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The abundance of eleven cyanobacteria operational taxonomic units (OTUs) was assessed in six Piedmont North Carolina Lakes using real-time quantitative polymerase chain reactions (qPCR). Lakes (including five drinking water reservoirs) were sampled 18 times from June 2011 to October 2012. OTUs were present in varying abundance and followed expected seasonal trends with peak abundance occurring in warmer months. Based on available environmental data in three drinking water supply reservoirs, OTU abundance was found to be correlated with one or more environmental (temperature, pH, conductivity, dissolved oxygen, phycocyanins, chlorophyll *a*, and turbidity) or nutrient parameters (total phosphorus, total organic carbon, and total Kjeldahl nitrogen) in each lake, although most parameters did not show significant correlations. Temperature was consistently correlated with OTU abundance. The results suggest that qPCR has potential for monitoring cyanobacteria and can contribute to understanding and management of cyanobacteria in North Carolina lakes. However, improved cyanobacteria taxonomy and better development of primers and standards used in qPCR are necessary for this approach to become practical

ABUNDANCE OF SELECT CYANOABCTERIA IN SIX PIEDMONT NORTH CAROLINA LAKES

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Cyanobacteria are photosynthetic prokaryotic organisms that are found worldwide. They inhabit marine to fresh water systems, small ponds to large lakes, oligotrophic to eutrophic systems, and across temperatures ranging from hot springs to arctic lakes (Fristachi *et al*., 2008; Ward *et al*., 2008; Jungblut *et al*., 2012). Cyanobacteria can produce toxins and cyanobacterial harmful algal blooms (CHABs) can have effects on organisms in the environment. In the last few decades, increased awareness of the effects that cyanobacteria can impose on humans and other organisms has led to increased monitoring and efforts to control cyanobacteria.

Cyanobacteria abundance typically increases with warmer temperatures (>20ºC), sunlight, increased phosphorus and nitrogen availability, and still waters (Hudnell, 2010; Huisman and Hulot, 2005). This may be due in part to the early evolution of cyanobacteria during a time when the earth was much warmer, thus providing cyanobacteria with an adaptive advantage **(**Paul, 2008). Other factors that may influence cyanobacterial abundance include turbidity and pH (Cuichao *et al*., 2013; Posch *et al*., 2012). Increased turbidity tends to reduce abundance of cyanobacteria. pH can affect various stages of cyanobacterial growth and different organism optimal pHs vary depending on the species (Cuichao *et al*., 2013). Kosten *et al.* (2012) found a positive correlation between pH and the proportion of cyanobacteria, however, the authors note

that this could be due to an indirect nutrient effect. An increase in pH can be due to increases in photosynthesis, which can be an indication of higher nutrient availability (Kosten *et al.,* 2012).

Some cyanobacteria produce toxins when growing under the right conditions. Cyanotoxins can be hepatotoxic, neurotoxic or can just act as an irritant to the skin and/or gastrointestinal system (Pantelic *et al*., 2013). Microcystins, cylindrospermopsins, and anatoxins are among the most common types of cyanotoxins (Frisatchi *et al*., 2008). Microcystins and cylindrospermopsins are most often hepatotoxic and anatoxins act as neuromuscular blocking agents (Frisatchi *et al*., 2008). Toxic effects have been seen in multiple organs including the liver and kidneys following ingestion of cyanotoxins (Falconer and Humpage, 2006). A range of species can produce each cyanotoxin, and there are many congeners of each cyanotoxin as well. Different combinations of congeners will affect the overall level of toxicity. Congeners can also vary in their own toxicity level. For example, *Planktothrix agardhii* produces an increased amount of a more toxic congener of microcystin when exposed to increased light intensity (Tonk *et al.,* 2005).

Cyanotoxins have been detected in treated drinking water. Rapal *et al*. (2002) studied nine water treatment facilities in Finland, and found that up to 41% of cyanobacteria toxins in raw drinking water can pass through treatment. The detection of cyanotoxins in drinking water suggests that a better understanding of how to limit and treat the growth of the cyanobacteria is needed (Fristachi *et al*., 2008).

Guidelines have been established defining the allowable limit of cyanotoxin concentrations in treated drinking waters, however a country or even individual states can alter that guideline (Hoeger *et al*., 2005). The World Health Organization set the allowable amount of microcystins as 1.0 μg L^{-1} in treated drinking waters (WHO, 1998), although guidelines range from levels below 1.0 μg L^{-1} in New Zealand to 1.5 μg L^{-1} in Canada (Ministry of Health, 2002; Health Canada, 2003). Many countries do not have guidelines or means of monitoring cyanotoxins (Pantelic *et al*., 2013). In the US, only three states (Florida, Ohio, and Oregon) have guidelines for cyanotoxin amounts in drinking waters. Twenty-one states have listed guidelines for cyanobacteria or cyanotoxins in recreational waters. Some states set guidelines for one or multiple cyanotoxins, while other states set limits for maximum cyanobacteria cell counts (Hudnell *et al*., 2013).

Cyanotoxins have been implicated in livestock poisonings and human illnesses. The main exposure routes are from consumption of contaminated waters or dermal contact. Consumption is generally more serious. In 1996 in Brazil, 76 hemodialysis patients died after being exposed to poorly filtered water contaminated with microcystins and cylindrospermopsins (Carmichael *et al*., 2001). In the US few incidents have occurred from consumption of contaminated water, and no human deaths have been clearly attributed to cyanobacteria in the US (Carmichael, 1998). Dermal contact may be more common, but often goes unreported. Animal deaths have been reported. For example, in North Carolina the deaths of several dogs were attributed to cyanotoxins (NCPH, 2013) and an investigation in New Mexico found that more than 100 elk died

after drinking from a trough containing neurotoxic cyanotoxins (Albuquerque Journal, 2013, www.abqjournal.com).

Odors are often associated with cyanobacteria. Lake Kasumigaura, the second largest lake in Japan, used for drinking water, irrigation, and fishing was found to have 5 species of cyanobacteria that contributed to the musty odor of the lake (Sugiura *et al*., 1998). In a California water supply system, geosmin and 2-methylisoborneol, compounds produced by species of *Oscillatoria* and *Anabaena,* were shown to be the main contributors to the musty-taste and odor problems (Izaguirre *et al.,* 1982). The odors can also attract unwanted species. For example, nematodes are attracted by cyanobacteria odor compounds (Hockelmann *et al*., 2004).

Hypoxia can occur as a result of the degradation of cyanobacteria blooms. Hypoxia and anoxia can lead to fish kills. There have been reports of fish kills in Lake Peipsi (the largest transboundary lake in Europe) dating back to 1895 with the most recent fish kill occurring in 2002 as a the result of low dissolved oxygen caused by a massive lake wide cyanobacterial bloom (Kangur *et al.*, 2005). The cyanobacteria were so densely concentrated that the blooms were causing oxygen supersaturation during the day and then depleting the oxygen to lethal levels at night from respiration (Kangur *et al*., 2005).

Over the past few decades reports of cyanobacteria and their potential harmful effects has increased (Cheung *et al*., 2013), and in the last few decades new methods have been developed to detect cyanobacteria and cyanotoxins in water sources. However, despite recognition of the problem, testing for cyanobacteria is not routine. This could be

because cyanobacteria are still not perceived as being a severe risk to human health or that testing for cyanobacteria is simply not regulated or is too costly. With all the harmful effects cyanobacteria can have there is an increased need for effective monitoring, detection, and management of cyanobacteria.

Several methods have been developed over the past few decades to detect cyanobacteria and their toxins in water systems. Due to the expertise required for visual identification of cyanobacteria, visually inspecting water samples can be time consuming and costly. Satellite remote sensing of cyanobacterial blooms has been employed over the past decade. With the recent advancement in utilizing light absorption features specific to cyanobacteria it is now possible to distinguish CHABs from other algal species (Kutser *et al*., 2006). However, this method is not reliable in acting as an early warning tool because a minimum biomass is needed in order to distinguish the CHABs from other algal populations that are in higher abundance (Kutser *et al*., 2006).

Several molecular methods have been developed to evaluate cyanobacteria even when present in minimal amounts and act as better detectors for CHABs. The most common molecular methods for detecting and monitoring cyanobacteria include microarrays, polymerase chain reactions (PCR) and the use of enzyme-linked immunosorbent assay (ELISA) to detect cyanotoxins. Castiglioni *et al.* (2004) developed a microarray that utilized ligation detection reactions with 16S rRNA gene polymorphisms to evaluate 9 cyanobacterial groups in environmental samples. Microarrays have become more specific and can now be used to discriminate toxic and non-toxic species. Ahn *et al*. (2006) used fiber-optic microarrays in combination with

oligonucleotide probes to detect and enumerate three potentially harmful cyanobacteria species. Polymerase chain reactions can be used to monitor cyanobacteria in freshwater systems. Al-Tebrneh *et al*. (2010) used qPCR to identify specific saxitoxin-producing *Anabaena circinalis* strains. Another study used gene-directed multiplex PCR that relied on the amplification of several *mcy* gene fragments to monitor microcystin-producing species regardless of their taxonomic position (Valerio *et al*., 2010). ELISA assays are commonly used to detect cyanotoxins. Based on the binding of antibodies and the subsequent color change it is possible to determine the amount of toxin present in water samples. ELISAs can detect cyanotoxins at various concentrations and have even been used to test 170-year-old herbarium specimens for cyanotoxins (Metcalf *et al.,* 2012). With the continued refinement and development of molecular techniques, it is becoming more efficient and easier then ever to detect and monitor cyanobacteria in environmental samples.

Cyanobacteria are common residents of North Carolina lakes. Touchette *et al*. (2007) assessed 11 reservoirs in NC and found that cyanobacteria comprised 60 – 95% of the total phytoplankton cell numbers. Several toxin-producing species were found in most of the samples (Touchette *et al*., 2007). Glasgow and Burkholder (2003) found that cyanobacteria comprised > 90% of phytoplankton in NC reservoirs. Increased eutrophication of NC lakes has led to increased turbidity and nutrient availability for cyanobacteria.

Stewart (2011) analyzed sequences of cyanobacterial 16S rDNA derived from 883 clone small subunit ribosomal DNA (SSU rDNA) sequences from six NC lakes. He

identified 100 operational taxonomic units (OTUs: sequences ≥ 97.5% similarity) and developed primers to the OTUs based on consensus sequences. He then selected primers to 25 OTUs and used quantitative PCR to evaluate abundance and diversity of cyanobacteria in City Lake, NC from December 2007 to December 2008. The 25 primer sets were selected because: 1) those primers appeared to be OTU-specific; 2) some of the OTUs were similar to sequences in GenBank from known toxin producing species; 3) the OTUs selected included some of the most abundant in the clone library; and 4) empirical testing showed they amplified intended targets (Stewart, 2011). Of the original 100 OTUs identified only 3 were identified to a species level (Stewart, 2011). All other OTUs had < 97.5% similarity to any previously documented sequence in GenBank (Stewart 2011). Out of the 25 OTUs tested, three represented the majority of cyanobacteria OTUs detected in the samples. These OTUs did not have any similarity matches > 95% to GenBank sequences and were only identified as uncultured cyanobacteria or bacteria (Stewart, 2011). Additionally, he found that richness increased summer to fall months and that 11 OTUs were present in every sample. Finally, he found cyanobacteria in City Lake at all times of the year, including species that may produce toxins, although he did not determine if toxins were present. Stewart (2011) recommended that more lakes be sampled and more OTUs be probed for in each lake in order to better understand the diversity of cyanobacteria in reservoirs.

The overall objective of this study was to extend Stewart's (2011) study. First, ribosomal databases were examined to determine if recent entries could better identify the OTUs. Second, the primers previously identified by Stewart (2011) were tested to ensure

primer specificity (Table 1). Third, selected primers were used in qPCR to evaluate the distribution of target cyanobacteria in six Piedmont NC lakes, including City Lake (Table 2). Physical and chemical environmental parameters and physical lake characteristics were also used to evaluate correlations among lake parameters and cyanobacteria. The results of this study should contribute to the future management and detection of cyanobacteria in NC water reservoirs.

CHAPTER II

METHODS

Re-evaluation of OTUs and Primers

In order to re-evaluate OTU identification, the results of Stewart (2011) were compared to multiple databases. OTU consensus sequences were compared to existing sequences in the following databases: GenBank (GB) (Altschul *et al*., 1997), EzTaxon-e (Ez) (Kim *et al*., 2012), Ribosomal Database Project (RDP) (Cole *et al*., 2014), and Bioinformatic Bacteria Identification Tool (BIBI) (Devulder *et al*., 2003) (Table 3). In an effort to identify which OTU sequences are similar and to determine which OTU primers may cross-react with other OTU primers, MEGA 6 (Molecular Genetics Analysis 6.0) was used to construct a PHYLIP rooted tree of the OTU consensus sequences.

Primer specificity to OTU sequences was checked *in silico* (by computer) using BioEdit 7.1, biological sequence alignment editor, to align all OTU consensus sequences and each primer sequence was searched across all OTU consensus sequences. To evaluate how well the primers complement known GenBank sequences, primers were checked against published sequences from the GenBank database using the BLAST algorithm. *In vitro* testing was done using cloned standards and some cultured standards to determine if any OTU primers amplified multiple OTUs or cross-reacted.

Sample Sites and Collection

Six Piedmont North Carolina lakes were sampled in this study (Table 2). Samples were collected bimonthly during late spring to summer and monthly during colder months. If rain had occurred, the sampling was postponed for 2 days to allow suspended sediment to settle. First, light penetration was determined by Secchi disk. Next, two integrated water column samples (surface to 1.5 x Secchi depth) were taken and mixed to get a pooled sample of the euphotic zone. Each sample was stored in brown Nalgene jars and returned to the lab where 50 – 200 ml of sample was drawn through a 25 mm GFF glass fiber filter (0.7 μm nominal pore size, *c.f.* Sheldon, 1972; Chavez *et al*., 1995). The filters were then placed in 15 ml polyethylene centrifuge tubes containing 2 ml of CTAB (hexadecyltrimethyl ammonium bromide) buffer and stored at room temperature until DNA extractions were performed.

DNA Preparation and PCR

DNA was extracted following the procedure of Schaefer (1997). Briefly, each 15 ml polyethylene tube containing a sample filter was placed in a water bath at 65°C for 60 minutes with a brief vortex mixing at 30 minutes. Following heating 2 ml of chloroform : isoamyl alcohol mixture (24:1) was added. Sample tubes were then inverted to mix and centrifuged in a clinical centrifuge at maximum speed for 25 – 45 minutes until clear separation of phases. Two 850 μl aliquots of the aqueous supernatant layer were then removed and put into separate 1.5 ml microcentrifuge tubes. Next, 600 μl of 2-propanol was added to each tube, inverted to mix and centrifuged at 14000 rpm in a microcentrifuge for 25 minutes. The supernatant was then removed and the 1.5 ml tubes

were inverted and left to air dry for several hours. The DNA was then re-suspended in 20 µl of TE buffer of pH 8, and the concentration of DNA present in each aliquot was measured (NanoDrop spectrophotometer). All samples were then diluted to a concentration of approximately 5 ng DNA μl^{-1} to minimize interference problems when running qPCR.

qPCR was performed following the protocol used by Stewart (2011). Each reaction contained the following reagents: Applied Biosystems Power SYBR® Green Master Mix (10 µl), PCR forward primer (1 µl), PCR reverse primer (1 µl), dH_2O (8 µl) and 1 µl of genomic DNA template, cloned standard (positive controls), or water (negative controls). Reaction conditions were: 10 min at 95ºC; 40 cycles of 15 sec at 95ºC, 30 sec at 60ºC, 60 sec at 72ºC, and 15 sec at 80ºC with data collection; with a final melt-curve analysis step to validate proper target amplification.

Environmental Parameters and Lake Characteristics

Data from a Real-Time Remote Monitoring Systems (RTRMS) managed by the NC State University Center for Applied Aquatic Ecology (CAAE) at Oak Hollow Lake and City Lake were used to evaluate six lake variables in those two lakes. Parameters from the RTRMS database included: water temperature (Temp), pH, dissolved oxygen concentration (DO), chlorophyll *a* relative fluorescence (CHL*a* rf), phycocyanin relative fluorescence (PHYC), and conductivity (Cond). Twelve samples collected and analyzed by the CAAE Water Quality Lab that coincided with the sample dates used in this study for City Lake and Oak Hollow Lake were used to correlate four key nutrients with OTU abundances. The nutrient data provided by CAAE included total phosphorus (TP), total

organic carbon (TOC), and total Kjeldahl nitrogen (TKN) and chlorophyll *a* concentration (CHL*a* con). Temperature, pH, and turbidity data provided by Eric Davis, City of Burlington Water and Sewer Operations Manager, were used to assess correlations in Lake Mackintosh.

Statistical Analysis

Data generated by qPCR were used to: 1) determine if there were differences between 2011 and 2012 OTU relative abundance when pooling OTUs across lakes; 2) determine if there were differences between 2011 and 2012 OTU relative abundance per lake; 3) determine if and where there are differences in OTU relative abundances across lakes independent of year; 4) assess correlations of OTUs with various environmental parameters in City Lake, Oak Hollow Lake, and Lake Mackintosh and; 5) compare the results of this study with results from Stewart (2011) in City Lake. Because the data were not normally distributed (Shapiro-Wilk test for normality) even after various transformations were tried, only nonparametric analyses were used throughout this study. The Mann-Whitney U test was used to compare 2011 verses 2012 OTU abundance. To compare taxon abundance in lakes, a Kruskal-Wallis test was performed. When a significant difference was found (p-value $\leq .05$); pairwise comparisons were evaluated to determine which lakes were different in any given OTU abundance. A Spearman Rank Order Correlation was used to investigate correlations between OTU abundance and the environmental parameters. Results of OTU abundances in City Lake from Stewart (2011) were visually compared to results of this study for City Lake to investigate if there were differences in abundance of species in City Lake in 2008 and 2012. All statistical

procedures were conducted using the IBM SPSS 21 statistical program (SPSS Inc, Chicago, IL).

CHAPTER III

RESULTS

OTU Identification and Primer Specificity

Four public domain databases were used to check the identity of the eleven OTUs used in this study in six Piedmont of NC lakes (Table 3 and 4, Figure 1). Prior to comparing results for those 11 OTUs, three OTUs (2, 59, and 41) were used as "controls" to verify the accuracy of each database. OTUs 2, 41, and 59 were chosen because the primers to those OTUs amplified a known clonal SSU rDNA *Cylindrospermopsis raciborskii* standard (Stewart, 2011). GenBank (GB), EzTaxon-e (EZ), the Ribosomal Database Project (RDP), and the Bioinformatic Bacterial Identification (BIBI) identified all three OTUs as being 95 – 98% similar to *Cylindrospermopsis* and/or *Raphidiopsis* sequences. A possible explanation for matches to both *Cylindrospermopsis* and *Raphidiopsis* is that they may represent the same organism. Both genera are morphologically similar and both produce cylindrospermopsins and anatoxins (Li *et al.,* 2001 and Namikoshi *et al.,* 2003). Some reports suggest that *Raphidiopsis* species are environmental morphotypes of *Cylindrospermopsis* species (McGregor and Fabbro, 2000).

Three OTUs (09, 17, and 71) were matched to the same sequences as reported by Stewart (2011 and Table 4). Many OTUs were identified as "uncultured organism" across all databases, however, the specific clone identification varied. Some OTUs were

specifically matched to a class or genus. For example, EZ identified OTU 89 as an uncultured bacterium within the genus *Prochlorococcus*. Though there was no consensus across all four databases, four OTUs (09, 11, 17, and 49) were matched to the same sequences in GB and RDP.

GenBank sequence matches (Table 4) and the PHYLIP rooted tree (Figure 2) suggested OTU sequence overlap. *In vitro* testing of OTU primers against various cloned or cultured standards found several primers that identified multiple taxa or cross-reacted. For example, an earlier qPCR test showed that several primers designed to several OTUs (08, 12, 26, 47,and 58) all amplified a known *Anabaena* spp. standard (L. Fondario Grubbs, Biology Dept.; UNCG, personal communication). Similarly, primers designed to amplify OTUs 01 and 79 were also found to amplify the same *Anabaena* spp. standard. Another group of primers (designed to amplify OTUs 02, 41, and 59) were all found to amplify a *Cylindrospermopsis* standard. Since the taxa *Anabaena* and *Cylindrospermopsi*s were the focus of another study (Fondario Grubbs, 2014) they were not used in this study. Other pairs of primers were also found to cross-react. Primers for OTU 36 and OTU 43 both amplified the OTU 36 and OTU 43 clone standards; however, those primers did not appear to be amplifying the same product, as evident by different melting temperatures of the amplified PCR product. Therefore they were eliminated from this study for not being OTU specific. Primers for OTU 4b and 5a also cross-reacted, but appeared to be amplifying the same product, thus only one primer from this set was used. OTU 4b was chosen, as it appeared to be slightly more efficient at amplification. Primers for OTU 67 and OTU 71 also had some cross-reactions, however,

the primer for OTU 67 would amplify both OTU 67 and OTU 71 standard, but the primer for OTU 71 only amplified the standard for OTU 71. Therefore only the primer for OTU 71 was chosen, as it appeared to be OTU specific.

OTU Abundance

Ten OTUs were found in all six lakes, however, not every sample contained all OTUs. OTU 44 was not found in Oak Hollow Lake. OTU target DNA amplified by qPCR ranged from 0 – 357.16 pg ml^{-1} (Figure 3a-3k and Table 5). OTU 89 appears to be the most abundant OTU (Figure 3j). It was found in every sample (except July 2011, Lake Brandt) in all six lakes with target DNA ranging from 0 – 357.16 pg ml^{-1}. OTU 11 was found in all lakes and also had high abundance with target DNA ranging from 0 – 35.3 pg ml^{-1} (Table 5). OTU 98 was not found in many sample, however, it was found in high abundance (53 – 67 pg ml^{-1}) in Randleman Lake in August and September 2012 samples (Figure 3k). OTU 4b was found in every sample in all six lakes but in much lower abundance (Figure 3a). Target DNA for OTU 4b ranged from 0.0001 – 0.24 pg ml^{-1} (Table 4). It is important to note that the primers may vary in their efficiency to amplify the target DNA and small differences may not be real. In cases were there is a large difference in OTU abundance (like with OTU 89 and OTU 4b) this difference is likely real and represents two organisms that are present in different amounts.

Comparisons of OTU Abundance Across Years

No significant difference was found when comparing total OTU abundances pooled across lakes from 2011 and 2012 ($p = 0.78$, Table 6). Although, when comparing individual OTU abundance pooled across lakes, two OTUs were different between 2011

and 2012 (Table 6). OTU 09 and OTU 17 were more abundant in 2011 compared to 2012 (Mann-Whitney U test; OTU 09, $p = 0.043$ and OTU 17, $p = 0.006$).

OTU abundances between 2011 and 2012 for individual lakes showed some significant differences (Table 8). When combining all OTUs within lakes, Belews Lake had less total OTU abundance in 2011 ($p = 0.02$) (Table 7). When comparing OTU abundance in 2011 and 2012 for individual OTUs, two OTUs (83 and 89) had higher abundance in Belews Lake between 2011 and 2012 (Mann-Whitney U test; OTU 83, $p = 0.035$ and OTU 89, $p = 0.017$). OTU 09 abundance was higher in Lake Mackintosh ($p = 0.028$). Abundance of three OTUs was higher in 2011 compared to 2012 in Randleman Lake (OTU 4b, $p = 0.006$, OTU 17, $p < 0.001$, and OTU 61, $p = 0.028$). Lake Brandt, City Lake, and Oak Hollow Lake did not have any OTU abundances that were significantly different between 2011 and 2012.

Differences in OTU Abundance Across Lakes

OTU abundances varied across lakes. Pooled data comparing all lakes (Kruskal-Wallace H test), indicated several significant differences of OTU abundance (Table 8). For example, abundance of OTUs 4b, 09, 11, 17, 61, 71, and 89 were all different in at least two lakes ($p < 0.0005$, Table8). The abundance of three OTUs, 49, 83, and 98, were not significantly different among lakes (OTU 49, $p = 0.496$, OTU 83, $p = 0.06$, and OTU 98 $p = 0.087$).

Pairwise comparisons (Dunn's procedure with a Bonferroni correction) indicated that all OTUs were significantly less abundant in Belews Lake compared to at least two other lakes (Table 9). Two OTUs (4b and 71) were significantly less abundant in Belews

Lake than all other lakes (p-values 0.004 – < 0.001, Table 9). OTU 09 abundance was higher in Lake Mackintosh and Randleman Lake than Belews Lake, City Lake, and Oak Hollow Lake (p-values ≤ 0.001, Table 9). OTU 11 abundance is higher in Randleman Lake compared to all other lakes (p-values 0.05 – < 0.001, Table 9). OTU 61is less abundant in Oak Hollow Lake compared to Lake Brandt ($p < 0.001$), Lake Mackintosh ($p = 0.007$), and Randleman Lake ($p = 0.041$).

Correlations of OTU Abundance and Environmental Parameters

There were significant correlations among several OTUs and environmental parameters within lakes (Table 10). The Spearman Rank Order test indicated several significant correlations between OTUs and environmental parameters. Most notable, five OTUs (61, 71, 83, 89, and 98) showed significant correlation with temperature in City Lake, Oak Hollow Lake, or both lakes (p-values from 0.04 – < 0.001, Table 10). Chlorophyll relative fluorescence and chlorophyll *a* concentrations were significantly correlated with seven OTUs (09, 11, 49, 61, 71, 83, and 89) ($p = 0.043$ – <0.001, Table 10). Three of those OTUs, 71, 83, and 89, are also highly correlated with total organic carbon concentrations ($p = 0.048$ – 0.002). OTUs 61, 71, and 98 have significant negative correlations with dissolved oxygen (DO) in Oak Hollow Lake ($p = 0.014$, 0.012, 0.005, respectively). In Lake Mackintosh, significant correlations were found between OTUs 9,11, 49, and 89 and temperature ($p = 0.033$ – 0.004). OTUs 09 and 89 were also significantly correlated with pH ($p = 0.021$ and $p = 0.001$). OTUs 17 and 44 were not significantly correlated with any environmental parameters in City Lake, Oak Hollow Lake, or Lake Mackintosh (Table 10).

CHAPTER IV

DISCUSSION

Cyanobacteria are increasingly abundant in aquatic systems, including drinking water supply reservoirs. Additionally, recognition of the health risks associated with cyanobacteria has led to development of better methods to monitor them. This study was initiated to extend and evaluate previous work on the use of molecular approaches to monitor cyanobacteria (Stewart, 2011).

OTU Identification

Stewart (2011) identified 96 OTUs, and assessed the abundance of 25 of them in City Lake. This study reevaluated Stewarts (2011) OTU identities by comparing OTU consensus sequences to four sequence databases. Overall, GenBank, EzTaxon-e, RDP, and BIBI databases matched OTU sequences to uncultured cyanobacteria and bacteria (Table 5). However, most of these had sequence similarities below 97.5% and therefore are probably not species identities. The databases use different statistical approaches to evaluate sequence similarities, which can result in different matches (Table 4). Park *et al.* (2012) assessed the use of GenBank, EzTaxon, and BIBI databases for molecular identification of blood culture isolates using 16S rRNA gene sequences. They suggested that two or more databases should be used. They especially noted that GenBank should be used first and then verified by another peer-reviewed database. They found that using GenBank with EzTaxon resulted in the most discriminative results (Park *et al.,* 2012). It

is clear, however, that these databases have limitations. Primarily, they still do not contain enough cyanobacterial sequences to properly identify all, or even most, isolates found in environmental samples.

Primer Specificity

Stewart (2011) designed and used 30 primer pairs to check OTU abundance in City Lake, although only 25 proved useful. These primers were checked for specificity, and multiple primers initially planned for use in this study were eliminated because they were not OTU specific. These results suggest that some primers created from 450bp SSU rDNA were not unique enough to distinguish between closely related taxa. Primers with higher specificity likely could be generated using larger target areas, such as the entire SSU rDNA sequence. Other target sequences that could be used to generate primers for cyanobacteria include other parts of ribosomal genes (23S large subunit, ITS1 and ITS2) and repetitive sequences, such as long tandemly repeated repetitive sequences (LTRRs). 23S rRNA was used as target sequences to assess algal diversity in two eutrophic lakes and > 70% of all sequences found were identified as cyanobacteria or eukaryotic algae, though not at a species specific level (Steven *et al*., 2012). Internal transcribed spacer (ITS) regions that occur between the 16S and 23S rDNA genes have also been used to discriminate between species of cyanobacteria with high resolution (Janse *et al*., 2003). LTTRs are widespread and conserved in prokaryotic genomes and have been used to identify cyanobacteria found in freshwater at genus and species specific levels (Valerio *et al*., 2009). Nevertheless, primers based on SSU rDNA have generally been used because the most extensive databases available are based on SSU rDNA gene sequences.

OTU Abundance

As suggested by Stewart (2011), OTU abundances were assessed in additional lakes in this study. The results are consistent with results found in previous work (Fondario Grubbs, 2014; Touchette *et al*., 2007; Stewart, 2011). Fondario Grubbs (2014) assayed the same samples as this study and found peak abundance of SSU rDNA of potentially cyanotoxin producing cyanobacteria occurred in mid-summer, similar to this study. She also found select cyanotoxin gene abundance followed seasonal patterns with peak abundance in warmer months.

Total OTU abundance in 2011 compared to 2012 was not significantly different. However, the abundance of two individual OTUs pooled across lakes, and others within lakes, were greater in 2011 compared to 2012 (Table 6 and 8). Fondario Grubbs (2014) reported a higher abundance of potentially cyanotoxin producing cyanobacteria SSU rDNA in Oak Hollow Lake in 2011 compared to 2012. The higher abundance in 2011 may be explained by the drought conditions present in 2011. During many of the sampling dates in 2011, the Piedmont of NC was classified as abnormally dry (NC Drought Management Advisory Council, http://www.ncdrought.org/). For most sampling dates in 2012 there was no drought classification for the Piedmont area and on several occasions a rain event occurred a few days prior to sampling. These rain events possibly caused mixing of the water column and disruption of cyanobacteria populations resulting in lower cyanobacteria abundance in 2012. Destratification of the water column due to mixing can inhibit cyanobacterial growth (Bouvey *et al*., 2003; Reichwwaldt and Ghadouani, 2012). Other types of algal populations (phytoflagellates and diatoms) grow

better in destratified waters and may outcompete cyanobacteria (Dantas *et al.,* 2011). Destratification has also been reported to cause bloom collapse (Jacobsen and Simonsen, 1993). High levels of rainfall can also increase turbidity, which may reduce light available for photosynthesis, thus decreasing cyanobacteria populations (James *et al.,* 2008). Touchette *et al*. (2007) reported that cyanobacteria abundance in NC reservoirs increased during periods of drought.

All the lakes sampled in this study are eutrophic, except Belews Lake that is oligotrophic (NCDENR, 2009; 2010). The low levels of key nutrients utilized by cyanobacteria, such as phosphorus and nitrogen, likely explains the lower OTU abundance in Belews Lake compared to the other lakes (NCDENR, 2010). Additionally, Belews Lake water is used to cool the Belews Creek Steam Station, a coal-fired power plant, and generally has warmer water temperature due to thermal discharge, and is less turbid (NCDENR, 2010). Thus, low OTU abundance in Belews Lake may also be because cyanobacteria growth is inhibited due to unusually high water temperatures or because of chemical discharges from the steam station resulting in altered water composition (Lemly *et al*., 2002).

Comparison of City Lake Data

Stewart (2011) evaluated OTU abundance in City Lake in 2008. He found OTUs 89 and 11 to be the most abundant. In this study, these OTUs were also the most abundant, but were found in higher concentrations. Stewart (2011) reported 5 – 250 pg DNA 100 ml^{-1} for OTU 89 and 5 – 100 pg DNA 100 ml^{-1} for OTU11 throughout the 2008 sampling period. In this study OTU 89 and OTU 11 DNA ranged from 14 – 16242

pg DNA 100 ml^{-1} and 0 – 984 pg DNA 100 ml^{-1} (Figure 3). The pattern of OTU 89 and OTU 11 abundance was also similar. Most other OTUs were found in much lower abundance in both studies.

Correlations of OTU Abundance with Environmental Parameters

OTU abundance did not show consistent correlation with any environmental parameters, except temperature, in the three lakes (City Lake, Oak Hollow Lake, and Lake Mackintosh) where environmental data were available (Table 10). Two OTUs (17 and 44) were not correlated with temperature, although OTU 17 appears to have a seasonal pattern of peak abundance in summer, and OTU 44 was only found in 5 of 19 samples, likely too few for statistical relevance. Fondario Grubbs (2014) also reported correlations of several taxa with temperature in these lakes. Touchette *et al.* (2007) reported correlations of cyanobacteria abundance with chlorophyll *a*. Correlations of OTU abundance with chlorophyll relative fluorescence or chlorophyll *a* concentrations were found in this study; however, there were no consistent patterns. Brient *et al.* (2008) found a significant correlation between phycocyanin and OTU community biomass using a phycocyanin specific fluorescence sensor. Only two OTUs (49 and 71) in this study showed positive correlations with phycocyanin relative fluorescence in City Lake and Oak Hollow Lake (Table 10)

Many correlations were found among OTUs, but there was no overall consistency across lakes. Three OTUs (71, 83, and 89) were positively correlated in 5 lakes (Table 11). Likely these OTUs all respond to warm temperatures.

Future Studies

The results of this study agree with other studies that found similar cyanobacterial communities in many lakes. Castiglioni *et al.* (2004) was able to identify cyanobacteria belonging to 19 cyanobacterial groups in European lakes. Eiler *et al.* (2004) also found that cyanobacteria communities are diverse and 25% of OTUs detected occurred in more than one lake in Sweden. Glasgow and Burkholder (2003) found that many potentially toxin producing species were frequent in NC reservoirs.

Improved methods are important for assessment of cyanobacterial communities. qPCR can be valuable for the identification and quantification of cyanobacteria, however there are limitations. Many sequences obtained from environmental samples cannot be identified at genus or species specific levels because database information is limited. Other studies have found many unidentified isolates using 16S rDNA genes sequences in environmental samples. (Brito *et al.,* 2012; Falcon *et al.*, 2002; Eiler *et al.,* 2013). Next generation sequencing (NGS) used to characterize the phytoplankton communities in 49 freshwater lakes found that more than 50% of the cyanobacteria had no closely related 16S rRNA sequences to isolated phytoplankton (Eiler *et al.* 2013). As cyanobacteria sequences are identified and databases grow, higher specificity primers can be developed. With an increase in identifiable sequences, better standards can be used as controls in qPCR reactions. Primer specificity is a concern, for example the universal CYA781Ra and b reverse primers were designed when there were only 174 published 16S rRNA sequences (Nubel *et al.*, 1997).

Characterizing copy number of the SSU rDNA genes in different cyanobacterial species would also improve assessment of cyanobacteria communities. Fogel *et al.,* (1999) identified multiple species of cyanobacteria that have more than 1 copy of SSU rDNA. Based on the number of SSU rDNA copies and amount of SSU rDNA in a sample, along with an estimate for genome size of organism in the sample, it is possible to determine the relative abundance of cyanobacteria in a sample (Fogel *et al*., 1999).

Sampling regime is also important for studies of cyanobacterial communities in aquatic ecosystems. The occurrence of blooms could easily be missed if samples are not collected at the right time. Species that are seasonally dependent could be missed if samples are not collected across all seasons.

This study suggests that real-time quantitative PCR has potential for monitoring cyanobacteria abundance in environmental samples, although there are limitations. For qPCR to be enhanced as a tool for monitoring and evaluating cyanobacteria communities in aquatic systems, expansion of cyanobacterial sequence information and careful database curation is needed to ensure cyanobacteria identification. By extending the work of Stewart (2011), this study provides insights that may lead to improved methodology for cyanobacteria identification and quantification using qPCR.

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APPENDIX A

TABLES AND FIGURES

Table 1. Primers Previously Identified by Stewart (2011) and Tested in This Study.

Primer sets with a forward primer to a single OTU paired with equimolar amounts of reverse primers CYA781Ra and CYA781Rb.

OTU	Primer Name	Primer Sequence
04	04bF	CCTTAGGAGGAGGATACAGCT
11	11F	CGGAAACGACTGCTAATACCTTATATG
44	44F	CCTTTAGGAAAGGGATACAATCGGAA
71	71F	GGTTAATTCTGCCTAGGATGAGCT
83	83F	CAGCTAGTTGGCGAGGTAAC
89	89F	GGTTTATCGCCTGAAGATGAGCT
98	98F	CCTCTAGGAAAGGGATACAATCGGAA
	CYA781R(a)	GACTACTGGGGTATCTAATCCCATT
	CYA781R(b)	GACTACAGGGGTATCTAATCCCTTT

OTU primer sets consisting of forward primers designed to multiple OTUs paired with unique OTU reverse primers.

OTU	Primer Name	Primer Sequence
09	F53-01/09/10/24/31/34/56/60/69/94	GGAAACGACTGCTAATACCCGATGT
	R293-09	GGYTTACAGCCCAGAGGCCTT
17	F30-17	CCTACAGACTCGGGGACAAAC
	R411-17	CCTTTACAGCCCAATCATTCCGGA
49	F81-19/49	GGAGGTGAAAAGAGTTTTGCCTA
	R323-21/06/49	GCTACCGTCATTATCTTCACAGA
61	F30-61	CCTACAGACTCGGGACAACAGT
	R413-61	GCTTTATGCCCAGTGATTCCGGA

Table 2. Piedmont North Carolina Lakes Sampled in This Study.

Name	Location	Size (acres)	Age (years)	Purpose
Belews Lake	Stokesdale	3864	39	Built to provide cooling water to Belews Creek steam coal-fire station power plant.
Lake Brandt	Greensboro	816	87	Greensboro water supply and recreation
Lake Mackintosh	Burlington	1100	21	Burlington water supply and recreation
Oak Hollow Lake	High Point	810	40	High Point water supply and recreation
City Lake	High Point	340	78	High Point water supply and recreation
Randleman Lake	Randolph Co.	3007	8	PTRWA water supply and recreation

Table 3. **Databases Used to Compare OTU Consensus Sequences.**

Database Name	URL	Statistics used	Description
leBIBI V5 Bioinformatic Bacteria Identification	https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi	BLAST and CLUSTAL W programs applied to GenBank sequences	Contains bacterial and archael sequences.
EzTaxon-e	http://www.ezbiocloud.net/eztaxon	BLAST and pairwise global sequence alignments for GenBank sequences	Contains 16s rRNA sequences of uncultured prokaryotic species found in ecological samples.
NCBI GenBank BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi	BLAST	Finds regions of similarity between sequences.
Ribosomal Database Project	http://rdp.cme.msu.edu/index.jsp	Sequences are aligned against a general bacterial rRNA model	Contains >2.9 million 16S rRNA bacterial and archael sequences.

Table 4. OTU Consensus Sequence Best Matches by Database.

OTU	Stewart (2011) GenBank Best Match	% Similarity	2014 GenBank Best Match	% Similarity	EZTaxon-e Best Match	% Similarity	Ribosomal database project Best Match	% Similarity	BIBI Best Match	% Similarity
04b	Unc. bacterium clone LK15m-37-16S	99%	Unc. organism clone 6m-91-27F	99%	*Teleaulax amphioxeia* clone SCCAP K0434 (chloroplast)	99.69%	Unc. bacterium	98.8%	Unc. cyanobacterium	99%
09	Unc. bacterium clone MFBC7A05	95%	Unc. bacterium clone MFBC3B08	94%	Uncultured bacterium clone AF400152_s MLS1228cl3 (Prochlorococcus)	94.70%	Unc. bacterium clone MFBC3B08	85.6%	Unc. Cyanobacterium (Prochlorococcus)	93%
11	Unc. cyanobacterium clone OO.P3.LT.46.ab1,	92%	Unc. bacterium clone EvoLake H.1	94%	(chloroplast) *Olisthodiscus luteus*	93.07%	Unc. cyanobacterium EvoLake H.1 and *Olisthodiscus luteus* (chloroplast)	77.3%	No Match Found	NA
17	Unc. cyanobacterium clone ND2_CYA_1_8	97%	Unc. cyanobacterium clone ND2_CYA_1_8	97%	Unc. bacterium clone AM259268_s Otu30s18 (Prochlorothrix-genus)	92.17%	Unc. cyanobacterium clone ND2_CYA_1_8	80.4%	Pseudoanabaena	97%
44	Unc. cyanobacterium clone OO.P2.OT.96.ab1	88%	Unc. bacterium clone EvoLake H.1	89%	Unc. bacterium clone FJ612351_s DP10.2.29 (Prochlorothrix-genus)	89.38%	Unc. cyanobacterium	57.8%	*Prochlorothrix hollandica*	93%
49	Unc. bacterium clone DP10.2.55	98%	Unc. bacterium clone DP10.2.55	98%	*Phormidium persicinum* SAG 80.79	91.05%	Unc. bacterium; DP10.2.55	88.1%	Unc. cyanobacterium	91%
61	Unc. cyanobacterium clone LW9m-1-3	93%	Unc. bacterium clone LK15m-37-16S	93%	*Teleaulax amphioxeia* SCCAP K0434 (chloroplast)	99.69%	Unc. cyanobacterium	80.1%	Unc. cyanobacterium	100%
71	Unc. Skeletonema sp. clone 2K19	94%	Unc. Skeletonema sp. clone 2K19	95%	*Oscillatoria neglecta* M-82	92.81%	Unc. cyanobacterium and Unc. Skeletonema sp. clone 2K19	81.5%	Unc. cyanobacterium and Unc. Plectonema	93%
83	Unc. Opitutae bacterium clone YL186	98%	Unc. bacterium clone LK15m-37-16S	99%	Unc. bacterium clone EU803302_s 5C230852 (Opitutae-class)	81.84%	Unc. Opitutae bacterium	77.5%	Unc. organism	98%
89	Unc. bacterium clone Reef_M07	95%	Unc. marine microorganism clone NB062806_306	95%	Unc. bacterium clone AJ347056_s TK09 (Prochlorococcus-genus)	93.96%	Unc. Cyanobacterium	90.4%	Unc. cyanobacterium	95%
98	Unc. bacterium clone XYHBP. 0912.47	98%	Unc. bacterium clone EvoLake H.1	91%	(chloroplast*) Geminigera cryophila* MBIC10567	97.50%	Unc. cyanobacterium	73.4%	No Match Found	NA

Table 5. Mean and Range of OTU Abundance (pg ml^{-1}). Each cell contains mean and range of observations in pg/mL.

	OTU 4b	OTU 09	OTU 11	OTU 17	OTU 44	OTU 49	OTU 61	OTU 71	OTU 83	OTU 89	OTU 98
Belews Lake	0.00046 0.0001-.0017	0.0004 0-.0015	0.5614 0-1.398	0.0135 0-.068	0.0250 0-.139	0.062 0-.32	0.00108 0-.0028	0.1511 .0046-.479	2.406 .123-8.77	1.4066 .125-3.01	0.1163 0-.416
Lake Brandt	0.0528 .001-.141	0.0097 0-.0306	5.629 .34-31.26	0.4649 0-2.365	0 0-0	0.101 0-.674	0.1389 0-.657	1.5878 .035-6.55	9.285 0-45.809	9.273 0-35.735	1.561 0-19.053
City Lake	0.039 .0042-.08	0.00188 0-.0096	4.4970 0-9.837	1.2035 0-5.223	0.349 0-1.986	0.0518 0-.2954	0.8238 0-3.2218	1.515 .077-3.8	2.8435 .250-8.61	36.1603 .14-162.1	1.8470 0-15.37
Oak Hollow Lake	0.0186 .001-.063	0.0026 0-.029	3.916 0-9.837	1.750 0-6.80	0 0-0	0.089 0-.35	3.923 0-12.44	3.649 .04-36.07	4.652 0-9.87	39.65 .11-137.1	0.226 0-2.36
Lake Mackintosh	0.0277 .0012-.23	0.053 .0001-.45	9.932 0-35.28	0.924 0-4.543	0.042 0-.802	0.064 0-.395	0.06 0-.27773	4.4314 0-27.295	3.211 .28-11.86	65.3242 .44-357.1	3.088 0-32.85
Randleman Lake	0.0651 .0039-.21	0.0513 0-.1459	1.5341 0-15.63	1.832 0-9.167	0.0023 0-.0441	0.314 0-2.757	0.1544 0-.553	4.231 0.13-23.9	3.2494 .285-11.8	19.9262 .249-83.2	9.317 0-.0664

Table 6. **2011 and 2012 Means and Ranges for OTU Abundance (pg ml^{-1}) for Data Pooled Across Lakes**. Total OTU abundance pooled across lakes is also provided. Mann-Whitney U test statistic and standardized test score z and p-value given. Negative z scores represent OTUs that had higher means in 2011 compared to 2012. Significant differences are highlighted.

OTU	Mean and Range 2011 DNA pg ml^{-1}	Mean and Range 2012 DNA pg ml^{-1}	Mann-Whitney test statistic *U* and z score	p-value
4b	0.0409 .00001-.23	0.028 .00004-.216	U = 34, z =-.898	.400
09	0.027 0-.45	0.013 0-145.90	U = 20, z = -2.041	.043
11	4.3916 0-31.26	4.30 0-35.28	U = 56, z = .895	.400
17	1.3625 0-9.16	0.73 0-6.80	U = 12, z = -2.694	.006
44	0.0056 0-.14	0.12 0-1.98	U = 69, z = 2.072	.053
49	0.082 0-.67	0.14 0-2.75	U = 57, z = 1.025	.315
61	1.0798 0-11.93	0.64 0-12.44	U = 26, z = -1.551	.133
71	2.8243 .0046-27.29	2.38 0-36.07	U = 30, z = -1.225	.243
83	4.0092 0-23.10	4.51 .1489-45.80	U = 49, z = .327	.780
89	28.01 0-357.15	29.17 .1122-367.57	U = 54.5, z = .779	.447
98	1.56 0-23.79	3.71 0-66.46	U = 41, z = -.327	780
Total	260.68 94.8-686.6	282.9 42-736.15	U = 49, z = .327	.780

Table 7. 2011 and 2012 Means and Ranges for Total OTU Abundance (pg ml^{-1}) for Data Combined Within Lakes. Mann-Whitney U test statistic and standardized test score z and p-value given. Negative z scores represent OTUs that had higher means in 2011 compared to 2012. Significant differences are highlighted.

	Mean and Range 2011 DNA pg ml^{-1}	Mean and Range 2012 DNA pg ml^{-1}	Mann-Whitney test statistic *U* and z score	p-value
Belews Lake	6.76 .25-12.46	2.93 .41-5.40	U = 17, z = -2.286	0.02
Lake Brandt	29.66 5.51-55.54	23.86 4.58-72.26	U = 39, z = -.490	0.66
City Lake	30.24 6.81-72.24	63.94 3.25-172.81	U = 55, z = .816	0.45
Oak Hollow Lake	58.47 24.67-125.60	58.47 8.33-155.24	U = 37, z = -.653	0.55
Lake Mackintosh	91.94 17.225-419.45	83.01 2.83-427.14	U = 36, z = -.735	0.50
Randleman Lake	43.60 11.98-73.14	50.70 1.32-121.86	U = 48, z = .245	0.84

Table 8. **Mann-Whitney U Test for Differences in OTU Abundance Between 2011 and 2012 Within Lakes**. Negative z scores indicate there was a higher abundance of OTU in 2011 compared to 2012. Cell contents include Mann-Whitney U test statistics (U), standardized test statistic (z) and p-value. Significant differences are highlighted.

OTU	Belews Lake	Lake Brandt	City Lake	Oak Hollow Lake	Lake Mackintosh	Randleman Lake
4b	22 -1.878 0.065	23 -1.545 0.136	47 0.163 0.905	60 1.225 0.4	44 -0.082 0.968	12 -2.694 0.006
09	32, -1.071 0.315	47 0.58 0.605	61 1.34 0.211	56 0.921 0.065	18 -2.205 0.028	30 -1.225 0.243
11	35, -0.816 0.447	30 -0.927 0.387	29 -1.306 0.211	22 -1.881 0.604	58 1.061 0.315	48 0.263 0.842
17	32 -1.063 0.315	37 -0.315 0.796	24 -1.718 0.095	38 -0.572 1	36 -0.736 0.497	7 -3.108 <.001
44	46 0.091 1	41 0 1	63 2.06 0.156	45 0 1	50 0.949 0.72	68 2.37 0.065
49	51 0.451 0.661	36 -0.485 0.666	61 1.454 0.211	45 0 0.243	46 0.099 1	64 1.633 0.133
61	30 -1.267 0.211	38 -0.236 0.863	44 -0.082 0.968	29 -1.307 0.211	31 -1.143 0.278	19 -2.168 0.028
71	23 -1.796 0.079	23 -1.545 0.136	36 -0.735 0.497	39 -0.49 0.661	38 -0.572 0.604	43 -0.163 0.905
83	19 -2.123 0.035	42 0.132 0.931	44 -0.082 0.968	35 -0.816 0.447	54 0.735 0.497	55 0.816 0.447
89	16 -2.368 0.017	25 -1.369 0.19	49 0.327 0.78	39 -0.49 0.661	30 -1.225 0.243	34 -0.898 0.4
98	47 0.167 0.905	44 0.476 0.796	59 1.475 0.278	44 -0.153 0.968	59 1.274 0.278	45 0 1

Table 9. **Kruskal-Wallace H Test for Differences in OTU Abundance.**. Larger H values indicate where there is a larger difference in OTU abundance between at least two lakes. Significant values are highlighted.

OTU	Kruskal-Wallace test statistic *H*	p-value
4b	58.846	<.0005
09	52.645	<.0005
11	41.395	<.0005
17	31.464	<.0005
44	19.609	0.001
49	4.381	0.496
61	44.801	<.0005
71	41.961	<.0005
83	11.056	0.06
89	33.3	<.0005
98	9.605	0.087

Table 10. Pairwise Comparison of OTU Abundance. Each cell contains the test statistic over the p-value. Significant results are highlighted. Note: OTUs 49, 83, and 98 had no pairwise comparisons performed due to non-significant results in the Kruskal-Wallace H test (Table 8)

OTU	4b	09	11	17	44	61	71	89
Belews Lake-Lake Brandt	-64.5 <.001	-26.7 0.183	-40.2 0.003	-19.3 1	-23.8 0.007	-11.8 1	-39.1 0.004	-24.7 0.32
Belews Lake-City Lake	-64.3 <.001	-7.2 1	-39.4 0.003	-39.2 0.004	-10.3 1	-41.3 0.002	-41.4 0.002	-44.4 0.001
Belews Lake-Oak Hollow Lake	-41.9 0.001	-5.9 1	-30.7 0.062	-48.4 <.001	-23.8 0.007	-64.5 <.001	-45.7 <.001	-50.1 <.001
Belews Lake-Lake Mackintosh	-42.8 0.001	-49.5 <.001	-41.8 0.001	-35.4 0.014	-20.6 0.038	-27.1 0.171	-52.5 <.001	-50.4 <.001
Belews Lake-Randleman Lake	-69.9 <.001	-57.9 <.001	-65 0.05	-48.8 <.001	-10.3 1	-32.4 0.036	-64.9 <.001	-40.7 0.002
Lake Brandt-City Lake	-0.211 1	-19.6 0.997	-0.842 1	-19.9 0.938	-13.5 0.716	-29.5 0.087	-2.3 1	-19.7 0.996
Lake Brandt-Oak Hollow Lake	-22.6 0.522	-20.8 0.77	-9.6 1	-29.1 0.098	0 1	-52.7 <.001	-6.7 1	-25.4 0.268
Lake Brandt-Lake Mackintosh	-21.7 0.64	-22.8 0.49	-1.6 1	-16.1 1	-3.2 1	-15.3 1	-13.5 1	-25.7 0.249
Lake Brandt-Randleman Lake	-5.4 1	-31.2 0.052	-46.7 <.001	-29.5 0.087	-13.5 0.71	-20.6 0.804	-25.9 0.236	-16 1
City Lake-Oak Hollow Lake	-22.4 0.548	-1.2 1	-8.7 1	-9.2 1	-13.5 0.716	-23.2 0.449	-4.4 1	-5.7 1
City Lake-Lake Mackintosh	-21.5 0.671	-42.4 0.001	-2.4 1	-3.8 1	-10.2 1	-14.2 1	-11.2 1	-6 1
City Lake- Randleman Lake	-5.6 1	-50.8 <.001	-45.9 <.001	-9.6 1	-0.026 1	-8.8 1	-23.6 0.418	-3.7 1
Oak Hollow Lake-Lake Mackintosh	-0.895 1	-43.6 0.001	-11.1 1	-13 1	-3.2 1	-37.4 0.007	-6.8 1	-0.289 1
Oak Hollow Lake-Randleman Lake	-28 0.135	-52 <.001	-37.2 0.008	-0.4 1	-13.5 0.71	-32.1 0.041	-19.2 1	-9.4 1
Lake Mackintosh- Randleman Lake	-27.1 0.172	-8.4 1	-48.3 <.001	-13.4 1	-10.3 1	-5.4 1	-12.4 1	-9.7 1

Table 11. Significant Correlations Among OTUs and Environmental or Nutrient Parameters. M = Lake Mackintosh; CL = City Lake; OH = Oak Hollow Lake. Spearman Rank Order coefficient and p-value provided.

OTU	Temp	CHL*a* rf	PHYC	[DO]	TKN	Turbidity
4b	M(r=-.492,p=.032)					
09	M(r=.502,p=.029)	CL(r=.599,p=.009)				
11	M(r=.528,p=.020)	OH(r=.796,p<.001)				
17						
44						
49	M(r=-.490,p=.033)	OH(r=-.563,p=.012)	OH(r=.655,p=.002)		C(r=.439,p=.032)	
61	CL(r=.485,p=.041)	OH(r=.469,p=.043)		OH(r=.555,p=.014)		
71	CL(r=.558,p=.016) OH(r=.847,p<.001)		CL(r=.552,p=.018)	OH(r=-.565,p=.012)		
83	OH(r=.516,p=.024)					
89	OH(r=.712,p=.001) M(r=.625,p=.004)					
98	OH(r=.712,p=.001)			OH(r=-.614,p=.005)		

OTU	CHL *a* conc.	TOC	pH	Cond	TP
4b					
09			M(r=.526,p=.021)		
11					
17					
44					
49					CL(r=.616,p=.033)
61					OH(r=.616,p=.033)
71	CL(r=.592,p=.043) OH(r=.692,p=.013)	CL(r=.804,p=.002) OH(r=.664,p=.018)	CL(r=-.486,p=.041)	OH(r=.492,p=.032)	
83	OH(r=.748,p=.005)	CL(r=.615,p=.033) OH(r=.720,p=.008)			
89	CL(r=.806,p=.002)	CL(r=.580,p=.048)	M(r=.686,p=.001)		
98		OH(r=.615,p=.033)		OH(r=.508,p=.026)	

Table 12. **Correlations of OTUs**. B:Belews Lake, BR: Lake Brandt, CL: City Lake, OH: Oak Hollow Lake, M: Lake Mackintosh, R: Randleman Lake, P: Pooled data. Correlation coefficient (r) and p-value given.

OTU	4b	09	11	17	44	49
4b			B(r=.667,p=.002)	B(r=.466,p=.044) OH(r=.557,p=.013)	R(r=-.554,p=.014)	
09			B(r=.474,p=.040) OH(r=.508,p=.026) P(r=.511,p=.026)	M(r=.596,p=.007) P(r=.530,p=.02)		B(r=.676,p=.002)
11	B(r=.667,p=.002)	B(r=.474,p=.040) OH(r=.508,p=.026) P(r=.511,p=.026)		B(r=.543,p=.016)	B(r=.512,p=.025)	
17	B(r=.466,p=.044) OH(r=.557,p=.013)	M(r=.596,p=.007) P(r=.530,p=.02)	B(r=.543,p=.016)			BR(r=.551,p=.019
44	R(r=-.554,p=.014)		B(r=.512,p=.025)			CL(r=.520,p=.022) R(r=.582.p=.009) P(r=.608,p=.006)
49		B(r=.676,p=.002)		BR(r=.551,p=.018)	CL(r=.520,p=.022) R(r=.582.p=.009) P(r=.608,p=.006)	
61	B(r=.470,p=.042) M(r=.553,p=.014)		B(r=.552,p=.014) OH(r=.489,p=.034	B(r=.544.p=.014),	P(-.484,p=.036)	
71	B(r=.804,p<.001)	M(r=.732,p<.001) R(r=.556,p=.013) P(r=.679,p=.001)	M(r=.751,p<.001) P(r=.547,p=.015)			
83	B(r=.642,p=.003) CL(r=.484,p=.036)	R(r=.658,p=.002)	B(r=.698,p=.001) M(r=.635,p=.003)	B(r=.692,p=.001) OH(.575,p=.010)		R(r=.491,p=.033)
89	B(r=.667,p=.002)	BR(r=.566,p=.014) M(r=.781,<.001) R(r=.500,p=.029)	B(r=.568,p=.011) M(r=.702,p=.001) P(r=.730,p<.001)	B(r=.714,p=.001) BR(r=.518,p=.028)		
98		B(r=.506,p=.027) CL(r=.526,p=.021)	B(r=.576,p=.010) M(r=.618,p=.005) P(r=.458,p=.049)		B(r=.783,p<.001) CL(r=.869,p<.001) P(r=.648,p=.003)	P(r=.605,p=.006)

OTU	61	71	83	89	98
4b	B(r=.470,p=.042) M(r=.553,p=.014)	B(r=.804,p<.001)	B(r=.642,p=.003) CL(r=.484,p=.036)	B(r=.667,p=.002),	
09		M(r=.732,p<.001) R(r=.556,p=.013) P(r=.679,p=.001)	R(r=.658,p=.002)	BR(r=.566,p=.014) M(r=.781,p<.001) R(r=.500,p=.029) P(r=.611,p=.005)	B(r=.506,p=.027) CL(r=.526,p=.021)

11	B(r=.552,p=.014) OH(r=.489,p=.034)	M(r=.751,p<.001) P(r=.547,p=.015)	B(r=.698,p=.001) M(r=.635,p=.003)	B(r=.568,p=.011) M(r=.702,p=.001) P(r=.730,p<.001)	B(r=.576,p=.010) M(r=.618,p=.005) P(r=.458,p=.049)
17	B(r=.544.p=.014)		B(r=.692,p=.001) OH(.575,p=.010)	B(r=.714,p=.001) BR(r=.518,p=.028)	
44	P(r=-.484,p=.036)				B(r=.783,p<.001), CL(r=.869,p<.001), P(r=.648,p=.003)
49			R(r=.491,p=.033)		P(r=.605,p=.006)
61			B(r=.752,p<.001)	B(r=.621,p=.005),	
71	C(r=.362,p<.001)		B(r=.533,p=.019) BR(r=.699,p=.001) CL(r=.668,p=.002) OH(r=.689,p=.001) R(r=.611,p=.005)	B(r=.684,p=.001) BR(r=.798,p<.001) CL(r=.468,p=.043) OH(r=.542,p=.016) M(r=.807,p<.001) R(r=.493,p=.032) P(r=.600,p=.007)	
83	B(r=.752,p<.001)	B(r=.533,p=.019) BR(r=.699,p=.001) CL(r=.668,p=.002) OH(r=.689,p=.001) R(r=.611,p=.005)		B(r=.863,p<.001) BR(r=.569,p=.014) R(r=.489,p=.033)	M(r=.541,p=.017)
89	B(r=.621,p=.005) C(r=.372,p<.001)	B(r=.684,p=.001) BR(r=.798,p<.001) CL(r=.468,p=.043) OH(r=.542,p=.016) M(r=.807,p<.001) R(r=.493,p=.032) P(r=.600,p=.007)	B(r=.863,p<.001) BR(r=.569,p=.014) R(r=.489,p=.033)		M(r=.510,p=.026)
98			M(r=.541,p=.017)	M(r=.510,p=.026)	

Figure 1. Location of Lakes Used in This Study. Darker areas represent urbanization.

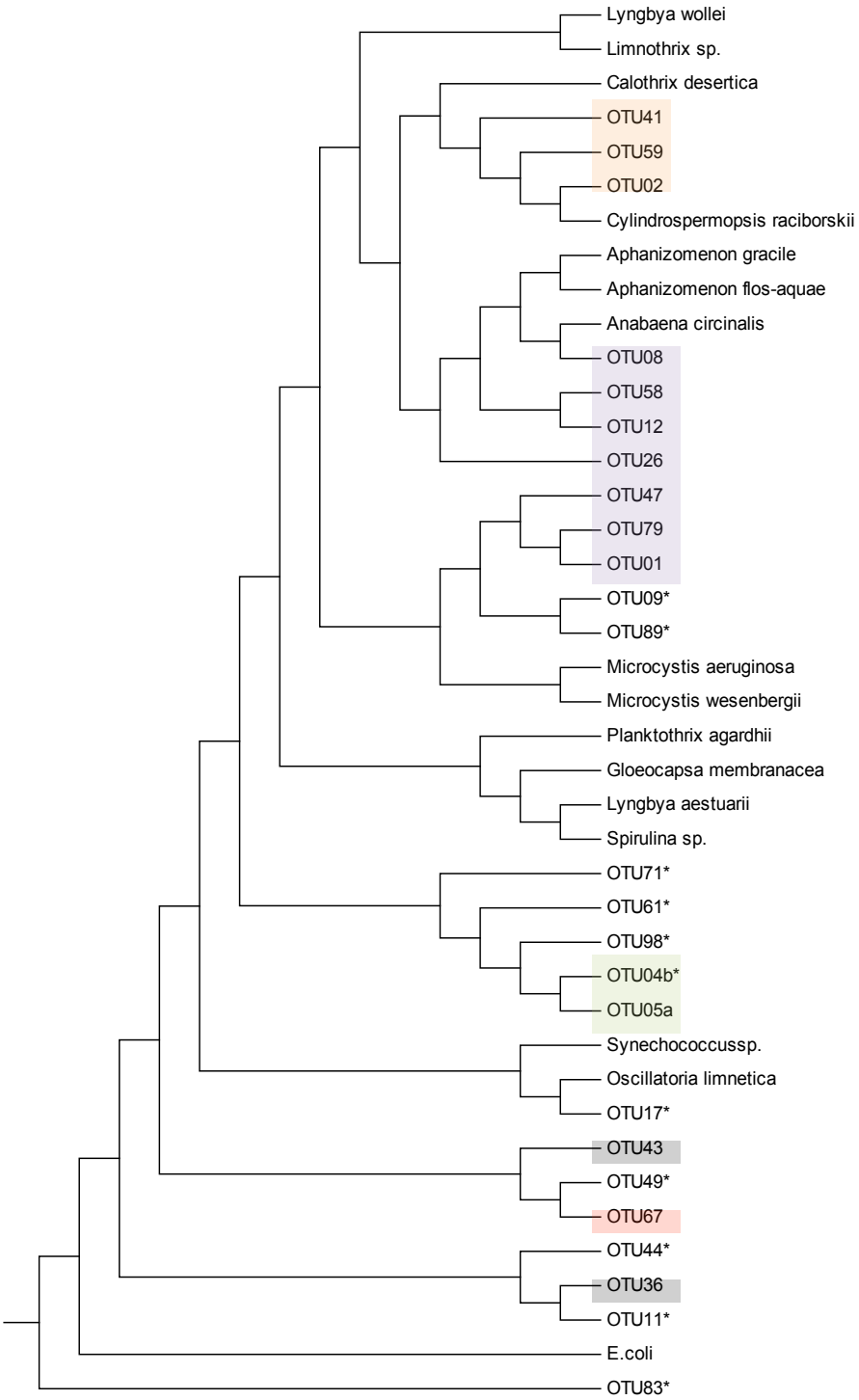

Figure 2. PHYLIP Rooted Tree of Initial 25 OTUs Considered in This Study and Known Sequences. * indicates the eleven OTUs used in this study. Highlighted areas of same color indicate OTUs whose primers cross reacted.

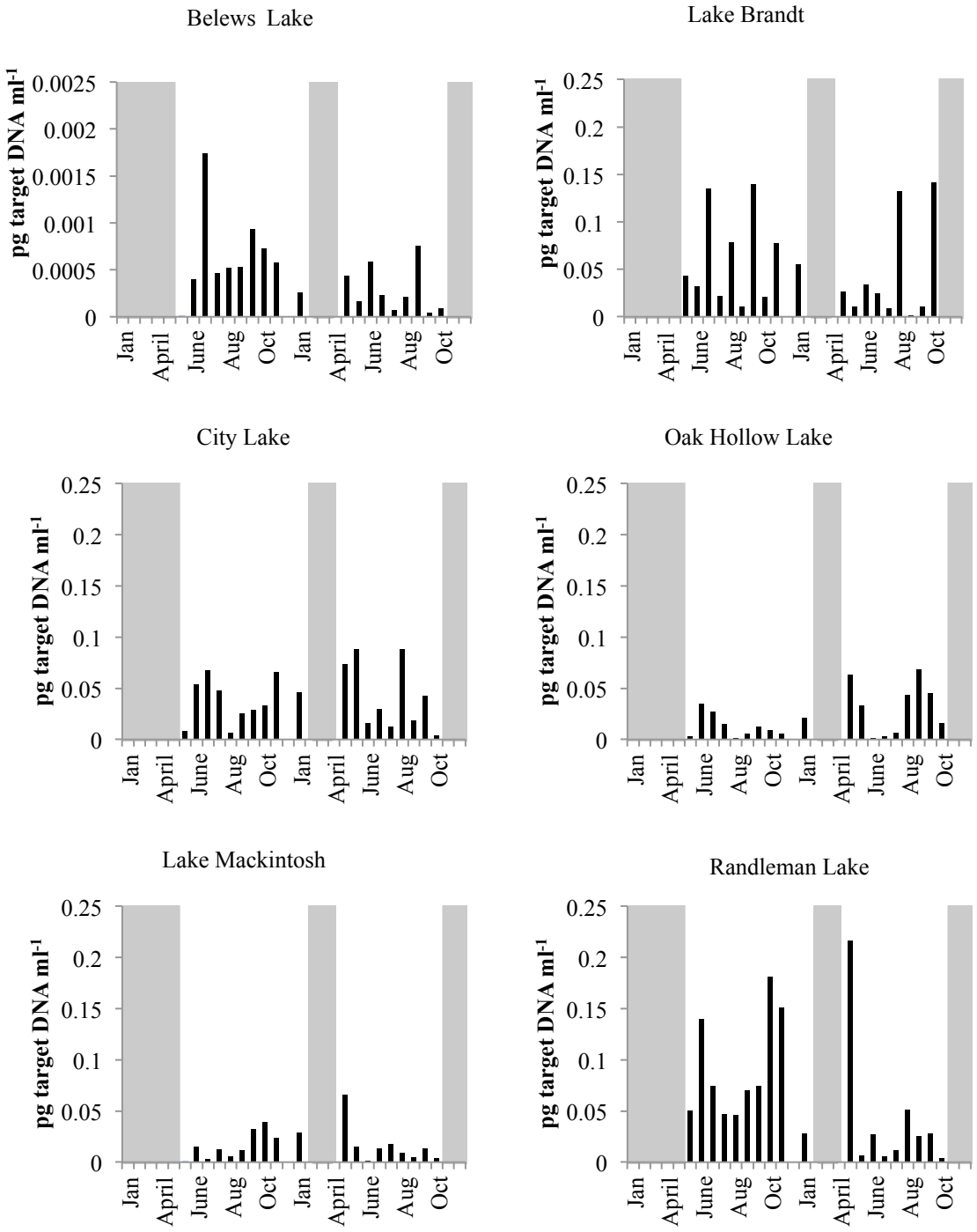

Figure 3a. **OTU4b Abundance for Samples Taken Between June 2011 and October 2012**. Gray bars indicate months when samples were not collected. Note: scale for Belews Lake is differen

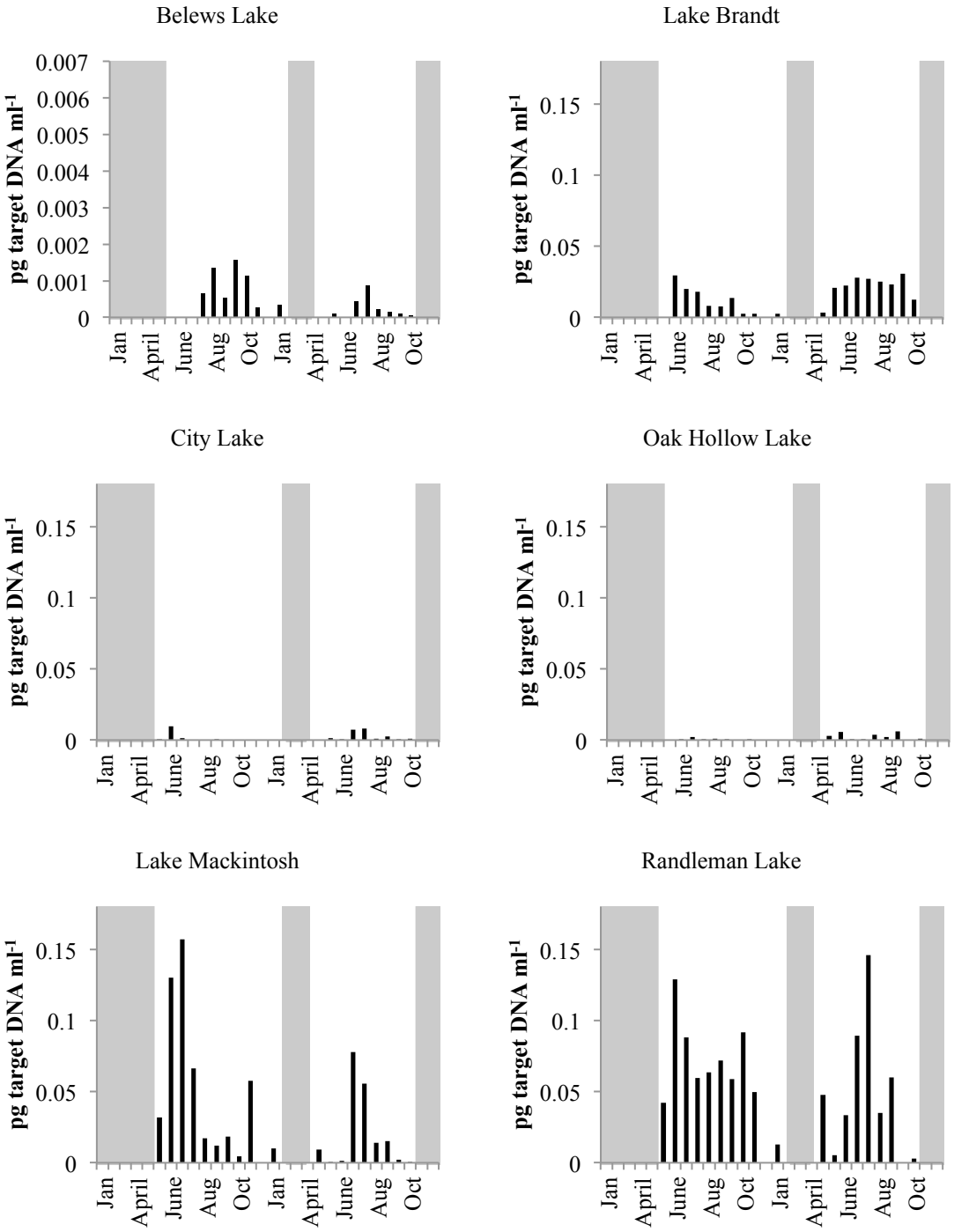

Figure 3b. **OTU09 Abundance for Samples Taken Between June 2011 and October 2012**. Gray bars indicate months when samples were not collected. Note: scale for Belews Lake is different

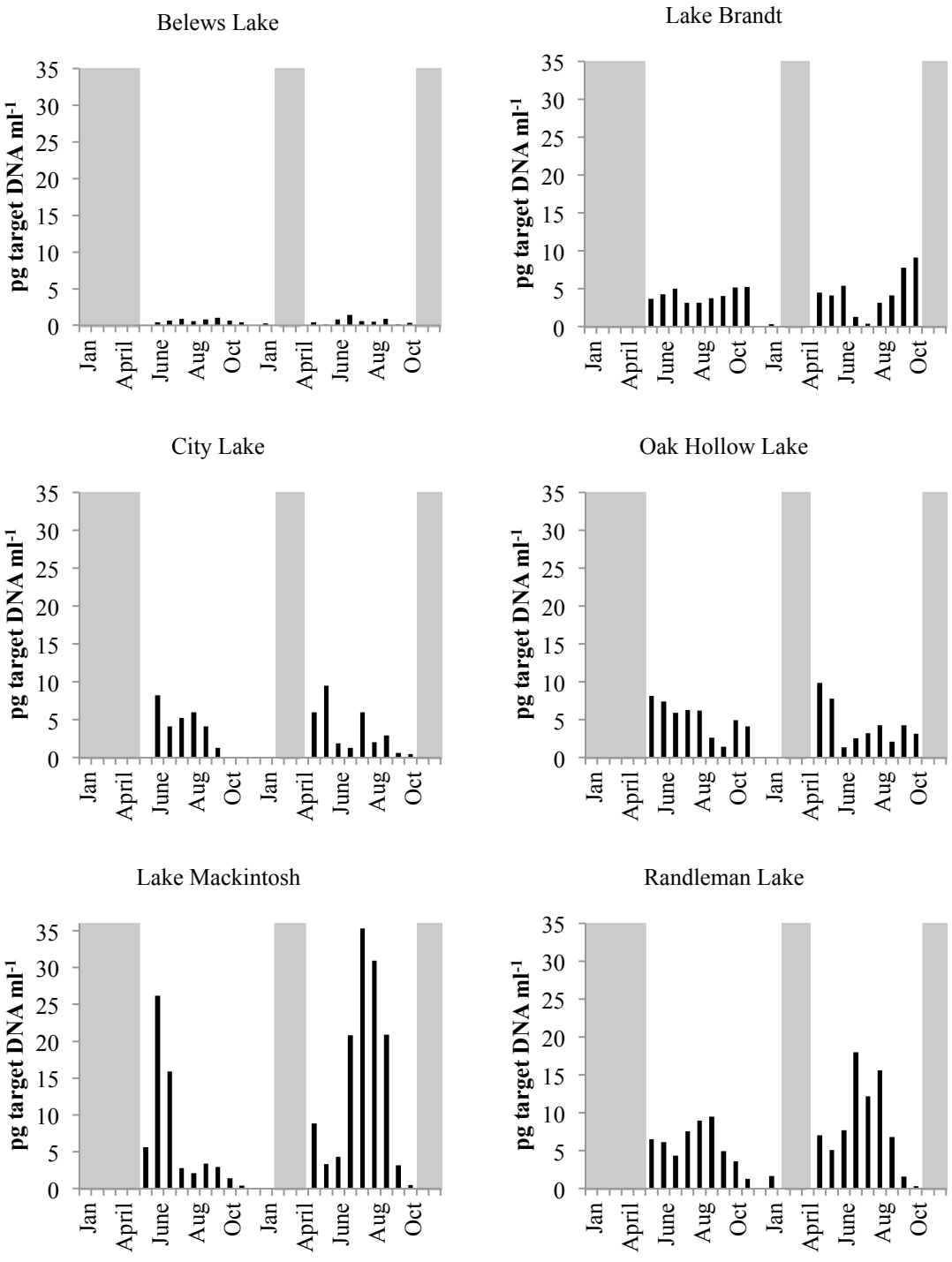

Figure 3c. **OTU11 Abundance for Samples Taken Between June 2011 and October 2012**. Gray bars indicate months when samples were not collected

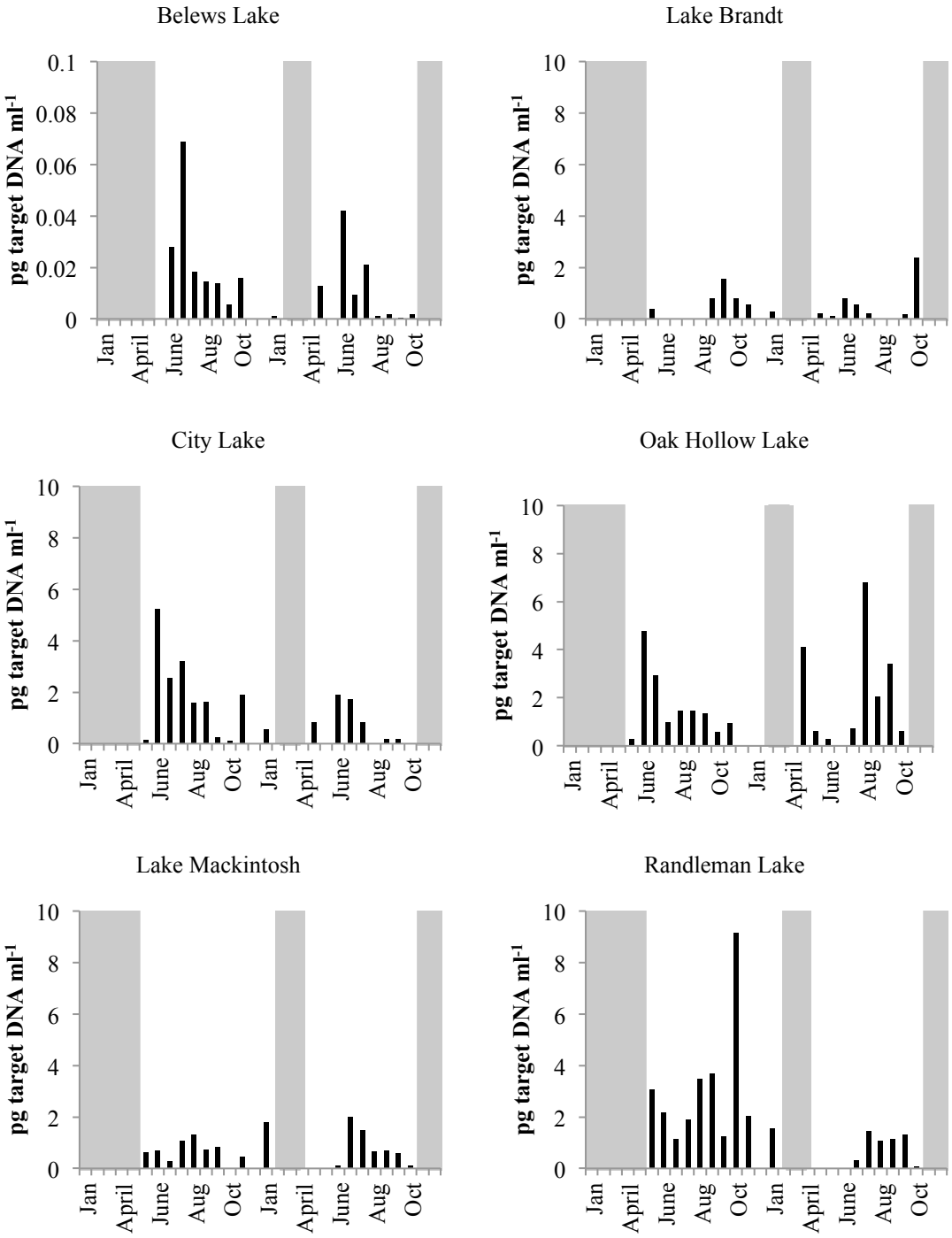

Figure 3d. OTU17 Abundance for Samples Taken Between June 2011 and October 2012. Gray bars indicate months when samples were not collected. Note: scale for Belews Lake is different.

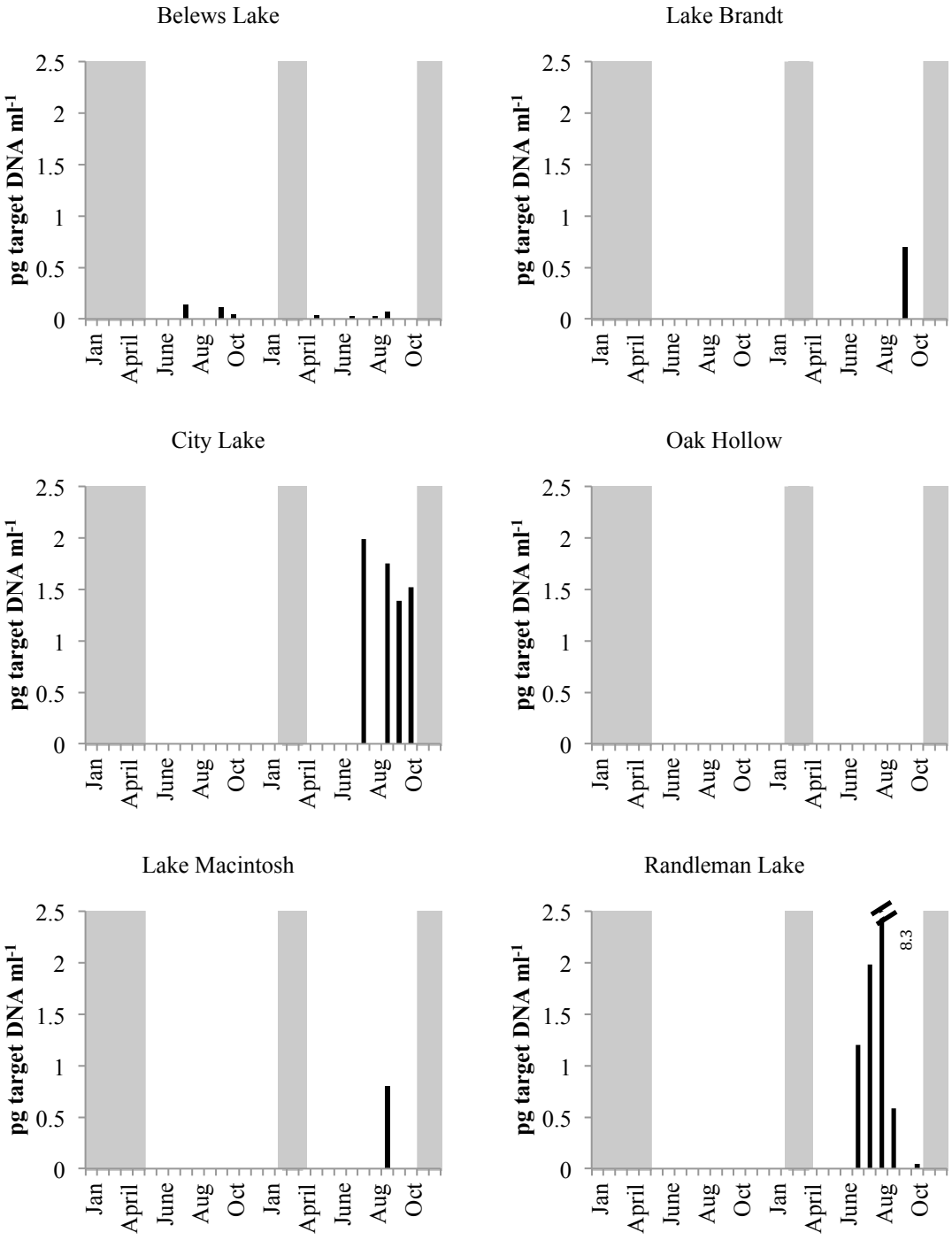

Figure 3e. **OTU44 Abundance for Samples Taken Between June 2011 and October 2012**. Gray bars indicate months when samples were not collected.

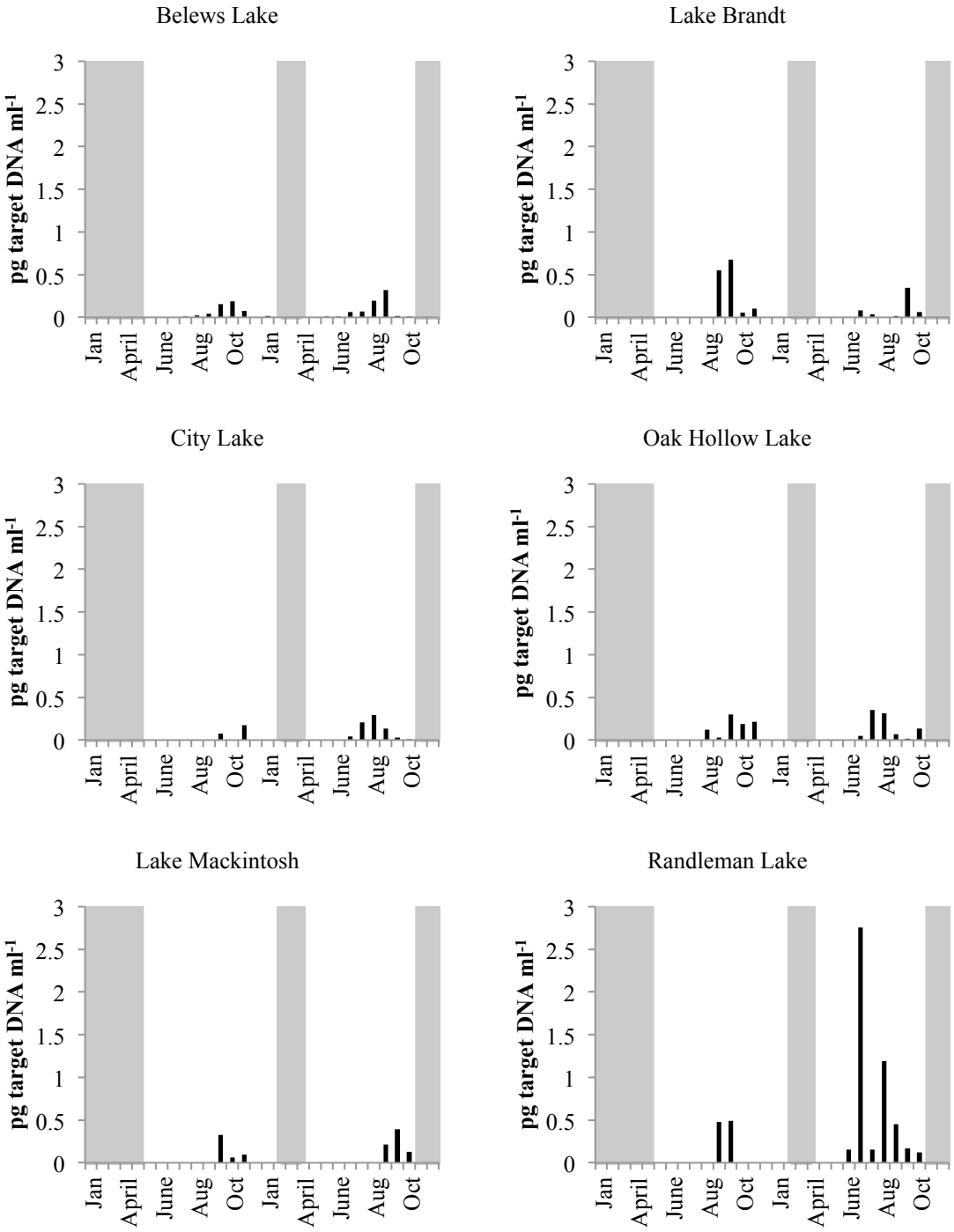

Figure 3f. OTU49 Abundance for Samples Taken Between June 2011 and October 2012. Gray bars indicate months when samples were not collected.

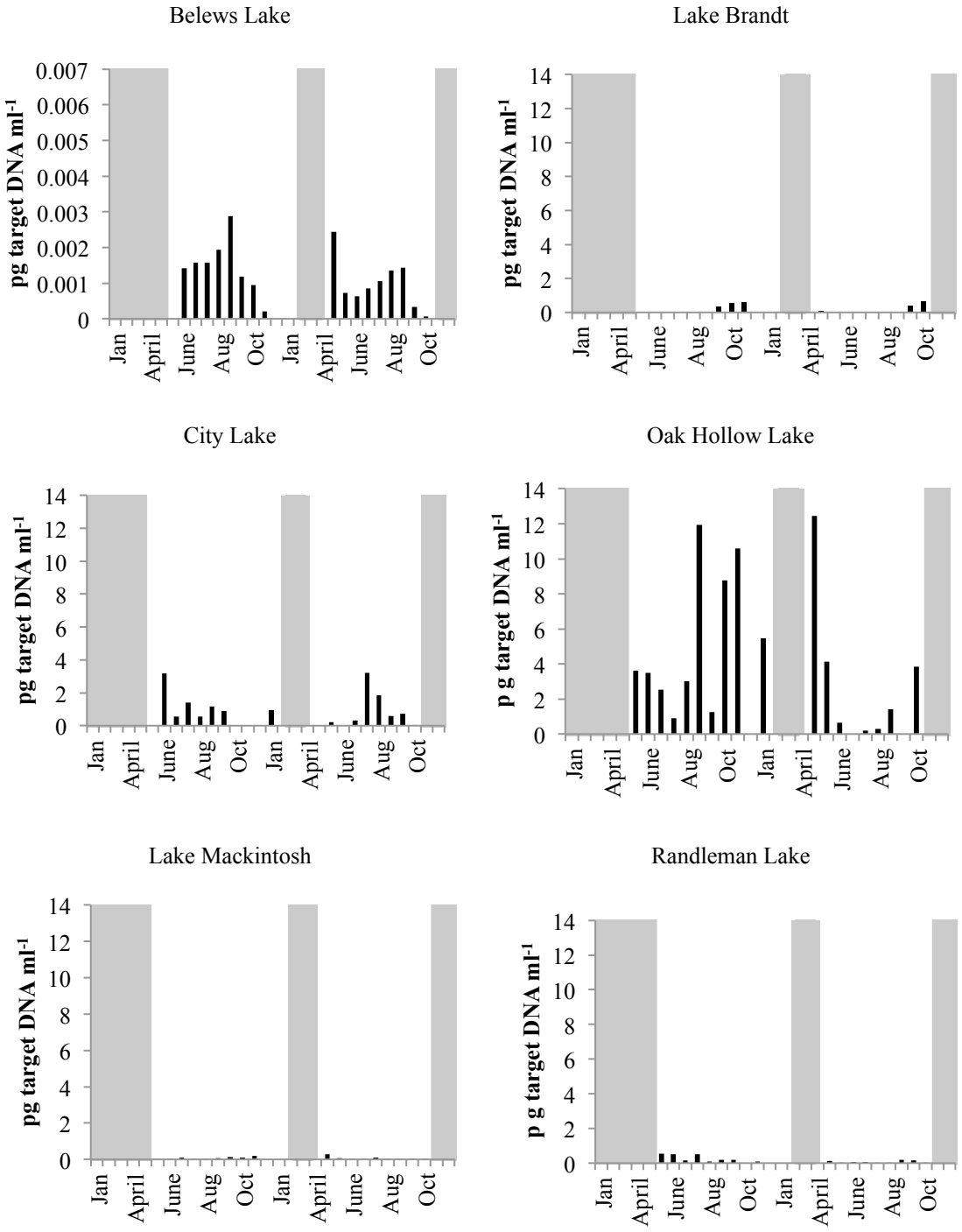

Figure 3g. **OTU61 Abundance for Samples Taken Between June 2011 and October 2012**. Gray bars indicate months when samples were not collected. Note: scale for Belews Lake is different.

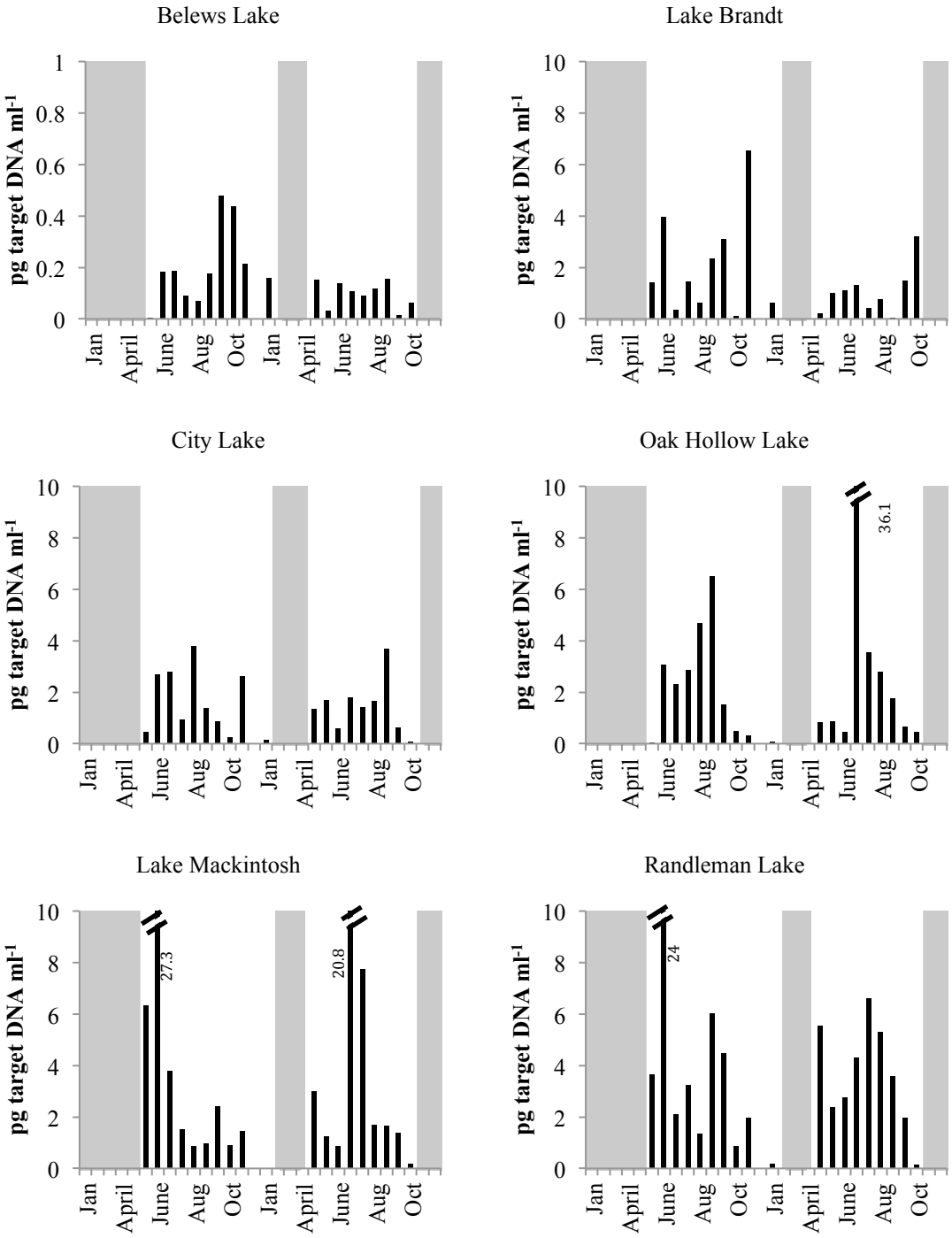

Figure 3h. OTU71 Abundance for Samples Taken Between June 2011 and October 2012. Gray bars indicate months when samples were not collected. Note: scale for Belews Lake is different.

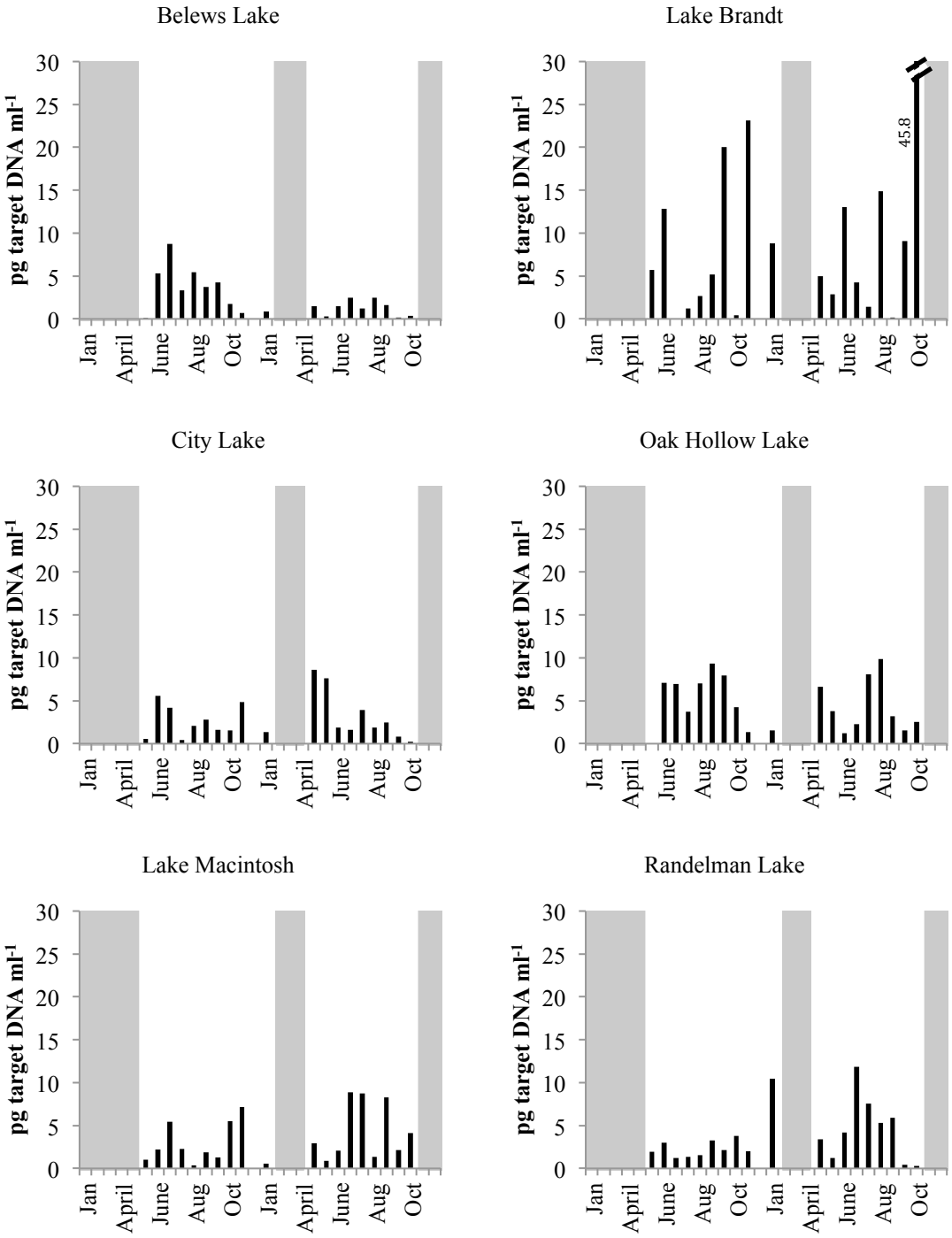

Figure 3i. OTU83 Abundance for Samples Taken Between June 2011 and October 2012. Gray bars indicate months when samples were not collected.

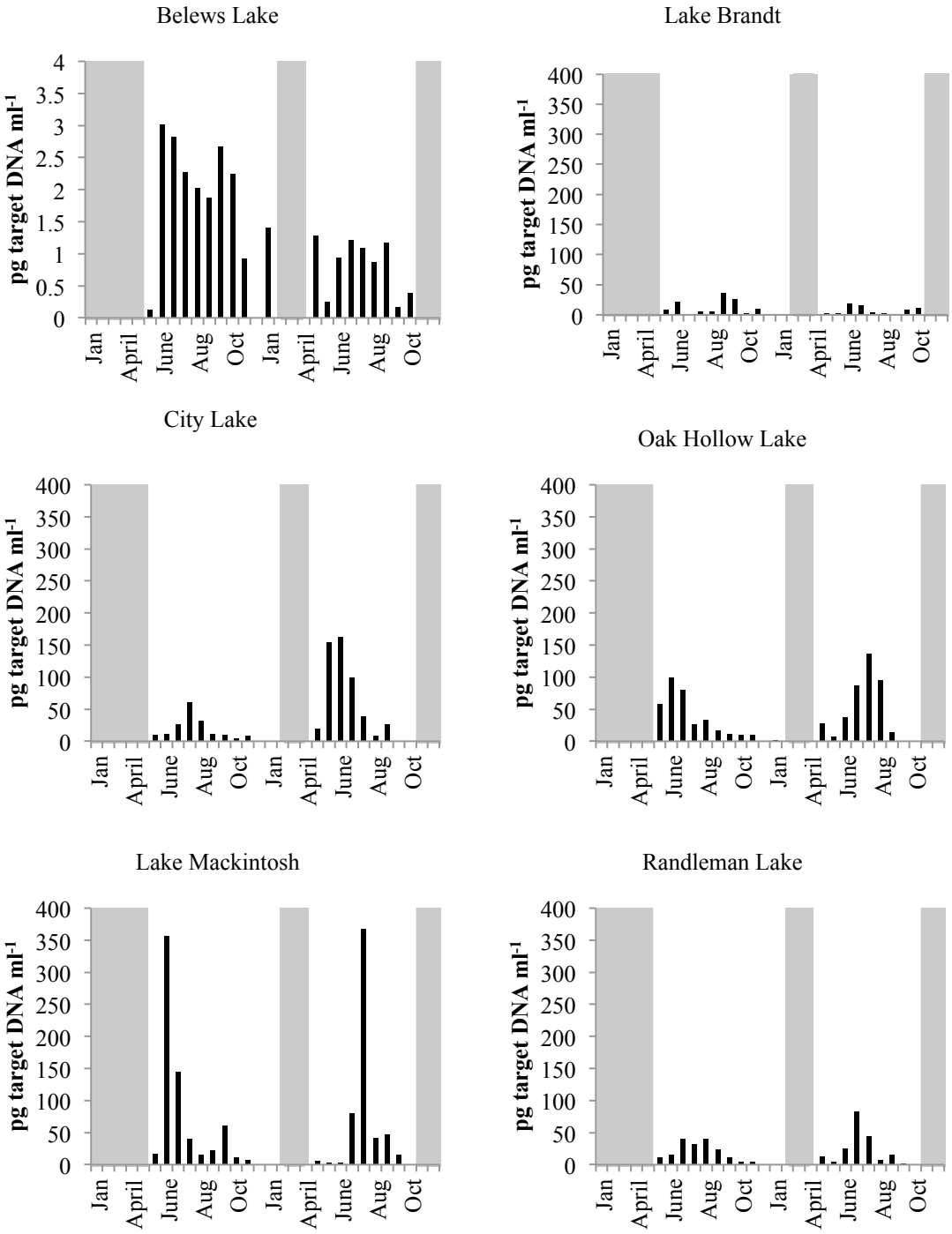

Figure 3j. OTU89 Abundance for Samples Taken Between June 2011 and October 2012. Gray bars indicate months when samples were not collected. Note: scale for Belews Lake is different.

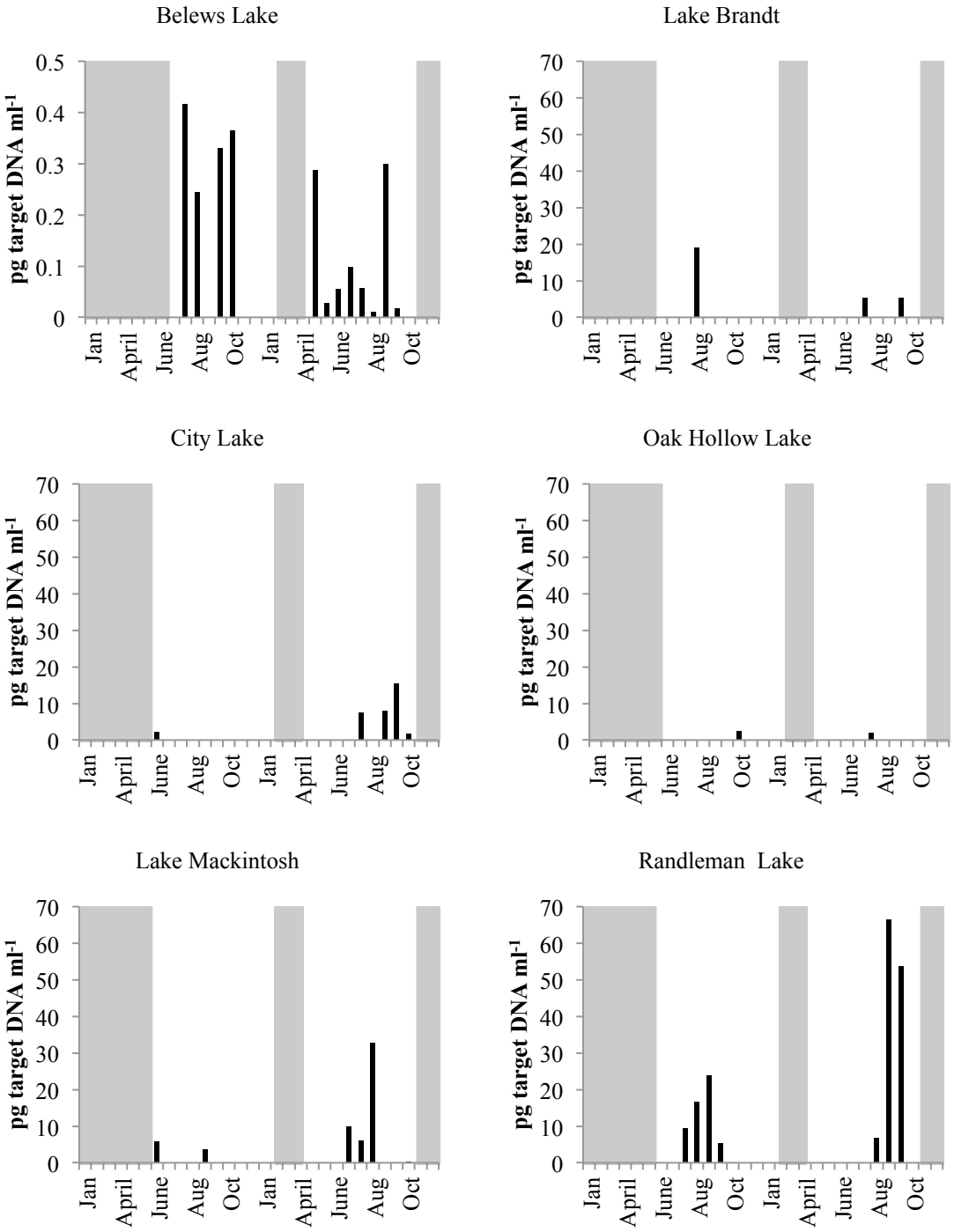

Figure 3k. OTU98 Abundance for Samples Taken Between June 2011 and October 2012. Gray bars indicate months when samples were not collected. Note: scale for Belews Lake is different.

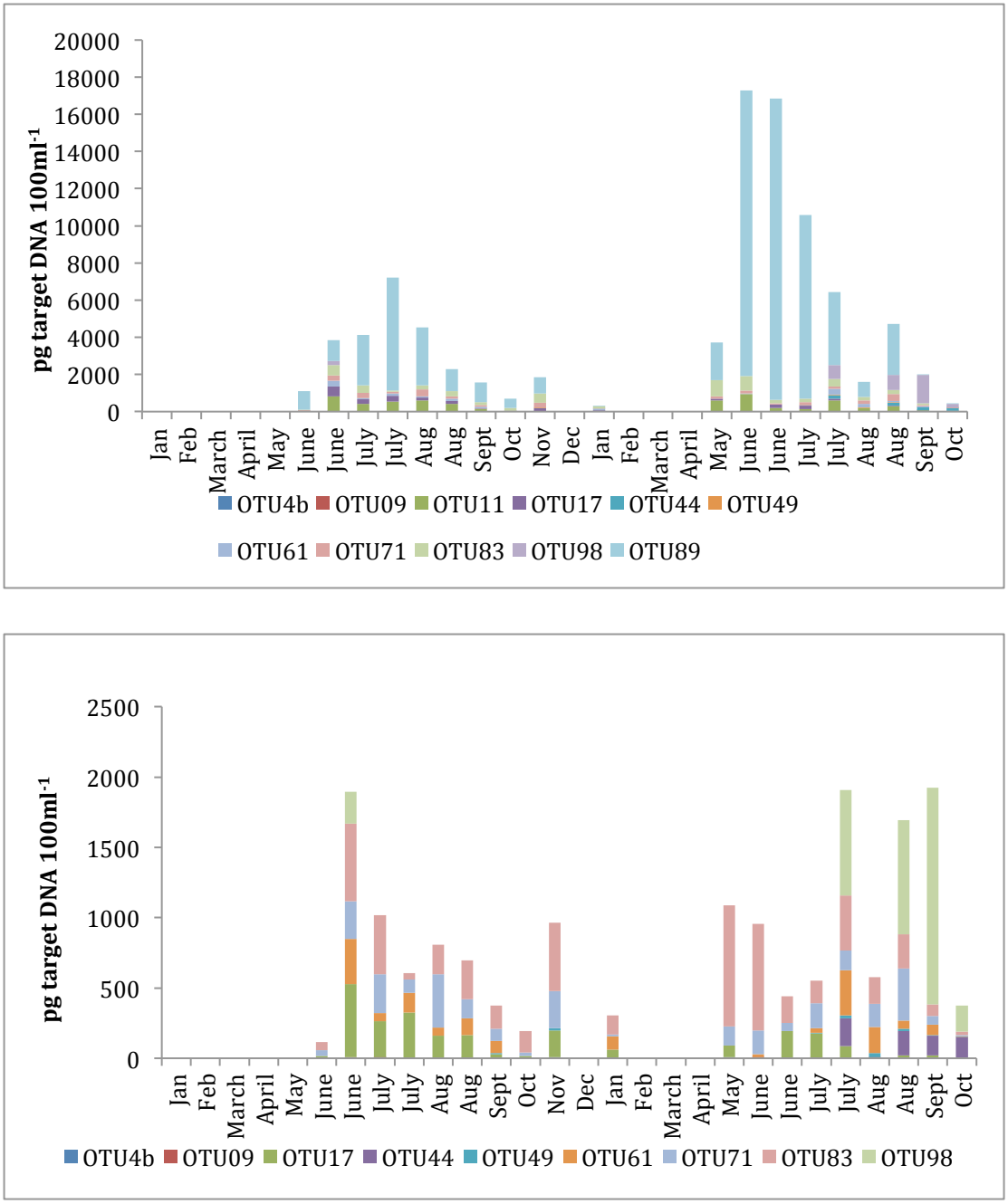

Figure 4. City Lake OTU Abundances for 2011-2012 Sampling Period. OTU abundances with OTUs 89 and 11 removed are shown in the bottom graph. Note scales are different.