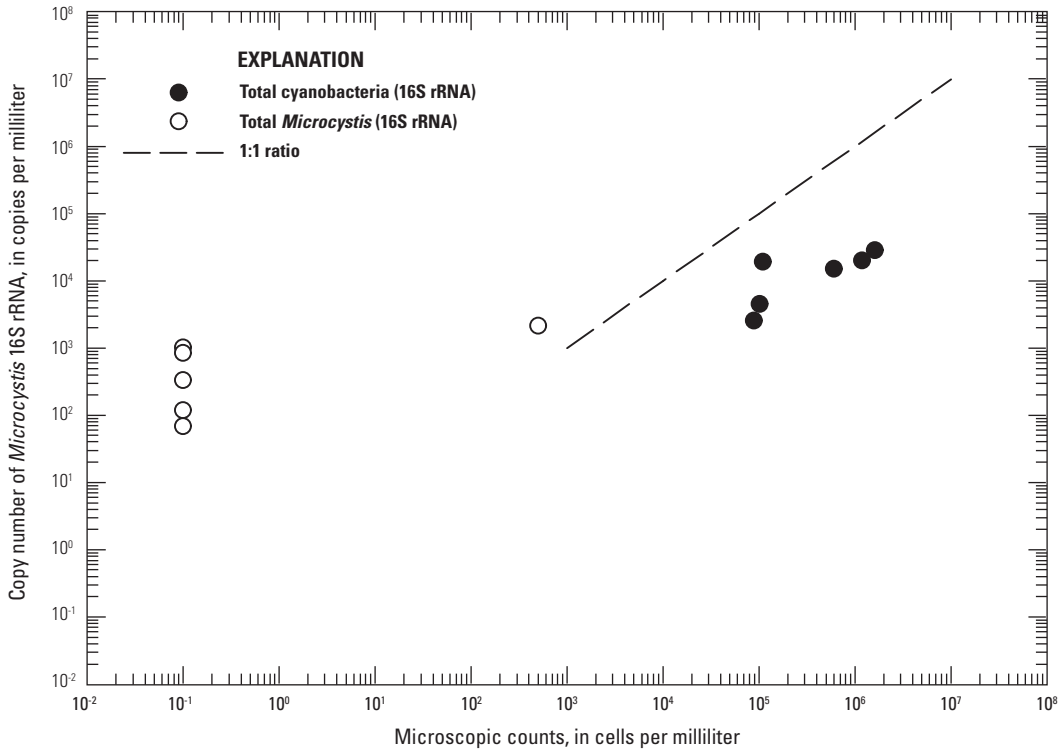


Figure 15. Comparison of qPCR-estimated results to cell counts by microscopy, 2013. The 1:1 line shows agreement between both methods.

Methods: qPCR Analysis of *Aphanizomenon* and *Microcystis*

Sample collection and preservation, DNA extraction, and qPCR analysis of *Microcystis* and total cyanobacteria (2013 and 2014) are previously described. In 2014, samples split from the composite samples collected for microcystins and qPCR analysis of *Microcystis* and total cyanobacteria were concurrently collected and shipped overnight to the laboratory of Dr. Theo Dreher at Oregon State University (Corvallis, Oregon). Samples were filtered in triplicate upon arrival through 1.2-µm glass fiber (GF/C) filters (Whatman/GE Healthcare, Piscataway, New Jersey). Filters were stored in microcentrifuge tubes at -80 °C until further analysis. DNA was extracted from filters with the SurePrep® soil DNA isolation kit (Fisher Scientific, Pittsburgh, Pennsylvania) using the optional humic acid removal step and two elution spins to obtain a final volume of 150 µL.

qPCR Design to Target *Aphanizomenon* from Upper Klamath Lake, Oregon

The primers and probe used for qPCR were designed from a 103-bp segment of the *Aphanizomenon cpc*A gene identified from deep sequencing of a culture from Upper Klamath Lake isolated from site MDT in 2013. This gene encodes the alpha subunit of the phycocyanin accessory pigment complex in an operon that has been widely used for qPCR and phylogenetic studies of cyanobacteria (Janson and Graneli, 2002; Kurmayer and Kutzenberger, 2003; Bozarth and others, 2010; Wang and others, 2013). Selection of the optimal primers and probe was based on the genome of an *Aphanizomenon* culture from Upper Klamath Lake, metagenome sequences from a Copco Reservoir (site KR19874; lower Klamath River) bloom sample collected on August 21, 2012, and *Aphanizomenon* spp. and *Dolichospermum* spp. DNA sequences in the GenBank® database (http://www.ncbi.nlm.nih.gov/genbank/).

The selected primers and probe were identical to *Aphanizomenon* metagenomic sequences from Upper Klamath Lake and to the Copco Reservoir sequences. However, because *Aphanizomenon* cannot be distinguished genetically from *Dolichospermum*, there was potential for amplification of both genera together. To address this, off-target sequence similarities of the primers and probe were identified using a basic local alignment search tool (BLAST). The target probe contained a 5 ft-FAM™ fluorescent reporter and a 3-non-fluorescent quencher. To normalize the fluorescent reporter signal, the ROX® passive reference dye (Thermo Scientific, K0232, Williamton, Delaware) was included in the qPCR master mix. Sample copy numbers were obtained from comparisons with a standard curve made from known copies of recombinant *cpc*A run in triplicate (6 points ranging from 106 to 10 copies serially diluted 10×).

Cloned target sequences were used to generate the standard curves for qPCR. *Aphanizomenon cpc*A gene sequences were cloned into vector pJET1.2/blunt, provided in the CloneJet® PCR cloning kit (Thermo Scientific, Williamton, Delaware). Plasmids were then purified, linearized with an XbaI restriction digest, purified by gel electrophoresis, and quantified using the Qubit fluorometer dsDNA assay. Plasmid concentrations were converted to copy numbers by using the following equation:

$$\frac{\mathrm{DNA\,(ng)} \times \left(6.022 \times 10^{23}\ \mathrm{molecules/mole}\right)}{\mathrm{Length\ of\ plasmid\ and\ gene\,(bp)} \times \left(1 \times 10^{9}\ \mathrm{ng/g}\right) \times 660\ \mathrm{g/mole-bp}} = \mathrm{copies/\mu L} \quad (1)$$

Recombinant plasmids were stored in working solutions at 2×10^7 copies/µL concentration in a Tris-EDTA buffer to prevent degradation. Serial dilutions of working plasmid stocks were made in nuclease-free water from 10^6 copies/5 µL to 10 copies/5 µL in 1/10 dilutions. For qPCR, 5 µL of each dilution was used. Working stocks of the primers and probe were made as with the plasmid standards to a final concentration in the qPCR reactions of 0.3 micromolar (µM) for the primers and 0.2 µM for the probe. The master mix constituents and the thermal cycler conditions used for the *Aphanizomenon* qPCR are shown in tables 9 and 10, respectively.

Amplification efficiencies for each qPCR reaction were calculated from standard curves ranging from 10^6 to 10 gene copies per well. Efficiency was calculated by using the equation:

$$-1 + 10^{-1/\text{slope of standard curve}} \times 100 = \text{percent efficiency} \quad (2)$$

Efficiencies ranged from 94 to 104 percent, within the range considered acceptable for qPCR (90–110 percent). Sample copy numbers were calculated as follows, correcting for dilutions and extraction efficiencies and using the standard curve linear equation and filtered sample volumes:

$$\frac{\left(10^{\frac{C_t - \text{y-intercept}}{\text{slope}}} \times \text{sample dilution factor}\right)}{\text{volume of filtered sample (mL)}} \times \text{extraction correction} = \text{gene copies/mL} \quad (3)$$

To test the efficiency of the SurePrep® DNA extraction kit, a validated qPCR assay for *Microcystis cpc*B (Kurmayer and Kutzenberger, 2003) was run in parallel to the *Aphanizomenon cpc*A assay. *Microcystis* DNA was extracted using the kit in triplicate from 1.5 mL of a GF/C filtered PCC299 culture of *Microcystis*. An additional 1.5 mL of the same *Microcystis* culture was pelleted in triplicate, and DNA was extracted using a traditional phenol-chloroform extraction method. qPCR was then used to obtain gene copy numbers for both genera. On average, the SurePrep kit was only 8.6 ±3 percent as efficient as the phenol-chloroform extraction, based on the efficiency calculation performed for *Aphanizomenon cpc*A. This was accounted for by applying a 11.6× correction factor to the final estimates. One explanation for the low extraction efficiency is difficulty in achieving complete cellular disruption from the filter mass, probably due to either adsorption to the filter, moisture retained in the filter, or to inadequate cell disruption. The limit of detection corrected for extraction efficiency was 116 gene copies per reaction. This was calculated using the lowest copy number on the standard curve and multiplying by the extraction efficiency correction factor.

Because determining changes in *Microcystis* and microcystin concentrations in relation to the *Aphanizomenon*-dominated bloom cycle was desired, the specificity of the *Aphanizomenon cpc*A qPCR assay was compared with those of the *Microcystis cpc*B gene. To test non-specific amplification, recombinant plasmids carrying *Microcystis cpc*B from the Copco Reservoir were spiked into samples containing recombinant plasmids carrying *Aphanizomenon cpc*A from Upper Klamath Lake. Conditions of high *Microcystis* to *Aphanizomenon* ratios were mimicked

Table 9. Master mix constituents for qPCR of *cpc*A gene sequences in *Aphanizomenon*, Upper Klamath Lake, Oregon, 2014.

[**Abbreviations:** *cpc*A, alpha subunit of the c-phycocyanin photopigment gene; qPCR, quantitative polymerase chain reaction; µM, micromolar concentration; µL, microliters; –, none]

Reagent	Stock solution	Added to each 25 µL reaction	Master mix volume (100 reactions)
Maxima probe/ROX master mix	2×	12.5 µL	1,250 µL
TaqMan exogenous IPC mix	10×	2.5 µL	250 µL
TaqMan exogenous IPC DNA	50×	0.5 µL	50 µL
Forward primer	18.75 µM	0.4 µL	40 µL
Reverse primer	18.75 µM	0.4 µL	40 µL
Probe	5.0 µM	1.0 µL	100 µL
Water	–	2.7 µL	270 µL
Temperature DNA	–	5.0 µL	–

Table 10. qPCR conditions to quantify *cpc*A gene sequences in *Aphanizomenon*, Upper Klamath Lake, Oregon, 2014.

[**Abbreviations:** *cpc*A, alpha subunit of the c-phycocyanin photopigment gene; qPCR, quantitative polymerase chain reaction]

Temperature (degrees Celsius)	Time	Cycles
50	2 minutes	1
95	10 minutes	1
95	15 seconds	40
60	30 seconds	40
72	30 seconds	40

by adding *Microcystis cpc*A in 100-fold excess relative to *Aphanizomenon cpc*A (10,000 copies:100 copies). Spiked wells were compared to non-spiked wells containing the same number of *Aphanizomenon cpc*A copies. The results revealed no significant difference in amplification of non-spiked as opposed to spiked wells, showing that this assay included no significant non-specific amplification of *Microcystis* ($p = 0.64$, two-sample t-test). Lack of amplification was likely due to differences in primer/probe annealing sites between *Microcystis* CR73 plasmid and the *Aphanizomenon* culture from Upper Klamath Lake where 27 out of 68 bases were mismatches (Connor Driscoll, Theo Dreher, and others, Oregon State University, written commun., 2016).

The specificity of *Aphanizomenon* against *Anabaena*/*Dolichospermum* was tested by combining equal concentrations of *Aphanizomenon* and *Anabaena*/*Dolichospermum* DNA extracted from *Aphanizomenon* from Upper Klamath Lake and *Anabaena*/*Dolichospermum* from Anderson Lake, Washington. Results from this specificity test revealed no significant difference in the concentrations of each genera treatment ($n = 3$; $p = 0.53$, two-sample t-test). Similar to *Microcystis*, lack of *Anabaena*/*Dolichospermum* amplification was likely due to differences between primer/probe annealing sites where 11 out of 68 bases were mismatches.

To test for inhibition, an internal positive control (containing control DNA as well as control-specific primers and probe) was included in each reaction (Bozarth and others, 2010). Ct values for these controls ranged from 28.5 to 27.8, which represented variation of less than a single cycle. The higher values in the range were associated with standard curve points containing the highest *Aphanizomenon* gene copy numbers. Results of this test indicated that no PCR inhibition was present in the samples.

qPCR of *Microcystis cpc*B

To compare qPCR quantification of *Aphanizomenon* with results of *Microcystis* quantification, qPCR targeting the *cpc*B region specific to *Microcystis* was performed at Oregon State University using the same filters analyzed for *Aphanizomenon cpc*A; qPCR quantification of total *Microcystis* targeting 16S rRNA gene sequences (previously described) was performed at OWML on samples split from the same composite water sample. Both *Aphanizomenon* and *Microcystis* have a single copy of the *cpc* gene per genome, and results of PCR or qPCR could vary between gene targets (16S rRNA and *cpc*B, for example) due to variations in the PCR reactions or run conditions. Therefore, comparisons were made between *Aphanizomenon cpc*A abundance and both *Microcystis* 16S rRNA (analyzed at OWML) and *Microcystis cpc*B (analyzed at Oregon State University). Reaction mixtures, cycle conditions, and preparation of plasmid DNA standards and primer/probe working solutions were as described in Bozarth and others (2010). The primers and probe used were described in Kurmayer and Kutzenberger (2003). Samples collected July 21 to September 15, 2014, were included in the analysis.

Results: qPCR Analysis of *Aphanizomenon cpc*A and Comparisons with Relative Copy Numbers of *Microcystis cpc*B, 2014

In 2014, results of qPCR targeting the *Aphanizomenon cpc*A gene ranged from 560 copies/mL (September 8) to 58,002 copies/mL (August 11; table 5). After August 11, copy numbers were higher for *Microcystis cpc*B, which peaked at 102,000 copies/mL on September 2. Changes in *Aphanizomenon* copies over time (fig. 16) suggested a moderate, steady bloom from June 16 to August 6 corresponding to chlorophyll *a* concentrations between approximately 75 and 200 µg/L (chlorophyll *a* data not shown). Concentrations declined after that date and remained less than that of *Microcystis cpc*B for the rest of the season. These results agreed with high densities of *Aphanizomenon* observed directly by field crews through August 11. Coincident with the decline in *Aphanizomenon cpc*A, *Microcystis cpc*B copy numbers peaked to almost twice the seasonal maximum estimated by qPCR for *Aphanizomenon cpc*A. *Microcystis cpc*B copy numbers were higher than *Aphanizomenon cpc*A copy numbers on every sample date after August 18, indicating that the *Aphanizomenon* bloom was overtaken by a stronger *Microcystis* bloom late in the season.

Ratios of *Microcystis* to *Aphanizomenon* based on copy numbers were greater than 1 after August 18 (fig. 17), which further suggested a dominance of *Microcystis* over *Aphanizomenon* during that time. Ratios of *mcy*+ *Microcystis* to *Aphanizomenon* followed the same seasonal pattern, but the proportion of *mcy*+ *Microcystis* was relatively consistent through the season (fig. 17). This suggested that *mcy*+ cell densities increased in the environment as the *Aphanizomenon* population declined and that this increase was a result of increased *Microcystis* abundance and not necessarily a result of a greater proportion of *Microcystis* cells becoming toxigenic (*mcy*+).

Discussion: qPCR as a Tool for Quantifying Populations of Bloom-Forming and Toxigenic Cyanobacteria

Blooms of toxigenic cyanobacteria are a primary cause of poor water quality in eutrophic, freshwater lakes. The global distribution of toxigenic cyanobacteria is a testament to their ecological success, particularly taking into account current climate change scenarios that are predicted to act synergistically to promote cyanobacterial dominance and persistence (Kosten and others, 2012; Paerl and Paul, 2012; Wells and others, 2015). Detecting and monitoring changes in these potentially toxic blooms is, therefore, fundamental to water resource management. Increased knowledge of

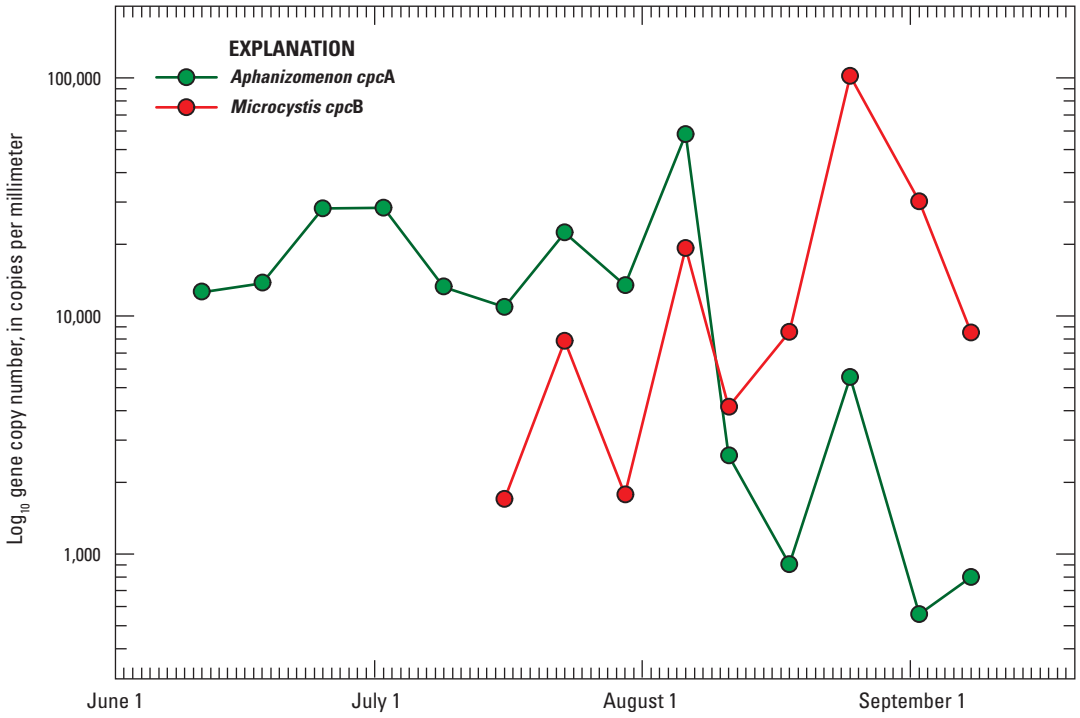


Figure 16. Log concentrations of *Aphanizomenon cpc*A and *Microcystis cpc*B gene targets determined by qPCR in water-column samples collected from site MDT, Upper Klamath Lake, Oregon, 2014.

the genetic basis for cyanotoxin production has led to the development of more sensitive and rapid monitoring tools that can be used to determine seasonal and temporal shifts in the proportions of microcystin- and non-microcystin-producing sub-populations. Ultimately, patterns revealed by these tools also may advance the understanding of factors that promote growth of toxigenic strains and the succession of cyanobacterial genera in natural aquatic systems in addition to helping understand the factors (chemical or biological) that promote toxin production, bloom formation, toxin cessation, and bloom collapse or senescence.

Results of this work agreed with previous studies in other aquatic systems that have shown direct correlations between qPCR-estimated quantifications of potentially toxigenic *Microcystis* cell densities and microcystin concentrations (Davis and others, 2009; Ha and others, 2009; Rinta-Kanto and others, 2009; Srivastava and others, 2012) and illustrated the use of qPCR as a tool for monitoring toxigenic blooms and for predicting when elevated microcystin concentrations are likely to occur. In addition, quantifications by qPCR of total and *mcy*+ *Microcystis* in this study revealed an unprecedented bloom of this species in 2014 that was observed directly by

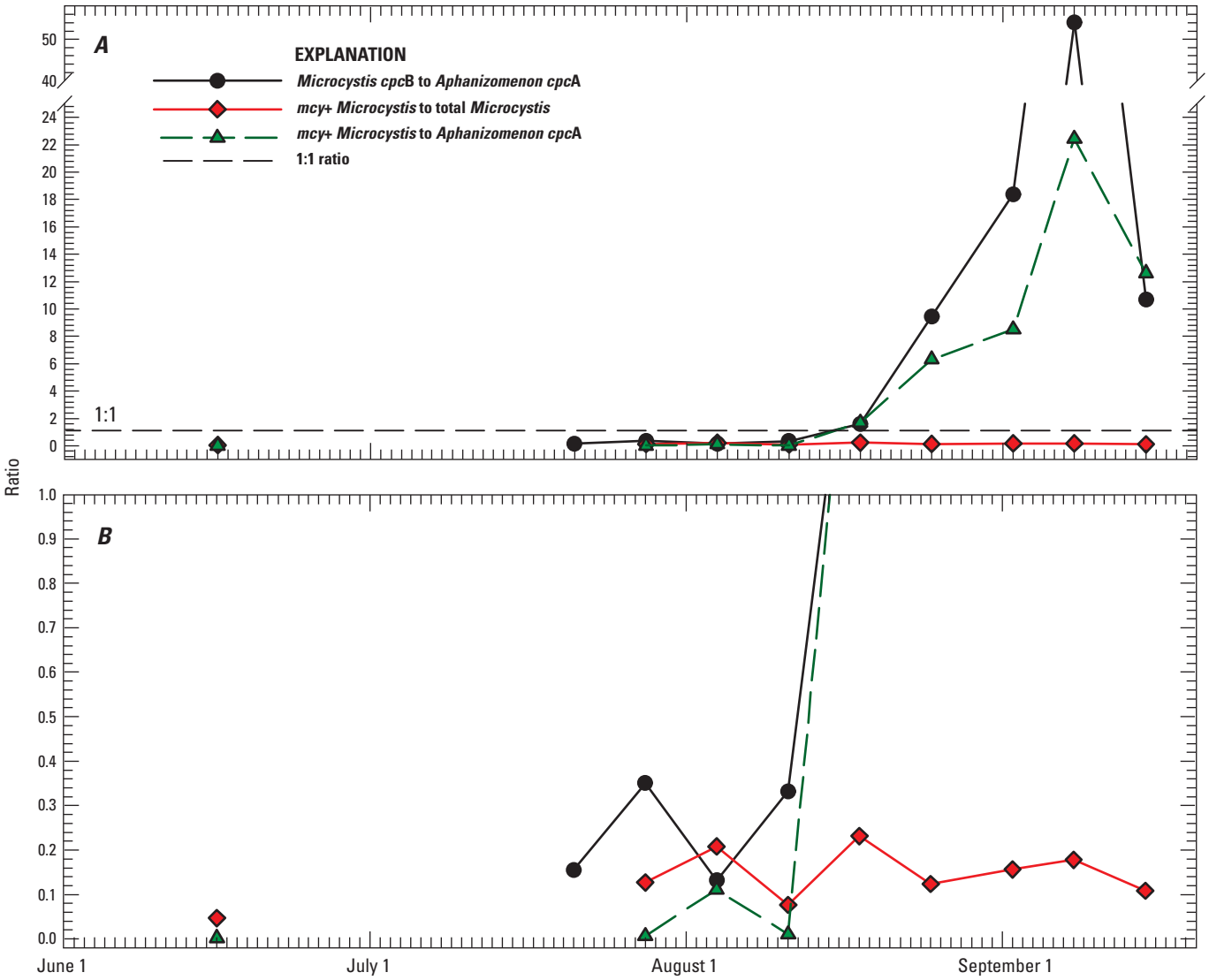


Figure 17. Ratios of *Microcystis cpc*B to *Aphanizomenon cpc*A, *mcy*+ *Microcystis* to total *Microcystis*, and *mcy*+ *Microcystis* to *Aphanizomenon cpc*A on different scales, 0 to less than 50 (*A*) and 0.0–1.0 (*B*), determined by qPCR in samples collected from site MDT, Upper Klamath Lake, Oregon 2014. *mcy*E copies per milliliter were lower than the detection limit on June 11, 2014 and from June 23 to July 21, 2014. *Microcystis cpc*B was not analyzed prior to July 21, 2014.

field crews and lab personnel handling water samples and that corresponded with elevated microcystin concentrations in the lake. Time-series trends in qPCR-determined copy numbers and results of correlation analyses in 2013 and 2014 showed a correspondence between microcystin concentrations and total *Microcystis* copies/mL over time that was not observed between microcystin concentrations and the proportion of *Microcystis* cells that were potentially toxigenic (*mcy*+) when the total *Microcystis* population was low. More *Microcystis* cells were *mcy*+ when the *Microcystis* population was smaller overall, but the total *Microcystis* population density proved to be a better predictor for elevated microcystin levels earlier in the sample season. Further investigation of overlapping nutrient dynamics and other environmental parameters will likely help to identify potential *Microcystis* growth factors and may explain some of the major contrasts in population sizes and microcystin levels observed between 2013 and 2014.

Despite the numerous benefits of using qPCR as a tool for water-quality monitoring, the method does possess some drawbacks that must be considered in the study design. For one, quantity estimates of cells carrying specific gene targets are based on genomic DNA of corresponding isolates for qPCR analysis. However, variations in the number of copies of the same gene, particularly of the 16S rRNA genes, occur within the genomes of different species, and these copies can vary from one to four or more per genome (Rinta-Kanto and others, 2005), although they do not vary over time. Therefore, a major caveat to using qPCR-based estimates of copy abundance or number to infer cell abundance or density is that some variation in copy number per genome within natural populations of any species is inherent and will be a source of error. This error may vary by gene target, so comparisons between populations of different species (or mixed communities) may be complicated by the error associated with variable gene copies per genome. Genomic analysis of *Microcystis aeruginosa* strain NIES-843 isolated from the Microbial Culture Collection of the National Institute for Environmental Studies revealed two sets of rRNA genes and a single *mcy* gene cluster (Kaneko and others, 2007). For *Aphanizomenon flos-aquae*, the near-complete genome sequencing of a culture established from Upper Klamath Lake, site MDT, collected in August 2013, indicated the presence of four rRNA gene copies per genome (GeneBank accession LJOY00000000; Connor Driscoll, Theo Dreher, and others, Oregon State University, written commun., 2016). Other studies using qPCR copy number estimates have adjusted for this copy number variation by converting target gene copy numbers to cell densities based on genomic DNA standards generated from laboratory-cultivated strains in which the number of copies of the desired target genes are known (Rinta-Kanto and others, 2009). In other studies focusing on *Microcystis* 16S rDNA targets, gene copy estimates obtained by qPCR were divided by two to determine the total *Microcystis* cell equivalents, given that *Microcystis* is known to have two copies of this gene (Baxa and others, 2010; Beversdorf and others, 2015). Translation of qPCR-estimated copy number to the density of cells in the environment may be improved by such conversions, but using target gene abundances or numbers converted to cell quantities could introduce potential bias in statistical analyses (Rinta-Kanto and others, 2009), and determining what to use as a conversion factor for complex communities may be difficult or impossible.

Another potential complication with qPCR is that, assuming genes for microcystin production are present and being expressed, it is still possible to see discrepancies between the copy number or abundance of toxigenic genotypes and concentrations of the associated toxin. This issue is not exclusive to qPCR, but is a major criticism of the technique. Though this was not the case in the current study, this lack of correspondence led Beversdorf and others (2015) to conclude that the presence of microcystin synthetase genes is not a good indicator of toxins in the environment. In that study, based on analyses of five gene targets across four different lakes, the copy numbers of all genes in collected samples were highly variable temporally and, in some cases, correlated more with increased temperature and nutrients than with microcystin concentrations. Such discrepancies may be due to non-toxigenic strains containing microcystin synthetase gene fragments or mutations that amplify with PCR or to the presence of other toxin-producing genotypes that are not identified or targeted in the qPCR study. Beversdorf and others (2015) used liquid chromatography-mass spectrometry (LC-MS) to evaluate microcystins but targeted only four congeners. So, it is possible that some microcystin congeners were not captured in that study or that those that were captured were differentially produced by different strains. In Upper Klamath Lake, for example, *Microcystis* spp. is thought to be the dominant toxigenic species, but other genera known for microcystin production, based on laboratory studies or work in other systems, also are present (and are highly variable), including *Dolichospermum* (*Anabaena*) and *Gloeotrichia*. Because qPCR probes are designed to target a specific population, the presence of other toxin producers may not be detected by some qPCR assays. General cyanotoxin assays are available to detect total microcystin producers or producers of other cyanotoxins; but because these assays are not organism-specific, it is not possible to identify which organism produced the toxin in the sample being analyzed. The identification of these organisms will, however, appear in sequencing data, which illustrates the benefit of using more than one type of analytical tool for describing cyanobacteria community dynamics.

Technologically advanced genetic monitoring tools, such as qPCR, enable rapid, reliable, and accurate identification of toxigenic cyanobacteria; so they are essential for improving the protection of water users, in that they help resource managers make more informed decisions regarding the potential risks to humans and local wildlife. As an early-warning monitoring tool, qPCR enables detection of low-abundance toxigenic genotypes long before the bloom becomes apparent. It is also a useful tool for

monitoring restoration projects that may affect cyanobacterial communities by quantifying shifts in community structure and(or) potentially toxigenic strains (Vaitomaa and others, 2003). If used routinely, qPCR-based estimates may be used to refine current standards or "action limits" for drinking or recreational water and may provide water quality or public health managers with a low-cost screening tool in place of more laborious, expensive, and less-sensitive microscope counts (Otten and others, 2015).

Quality Control for Bias and Variability in Sampling and Analysis

Field Sampling Variability

Sample collection for quality control (QC) in this study consisted of split replicate samples (hereinafter referred to as "split" samples) and sequential replicate samples (hereinafter referred to as "replicate" samples) collected from site MDT for HTS and qPCR analysis targeting total cyanobacteria, total *Microcystis*, and the *Microcystis*-specific *mcy*E gene (Eldridge and others, 2017). In 2013, 22 percent of the collected water samples were split samples, and an additional 22 percent were collected as replicate samples. In 2014, 20 percent of collected samples were split samples, 10 percent of the samples were replicates for HTS, and 11 percent of the samples were collected as replicates for qPCR analysis. In each year of the study, split and replicate samples were collected on approximately alternate weeks. Split samples were collected by dividing a single composite volume of lake water with a churn splitter (bottles were filled sequentially from the same churn), and they were analyzed to determine the variability in sample splitting and analyses. Replicate samples were collected in rapid succession from the same location (the entire sample collection procedure was completed twice), and they were analyzed to determine variability of the system, variability of collection, and variability in the analyses. In general, replicate samples are collected to measure variability in field and laboratory procedures, and split samples isolate sources of analytical variability. This distinction between the two types of QC samples helps to determine how much variability is due to sampling as opposed to analysis.

Results of split and replicate sampling for HTS (at the levels of phylum and all taxonomic levels below cyanobacteria) and qPCR analyses in this study are shown in Eldridge and others (2017). The relative percent differences (RPD) were calculated for each type of analysis as the absolute value of the difference between the primary and QC sample results divided by the average of the primary and QC sample results (multiplied by 100 to convert to percentage). In 2013 and 2014, median RPDs for HTS samples at both the phylum level and at taxonomic levels below cyanobacteria were higher in replicates than in splits. This was an expected result, given that replicate sampling is designed to capture more sources of variability. The median RPDs among HTS split and replicate samples were also higher in 2014 (13.6 percent at the phylum level for split samples, 5.95 percent within the cyanobacteria in split samples, 26.9 percent at the phylum level for replicates, and 19.7 percent among cyanobacteria in replicates) than in 2013 (8.85 percent, phylum level, in split samples, 5.98 percent within cyanobacteria in split samples, 17.8 percent, phylum level, in replicates, and 11.5 percent among cyanobacteria in replicates). This difference in RPD values between 2013 and 2014 was likely a result of the higher bacterial diversity (more different types and more of each type) observed in 2014. More rare (low abundance) groups were identified in the 2014 samples, and larger RPDs were calculated that year because the differences in these rare taxa between primary and QC samples were based on fewer than five reads.

Quality control analysis for qPCR sampling was based on the absolute value $\log_{10}$ differences (AVLD) for each sample set (primary and split or replicate; Stelzer and others, 2013; Francy and others, 2015). Copy numbers generated by qPCR analysis can cover a wide range of values, so concentrations of gene targets were $\log_{10}$-transformed before QC analysis. AVLDs were then computed as the absolute value of the difference between concentrations (log copies/mL) in the primary and corresponding split or replicate sample (Stelzer and others, 2013). The RPD of each sample set was calculated from the AVLD. In 2013 split samples, median AVLDs were 0.07 log copies/mL (total *Microcystis*), 0.09 log copies/mL (total cyanobacteria), and 0.10 log copies/mL (*mcy*E, Eldridge and others, 2017). AVLDs were higher among 2014 split samples in all qPCR analyses (0.19 log copies/mL for *mcy*E, 0.37 log copies/mL for total *Microcystis*, and 0.40 log copies/mL for total cyanobacteria). In 2013, the highest median AVLD or RPD was found for the *mcy*E gene specific to *Microcystis*, but in 2014, the AVLD for total cyanobacteria was higher. Among replicate samples, total *Microcystis* (2013) and *mcy*E (2014) showed more variability. This higher variability among *Microcystis* samples and most AVLDs, in general, agrees with the results of the QC analysis in Francy and others (2015). In the current study, QC sampling was performed to measure within-bottle and between-bottle differences for replicate data analyzed twice by qPCR from the same filter, and more AVLD outliers were found for the *mcy*E assays (between-bottle); the highest AVLD outlier was 1.85 log copies/100 mL, which is higher than any AVLD found in the current study. Statistical analysis of AVLDs of paired qPCR, within-bottle, and between-bottle variability in Francy and others (2015) showed that distributions of within-bottle and between-bottle AVLDs were not statistically different for any assay, suggesting that sampling variability was small compared to sample processing and matrix heterogeneity. In the current study, AVLDs greater than 1.0 were observed only in 2014 split samples (1.14 for total *Microcystis* and 1.41 for *mcy*E, on July 21; Eldridge and others, 2017).

Analytical Variability

Quality control of qPCR laboratory methods for total cyanobacteria, total *Microcystis* (16S rRNA), and *mcy*E was discussed in Stelzer and others (2013). In brief, quality control procedures included analyses of filter blanks (sterile, buffered water), extraction blanks (sterile vials extracted along with DNA samples), and qPCR blanks (no-template controls) and duplicate qPCR runs. Matrix effects during qPCR were evaluated by analyzing a portion of each sample spiked with a known quantity of plasmid-borne target DNA. If matrix inhibition was detected, sample DNA extracts were diluted accordingly and reprocessed. Of the 18 filter blanks processed in 2013 and 2014, two samples showed detections for either total cyanobacteria or total *Microcystis* (data not shown). All other blanks, including extraction blanks and no-template controls, were lower than the detection limit. The two detections in the filter blanks were near the reporting limit and at least an order of magnitude lower than concentrations in the environmental samples. These detections were, therefore, used to set the low detection limit for the corresponding qPCR assays.

In development of the *Aphanizomenon cpc*A qPCR assay, laboratory quality control included filter blanks (filtered, sterile water) extracted and analyzed concurrently with environmental samples and triplicate qPCR analyses to measure variability in sample handling through filtration and DNA extraction. Results of filter blank qPCR analysis showed only single copies or less in each blank (data not shown), indicating that values were lower than the detection limit and no samples were contaminated during sample processing or analysis. Standard deviations among triplicate analyses were less than 10 percent of the mean in most samples and ranged between 0.7 and 15.2 percent. Higher variance was usually the result of single Ct values, which influenced both mean and standard deviation. Gene copy estimates for each set of triplicate filters indicated significant differences between triplicate filters from the same sample. This was likely due to biases introduced during either filtration or extraction. The buoyancy of cyanobacterial colonies in the water samples may have introduced bias during sample filtration, and the bead-beating step may not have adequately broken all colonies during DNA extraction. But the primary source of variation was likely caused by relatively low DNA extraction efficiency, which was discussed in section, "qPCR Design to Target *Aphanizomenon* from Upper Klamath Lake, Oregon."

Summary

Cyanobacterial blooms (characterized by chlorophyll *a* concentrations greater than 200 mg/L) in Upper Klamath Lake, Oregon, impair water quality and produce microcystins that may pose a threat to the health of humans and local wildlife. Detecting and monitoring changes in the growth and ecology of these blooms are fundamental to managing this water resource. Recent technological advances coupled with an understanding of the genetics underlying microcystin production have led to the development of more sensitive and rapid monitoring tools based on the detection and quantification of environmental DNA that can be used to determine seasonal and temporal shifts in the proportions of microcystin-producing populations. This report presents results of genetic fingerprinting, high-throughput DNA sequencing (HTS), and quantitative PCR analyses to characterize the cyanobacterial community structure and to detect and quantify bloom-forming *Aphanizomenon*, potentially microcystin-producing *Microcystis*, and total cyanobacteria in depth-integrated water column samples collected from Upper Klamath Lake from June to September 2013 and 2014. The study objectives were (1) to characterize the microbial community at one site in Upper Klamath Lake and determine changes in the community through time using T-RFLP genetic fingerprinting of total cyanobacteria and high-throughput DNA sequencing of the total bacteria community in comparison with light microscopy; (2) to determine the abundances of potentially toxigenic and non-toxigenic strains of *Microcystis* and the changes through time of these abundances using qPCR; and (3) to determine abundances of *Aphanizomenon*, *Microcystis*, and total cyanobacteria and the changes through time of these abundances using qPCR.

The T-RFLP fingerprinting method is a rapid, high-throughput means for comparing diversities among complex bacterial communities. In this study, T-RFLP analysis specific to cyanobacteria in water-column samples showed a dominance of one or two distinct genotypes with some temporal variation in the presence of those genotypes from early and late July through early August 2013.

This study was the first to describe the phytoplankton community in the Upper Klamath Lake using next-generation, high-throughput DNA sequencing (HTS) technology. The results presented here showed the value of this tool for water-quality studies and(or) monitoring of this system. HTS has many advantages over traditional microscopy in that it allows the simultaneous detection of organisms affecting water quality in a reproducible, high-throughput, and cost-effective manner. In general, the HTS approach for phytoplankton identification described changes in the phytoplankton community in 2013 and 2014 at a lower level of detection (greater sensitivity) while showing seasonal and interannual variations in the bloom cycle that fit with the general understanding of phytoplankton bloom dynamics in Upper Klamath Lake. HTS also tracked the presence of potentially toxigenic *Microcystis* during the spring and summer and showed this group to be more prevalent in water samples than shown in data previously collected and analyzed microscopically. Although the number of taxa detected by both methods on a given sample date was relatively low, more distinct taxa were detected at the order and genus levels by HTS than by traditional microscopy. Results of HTS in this

study also showed an abundance of heterotrophic bacteria in all collected samples, revealing a significant component of the pelagic microbial community that would have been missed if only phytoplankton were targeted for sequencing.

Detection of toxigenic and bloom-forming cyanobacteria in Upper Klamath Lake was historically based on microscopic techniques combined with the chemical detection of microcystins and the pigments chlorophyll *a* and phycocyanin in water samples, but it is not possible to determine whether a cyanobacterial strain may be toxigenic by observing morphology or by measuring pigments. As a water-quality monitoring tool, quantitative polymerase chain reaction (qPCR) is a method for detecting and quantifying total *Microcystis*, potentially toxigenic *Microcystis*, and total cyanobacteria in water samples. qPCR may also be used to predict dense or toxigenic blooms because this method enables detection of less abundant, toxigenic genotypes long before the bloom becomes apparent. qPCR was used in this study to detect and quantify the key cyanobacterial species thought to have the greatest effect on water quality in Upper Klamath Lake and to describe the temporal changes in the relative proportions of these species within the total cyanobacterial community in lake samples.

Along with microcystin concentrations, the copy numbers of cyanobacterial 16S ribosomal RNA (rRNA), *Microcystis* 16S rRNA, and *Microcystis* microcystin synthetase E gene (*mcy*E) target genes were higher in 2014 than in 2013. Comparisons of the relative percentages of these gene targets within total cyanobacteria indicated that total and *mcy*+ *Microcystis* proportions were more abundant in 2014 when microcystin concentrations were also higher.

Temporal changes in microcystin concentrations and the relative copy number ratios of *mcy*+ *Microcystis*, total *Microcystis*, and cyanobacteria in 2013 and 2014 showed that total *Microcystis* growth overall was a good predictor in this study for elevated microcystin concentrations. *Microcystis* and *mcy*E copy numbers were significantly correlated with microcystin levels in both years. Microcystin concentrations were significantly correlated with the percentage of *Microcystis* in total cyanobacteria, but not with the percentage of *mcy*+ cells in total *Microcystis*.

To compare changes in the relative copy numbers of *Microcystis* and *Aphanizomenon* directly and to relate the copy numbers of these species to changes in the total cyanobacterial community, a protocol for using qPCR to quantify *Aphanizomenon* specific to Upper Klamath Lake was developed at Oregon State University and used to analyze samples collected in 2014. Changes in qPCR-estimated *Aphanizomenon* copy numbers over time indicated a moderate, steady bloom (corresponding to chlorophyll *a* concentrations between approximately 75 and 200 µg/L) from June 16 to August 11 that declined after August 18. Coincident with this decline in *Aphanizomenon*, *Microcystis* gene copies increased after August 25 and peaked to almost twice the seasonal maximum estimated by qPCR for *Aphanizomenon*. *Microcystis* copy numbers were higher than *Aphanizomenon* copy numbers on every sample date after August 18, indicating that the *Aphanizomenon* bloom was overtaken by *Microcystis* late in the season. Relative copy number ratios of *Microcystis* to *Aphanizomenon* were greater than 1.0 after August 18, which further illustrated the dominance of *Microcystis* over *Aphanizomenon* during that time.

Future work will include statistical and time-series comparisons between the genetic taxonomic data or microcystin concentration results presented in this report and concurrently collected water-quality and environmental data to evaluate potential triggers for increased lake toxicity and to understand the changes in phytoplankton community composition observed between 2013 and 2014.

Acknowledgments

We thank the reviewers who provided comments on this manuscript and the personnel of the USGS Klamath Falls Field Station for their assistance with field equipment and laboratory space. Robert Blake, Blake Eldridge, Charles Goebel, Alix Lee, Celeste Melosh, Keith Sauter, and Bradley Smith from the USGS in Klamath Falls, Oregon, performed the fieldwork. We also thank Liam Schenk and Tamara Wood from the USGS Oregon Water Science Center (logistics, planning, and review), Alta Harris at the USGS Klamath Falls Field Station (IT support), Erin Stelzer, Rebecca Bushon, and personnel at the USGS Ohio Water Microbiology Laboratory (laboratory analysis), and Natasha Isaacs, Carrie Givens, and personnel at the USGS Michigan Bacteriological Research Laboratory (laboratory and bioinformatics analyses).

This work was funded by the U.S. Geological Survey Office of Water Quality and by the U.S. Geological Survey National Water-Quality Assessment Program (NAWQA).

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Glossary

16S ribosomal RNA A section of DNA, or gene, found in all bacteria and archaea that codes for ribosomal RNA, which makes up part of the ribosome used in the cell to make proteins. The 16S rRNA gene is a common tool for identifying bacteria.

Amplicon A piece of DNA (or RNA) that is the source and(or) product of amplification or replication events, usually using PCR or natural gene duplication.

Community An assemblage or association of populations of different species occupying the same geographical area during a particular time.

***cpc* genes** Genes within the phycocyanin (PC; photosynthetic accessory pigment specific to cyanobacteria) operon coding for two bilin (biological pigment) subunits and three linker polypeptides; the two bilin subunit genes in cyanobacteria are designated ß (*cpc*B) and α (*cpc*A).

Diversity (ecology) The degree of variation of living things present in a particular ecosystem. As a concept of species biodiversity, diversity pertains to a combination of species richness (the number of species in a community) and the evenness of species' abundances.

DNA Deoxyribonucleic acid. A macro-molecule that is the main component of chromosomes and is the material that carries and transfers genetic characteristics in all life forms; the genetic information of DNA is encoded in the sequence of nucleotide bases: adenine, guanine, cytosine, and thymine.

Gene A specific sequence of nucleotides in DNA or RNA that is the functional unit of inheritance controlling the transmission and expression of one or more traits by specifying the structure of a particular protein or controlling the function of other genetic material.

Genome The complete set of genes or genetic material present in a cell or organism.

Genotype All or part of the genetic constitution of an individual or group which determines a specific characteristic of that individual or group.

Heterotroph An organism that obtains its nutritional requirements from complex organic substances (in contrast to *autotroph*, which can make energy-containing organic molecules from inorganic substances using light or chemical energy).

High throughput DNA sequencing (HTS) Also referred to as "next-generation" sequencing; recently developed (since mid-to-late 1990s) techniques to sequence and analyze large genomes, producing thousands or millions of sequences concurrently, that generally involve the amplification of DNA templates by PCR and the physical binding of template DNA to a solid surface or to microbeads; intended to lower the cost of DNA sequencing while generating more DNA sequence data than traditional dye-terminator (Sanger-based) methods.

***mcy* gene cluster** Microcystin synthetase genes; the large gene cluster involved in microcystin production in *Microcystis* spp.

Morphotype An individual, specimen, or strain possessing a physical, or morphological, variation within a population.

Operational taxonomic unit (OTU) An operational definition of a species or group of species often used when only DNA sequence data is available; clusters of similar gene sequences used as basic diversity units in large-scale characterizations of microbial communities.

Operon A unit of linked genes that consists of structural genes and regulating elements.

PCR Polymerase chain reaction. A technique used to copy DNA (genes or parts of genes) by orders of magnitude so that it can be detected (presence/absence) within a mixture of DNA molecules.

Population In ecology, a group of individuals of the same species inhabiting the same area.

Primer In PCR, a strand of short nucleic acid sequences that acts as a starting point for DNA synthesis. Primers are designed to bind to the start and end of the DNA target.

qPCR Quantitative polymerase chain reaction. A technique used to copy DNA (genes or parts of genes) by orders of magnitude so that it can be detected and quantified within a mixture of DNA molecules.

T-RFLP Terminal restriction fragment length polymorphism. A technique for profiling of microbial communities based on digesting a mixture of genetic variants (after PCR amplification) using one or more restriction enzymes and detecting the size of each of the individual resulting terminal fragments.

Publishing support provided by the U.S. Geological Survey
Science Publishing Network, Tacoma Publishing Service Center

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Director, Oregon Water Science Center
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ISSN 2328-0328 (online)
https://doi.org/10.3133/sir20175026