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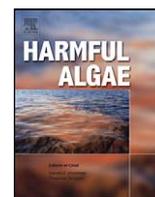
The relationships between nutrients, cyanobacterial toxins and the microbial community in Taihu (Lake Tai), China

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ABSTRACT

In recent years, excessive anthropogenic nutrient loading in Taihu (Lake Tai), China's third largest freshwater lake, has promoted the growth of harmful cyanobacterial blooms. Often composed of toxic species, these blooms threaten the supply of drinking water and fisheries-related food supplies for more than 8 million people. During a spatial survey in May 2009, surface water samples were collected from 14 stations located throughout this 2338 km^2 lake. Physicochemical and toxin measurements were compared to results from PCR based analyses of extracted DNA to examine microbial community diversity. Maximum concentrations of the hepatotoxin microcystin in this region were >600 μg g^{-1} dry weight. Statistical comparisons of lake chemistry demonstrate relationships between toxin concentrations and physicochemistry (e.g., temperature, nutrients) within the water column. Pyrosequencing of bacterial 16S rRNA gene fragments revealed a diverse community that includes potential fecal bacteria. However, subsequent source tracking of specific fecal bacteria (by quantitative PCR) indicated that fecal bacteria concentrations in the lake water bacteria were relatively low and likely not of human origin. In total the data suggest that the proliferation of cyanobacteria, but not the production of microcystin, is influenced (and perhaps regulated) by both nitrogen and phosphorus concentrations. Our observations further suggest that microcystin concentrations are correlated to the diversity of the eubacterial community, implying that specific bacteria may associate with bloom events and/or be associated with nutrient sources loading into this system.

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1. Introduction

The eutrophication of waterways worldwide is an ongoing problem commonly tied to anthropogenic over-enrichment of growth limiting nutrients (Schindler and Vallentyne, 2008; Vollenweider and Kerekes, 1982). Concurrent with this process, increases in both the frequency and duration of toxic cyanobacterial blooms have been documented on a global scale (Carmichael, 2008; Schindler and Vallentyne, 2008). These blooms are increasingly evident in large lakes and reservoirs around the world (e.g., Allender et al., 2009; Boyer, 2008; Kurmayer et al., 2002; Rantala et al., 2006; Rinta-Kanto et al., 2005). One such water body with a history of rapidly advancing eutrophication and toxic cyanobacterial proliferation is China's Lake Tai (here-in referred to by its Chinese name, Taihu[1]). Taihu is the third largest freshwater lake in China with a watershed providing a home to ~40 million residents. As a shallow lake (mean depth ~2 m), rapid development and concomitant increases in nutrient loading have driven the lake to a hypereutrophic state within a only a few decades (Guo, 2007; Qin et al., 2007). To address this problem, several groups have carried out examinations of nutrient dynamics, cyanobacterial diversity and microbial community composition (Tang et al., 2010; Ye et al., 2009a).

Environmental factors that control harmful algal blooms, including those attributable to cyanobacteria, are the subject of considerable debate. In particular, the relative importance of

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[1] "Hu" means 'lake' in Chinese so the common terminology of Lake Taihu, or Lake Lake Tai, is redundant.

nitrogen and phosphorus controls have been the most hotly discussed (Conley et al., 2009; Schindler et al., 2008; Scott and McCarthy, 2010). In North America's Lake Erie, Rinta-Kanto et al. (2009a) carried out a multi-year study to identify environmental controls of cyanobacterial populations and microcystin concentrations. In that study they concluded that there were no clear connections between factors promoting *Microcystis* proliferation and factors associated with the accumulation of microcystin. While this relationship may in part be due to the presence of other microcystin producers (e.g., Rinta-Kanto and Wilhelm, 2006), the periodic reintroduction of viable cells from sediment stores through resuspension (e.g., Rinta-Kanto et al., 2009b) or the surface accumulation of cells due to their buoyancy (Chen et al., 2003), these observations highlight the importance of considering all environmental factors when examining toxic cyanobacterial blooms and co-occurring toxins.

Here, we address the spatial variance in environmental factors associated with a bloom event that occurred in May of 2009 in Taihu, and include the potential influence of the co-occurring eubacterial community as well as the chemical speciation of the microcystins produced by the community. To identify the factors most strongly correlated to toxin production, we carried out a survey of stations across the entire lake to collect information on a suite of cyanobacterial toxin concentrations, cyanobacterial abundance, algal biomass and nutrient concentrations. Moreover, to determine how the co-occurring microbial community composition responded to these large bloom events, we used high-throughput pyrosequencing (*aka* 454 sequencing) to characterize the eubacterial community associated with 10 of the field sites. We present data on physical and chemical conditions coupled to biomolecular characterizations to examine relationships that are manifested as distinct populations of microbes (including toxic and non-toxic *Microcystis* species) that proliferate in this lake.

2. Methods

Surface water samples from 14 stations on Taihu were collected in May 2009 (Fig. 1). Depth at all stations ranged from 1 to 2 m (the latter being the maximum depth of the lake). One station (denoted "Channel") consisted of a man-made channel between two northern embayments of Taihu. On May 27, 2009 a full lake survey was completed during daylight hours and samples for chlorophyll *a*, cyanobacterial abundance, dissolved oxygen, *in situ* temperatures, conductivity, pH concentrations, toxin concentrations and biomass for DNA extraction were all collected. To enhance our ability to better resolve environmental relationships, nutrient data (ammonia, nitrite, nitrate, total dissolved N, total N, phosphate, total dissolved P, total P,) and ions (sodium, calcium, magnesium) collected 5 days earlier (May 22, 2009) as part of the regular Taihu biogeochemical survey are included in this analysis: samples were processed as previously described (McCarthy et al., 2007; Xu et al., 2010). Weather conditions were constant through the 5-day period and marked by minimal wind and cloud, consistent air temperatures and no precipitation.

Total microcystin activity in water samples, expressed as microcystin-LR equivalents, was determined by means of protein phosphatase inhibition assays. Samples were collected on GF/F (Whatman) filters for toxin extraction as in our previous work (Rinta-Kanto et al., 2009a) or by filtration through a 20 μm CellMicroSieves (BioDesign Inc., Carmel, NY). Cell biomass was dried by lyophilization prior to extraction with 50% methanol using ultrasound. Microcystins were assayed in 96-well plates containing 0.1 milliunits enzyme (recombinant protein phosphatase 1A, catalytic subunit, Roche Applied Science), 1.05 mg para-nitrophenyl phosphate (Sigma) and 10 μL of sample or microcystin-LR (Sigma Biochemical) using the method of Carmichael and An (1999). The rate of phosphate hydrolysis was calculated from the change in absorbance at 405 nm over 1 h, and then compared to the control (no added microcystin-LR) and to standards containing between 6 and 40 μg L^{-1} microcystin-LR. Toxin concentrations are therefore reported as microcystin-LR equivalents as other bioactive microcystin congeners are known to be present in these samples. Blanks (no enzyme, no toxin), unknowns, standards, and controls were all run in duplicate. The contributions of different microcystin congeners (and nodularin congeners, which were not present) to the total toxin pool were determined by LCMS using an Ace C18 column and an aqueous acetonitrile gradient containing 0.2% trifluoroacetic acid as previously described, coupled to a Micromass ZQ 4000 single quadrupole mass spectrometer operating in the positive electrospray full scan mode between
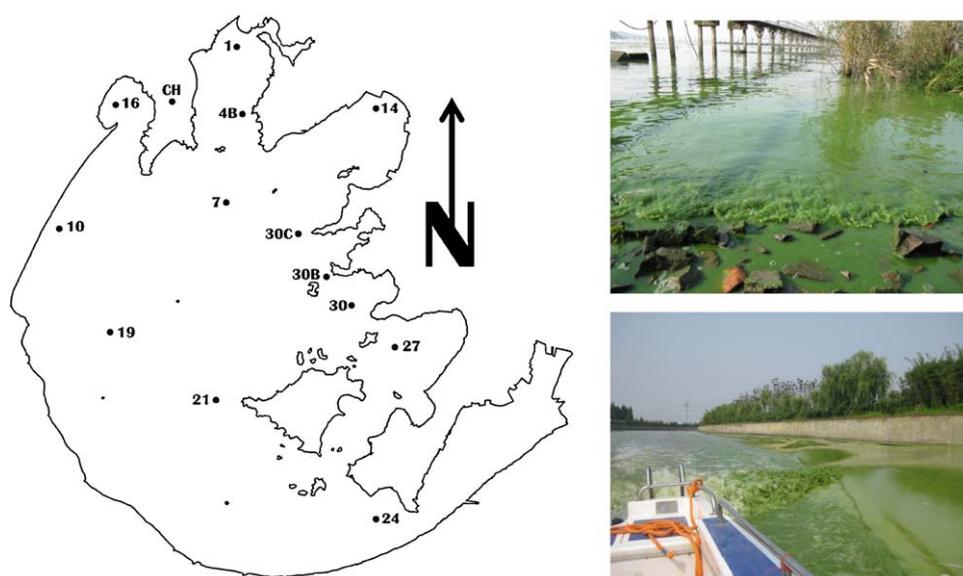
Fig. 1. Station locations in Taihu. Station notations were derived from long term monitoring sites occupied by researchers from the Nanjing Institute of Geography and Limnology. CH – denotes the location of a channel through this peninsula which was sampled as part of this study. Photographic images document the intensity of the bloom at the *Taihu Laboratory for Lake Ecosystem Research* (top right, east of station 4) and the channel site (bottom right).

m/z 800 and m/z 1350 (Boyer, 2007). Anatoxin-a and cylindrospermopsin were determined in a single run using a similar column and an aqueous acetonitrile containing a 0.1% formic acid gradient. B-Methyl amino-alanine (BMAA) was determined by positive electrospray LCMS using a TosoHass Amide-80 column eluted isocractically with 50% acetonitrile acidified with 0.1% formic acid. All toxins were quantitated against authentic standards (certified by mass spectrometry and 600 mHz NMR at the College of Environmental Science and Forestry, SUNY-ESF) using the ion intensities of their molecular ions and sodium adducts. Chl a concentrations in the dried powders were determined using the fluorometric method of Welschmeyer (1994).

For DNA analysis, 50 mL of surface water from each station was filtered through a 0.2-μm polycarbonate filter and the sample frozen (−20 °C) until processing. DNA was extracted using a MOBIO Ultra Clean Soil DNA kit. The presence of Microcystis and potential microcystin producers was assayed using diagnostic genes (Microcystis-specific 16s rDNA and the mcyA gene), respectively, as previously described (Ouellette et al., 2006; Ouellette and Wilhelm, 2003; Rinta-Kanto and Wilhelm, 2006). Where negative PCR results were observed, samples were reprocessed after being spiked with a positive control DNA fragment (DNA from Microcystis aeruginosa PCC 7806) to ensure there was no PCR inhibitors within the samples.

To examine the eubacterial community, amplicons were generated using universal eubacterial PCR primers targeting the hypervariable V3 region of the 16S rRNA gene (Huse et al., 2008), barcoded (Hamady et al., 2008) by station, pooled and submitted to facilities at the UT/ORNL Joint Institute of Biological Sciences for sequencing on a Roche 454 instrument. Sequence information was processed first through the proprietary Roche software to generate ~16,000 sequences whose score passed the manufacturers specifications for quality reads prior to analyses through the Ribosomal Database Pyrosequencing Pipeline (RDP, http://rdp.cme.msu.edu). Samples were aligned and initially classified using the classifier set at boot strap thresholds of 50% (as recommended). To further examine the community, sequences were clustered by the complete linkage method (set at 3% for variance in diversity) and the resulting data set dereplicated. Estimates of diversity (Chao1, Simpson's diversity (H') and evenness (E)) were all determined using the RDP. Comparisons between stations were completed using the UniFrac analysis service (http://bmf2.colorado.edu/unifrac/index.psp) as previously described for weighted environments (Lozupone et al., 2006, 2007). Sequences were realigned and phylogenetic reconstructions were created for the community using a Maximum Likelihood approach (Dereeper et al., 2008) prior to UniFrac analyses. Sequence data from this study were deposited in the CAMERA database.

Quantitative PCR (qPCR) was performed using assays designed for two fecal indicator bacteria, E. coli and total fecal Bacteroides (Bacteroides class). The gene targets for the E. coli and Bacteroides assays were the 23S rRNA gene and the 16S rRNA gene, respectively (Bernhard and Field, 2000; Knappett et al., 2010; Layton et al., 2006). All qPCR assays were performed in triplicate for each sample with an additional well for each sample containing a known amount of the standard as spike to monitor PCR inhibition. All qPCR reactions were prepared using 12.5 μL PCR mix, (QIAGEN, Valencia, CA or Stratagene, LaJolla, CA), 5 pmol of the forward primer and reverse primers, 15 pmol of the probe, 8 μL of sterile water and 2.5 μL of sample or standard. PCR amplification and fluorescent probe detection were performed using the Chromo4 Real-Time PCR Detection system (BioRad, Hercules, CA) and the following amplification protocol: 50 °C for 2 min, 95 °C for ten min, and 45 cycles of alternating 95 °C for 30 sec and an annealing temperature of 60 °C for the Bacteroides assay and 55 °C for the E. coli assay for 45 s. The standards used to calibrate the qPCR assays consisted of the target gene cloned into a plasmid for both the total Bacteroides and E. coli assays. For plasmid standards, serial 10-fold dilutions were performed from a starting concentration of 1×10^7 plasmid copies to 10 copies, and 2.5 μL of each plasmid dilution was placed in triplicate wells. Data for each sample and assay was calculated as copies ng^{-1} of total extracted DNA and then converted to copies mL^{-1} based on the volume of water filtered. The detection limit was determined to be when the copies of marker DNA was less than 1 copy ng^{-1} of extracted DNA.

2.1. Statistical analyses

Statistical analyses and figures for environmental data were completed using Microsoft Excel (2007), SigmaPlot (ver. 10) and Primer-E (Primer-E Ltd., Plymouth, UK). Stations were removed for comparisons where data were absent.

3. Results

3.1. Environmental conditions and distribution of biomass

Samples were collected during a significant algal bloom on Taihu. Water column chlorophyll a concentrations ranged from highs of ~35 μg chlorophyll a L^{-1} at northern locations in the lake to lows of ~1.5–2.0 μg L^{-1} at southern stations (Table 1). Differences in nutrient concentrations (N, P) in Lake Taihu were generally consistent with changes in biomass. The distribution and the predominance of Microcystis in the lake resulted in a strong and positive correlation (R^2 = 0.762) between chlorophyll a and total cyanobacterial abundance (Table 2). Other metrics (e.g., DO, pH) collected during sampling were consistent with a highly productive, warm (22–26 °C) and shallow lake system which experiences cyanobacterial blooms.

3.2. Cyanobacteria, Microcystis and microcystin

Total cyanobacterial abundance across the lake ranged from ~650 to >17,000 cells mL^{-1} and correlated positively (R^2 = 0.487) with the concentration of total microcystin present in the water column. Correlations of total cyanobacterial abundance vs. total N (R^2 = 0.828) and total P (R^2 = 0.888) concentrations demonstrated a strong relationship between the abundance of members of this group and major nutrients (Fig. 2A and B). Surprisingly, the relationship between toxin concentrations and these nutrients was weaker, suggesting that biomass and toxin production were not responding to the same environmental drivers (Fig. 2C and D). No other strong relationships between environmental parameters and toxin concentrations were observed.

Microcystis spp. were detected at all stations throughout the lake, although toxigenic strains (mcyA+ samples) were only detected at a three of the fourteen stations. The concentration of total microcystin in equivalents per unit algal biomass ranged from below detectable limits (<1.4 μg microcystin equivalents g^{-1} dry weight at Station 24) to 631.3 μg microcystin g^{-1} dry weight at the channel station (Table 2). Microcystin concentrations were highest at stations in the northern part of the lake, especially at the channel station where high amounts of biomass and a high toxin concentration per biomass were measured. These high toxin concentrations measured using the protein phosphatase inhibition assay also corresponded with the highest estimates of the microcystin-RR congener; a substantially higher percentage of the microcystin was in the -LA form in the more southern Taihu sites. Interestingly, this relationship also correlated with the concentration of sodium ions in the water column (Fig. 3). Microcystin-LR (the most toxic of the chemical forms) did not follow

Table 1
Environmental parameters at stations sampled for this study. Ranges for turbidity, chlorophyll and dissolved oxygen are given for n=2 replicate measurements.

Stn	Temp. (°C) (NTU)	Conductivity (μg L⁻¹)	pH (%)	Turb (mg L⁻¹)	Chl a (mg L⁻¹)	DO (mg L⁻¹)	N-NH₃ (mg L⁻¹)	N-NO₂ (mg L⁻¹)	N-NO₃ (mg L⁻¹)	Total dissolved N (mg L⁻¹)	Total N (mg L⁻¹)	P-PO₄ (mg L⁻¹)	Total dissolved P (mg L⁻¹)	Total P (mg L⁻¹)	Na	Ca	Mg
1	24.6	0.63	8.10	19.4±1.1	14.0±0.6	122.7±0.4	0.57	0.073	1.50	3.64	4.27	0.020	0.046	0.128	68.2	23.2	11.26
4B	24.1	0.61	8.07	19.9±1.1	14.2±0.26	123.6±0.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
7	22.1	0.53	8.11	19.7±0.8	5.9±0.1	100.0±0.0	0.50	0.014	1.54	2.85	3.31	0.010	0.023	0.074	33.4	12.1	5.36
10	22.7	0.72	7.49	60.1±1.0	16.7±0.3	72.9±0.6	2.24	0.197	0.99	4.83	6.10	0.063	0.096	0.375	62.0	19.5	8.63
14	26.0	0.40	7.54	16.0±0.3	5.0±1.8	113.3±0.2	0.42	0.058	0.99	2.41	2.80	0.043	0.064	0.149	32.9	12.4	6.42
16	25.7	0.72	7.86	25.1±5.5	35.1±0.2	142.3±1.0	2.83	0.207	0.86	5.01	6.45	0.102	0.139	0.388	73.8	19.3	9.58
19	22.5	0.50	8.24	47.5±0.8	8.9±0.4	102.8±0.4	0.27	0.010	1.80	3.26	3.92	0.009	0.019	0.087	46.4	20.1	9.68
21	22.6	0.53	8.34	26.5±0.2	8.1±0.5	105.6±0.1	0.54	0.008	0.98	2.64	3.22	0.007	0.016	0.075	49.8	23.9	10.21
24	22.7	0.44	7.84	5.7±0.0	2.6±1.2	93.7±1.0	0.36	0.014	0.78	1.73	2.04	0.006	0.013	0.035	41.2	20.3	8.64
27	23.9	0.49	8.00	0.1±0.0	1.6±0.6	134.1±0.1	0.40	0.010	0.71	1.87	2.05	0.007	0.015	0.023	52.7	20.8	10.66
30	24.7	0.53	7.85	3.4±0.1	1.9±0.4	121.2±1.6	0.26	0.007	1.39	2.70	2.94	0.011	0.018	0.038	50.8	21.7	10.11
30B	24.3	0.56	7.64	11.2±2.2	3.8±0.5	115.0±0.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
30C	24.2	0.58	7.66	7.8±2.4	3.0±0.60	107.9±0.9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CH	25.8	0.69	8.13	31.7±4.1	22.4±1.82	125.9±1.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

any discernable distribution patterns. Other common toxins produced by cyanobacteria including anatoxin-a ($<1 \mu g\ g^{-1}$ dry weight), cylindrospermopsin ($<10 \mu g\ g^{-1}$ dry weight) and BMAA ($<1 \mu g\ g^{-1}$ dry weight) were below the detection limit of the single quadrapole LCMS instrument used in these experiments.

3.3. Diversity of the eubacterial community

Given the important roles that bacteria play in shaping the geochemistry of aquatic systems, we felt it was important to establish the relationship between these communities and stations in Taihu. In total, 16,979 acceptable-quality reads were returned from the 454 sequencing run of the 10 lake stations examined in more detail. Across all stations, a combined 2096 unique 16s rDNA amplicons were identified, with the most highly represented sequences totaling 1444, 961, 568 and 450 individuals that were 100% identical (Fig. S1). We identified a total of 1252 singleton sequences, suggesting the presence of a potentially expansive "rare biosphere" (vis a vis Sogin et al., 2006) within this system.

Amongst sequences identified by the RDP classifier, proteobacteria (42% of all sequences), actinobacteria (29%), bacteroides (5.6%) and cyanobacteria (5.5%) were the most dominant groups (Fig. 4). Unidentified sequences (those that were of sufficient quality yet not classifiable by the RDPII analyzer) ranged from ~2% to 30% of the individual libraries.

When considered at the 97% identity level, a value commonly used for identification of operational taxonomic units (OTUs, Stackebrandt and Goebel, 1994), the libraries were divided into 175–480 different groups (Table 3). These estimates of population richness were high, but the Chao1 metric (Chao, 1984; Colwell and Coddington, 1994) suggested no linkages between the estimated richness of the eubacterial populations and either chlorophyll a, microcystin or cyanobacterial abundance (data not shown). Even though that the amount of 16S rDNA sequencing completed in this project was greater than in other studies (Tang et al., 2010; Wu et al., 2007), in only a couple stations did sequencing coverage from our samples approach saturating rarefaction (Fig. S2).

3.4. Quantification of fecal bacteria within the community

Due to their linkage to anthropogenic nutrient sources, their implications for human health and their abundance in the pyrosequencing data set, we wanted to further examine the potential fecal bacteria within the microbial community. Total Bacteroidetes populations ranged from below detectable limits to more than 450 copies mL^{-1} (Fig. 5). In contrast, the detectable E. coli copies mL^{-1} were lower in overall abundance (BDL to 60 mL^{-1}) but were strongly related with concentrations of ammonium ($R^2 = 0.832$), suggesting they may be tracking nutrient inputs into the lake. E. coli also related strongly to total phosphorus ($R^2 = 0.752$), nitrite ($R^2 = 0.696$) and predicted by concentrations of dissolved nitrogen ($R^2 = 0.608$). Measurement of specific host-types using qPCR suggested the E. coli was not closely associated with known isolates associated with human fecal contamination (data not shown). Examination of the Bacteroides group identified within the pyrosequencing dataset indicated that only 27 of 568 sequences belonged to the class Bacteroidetes, and none of these appeared to be of known human fecal origin.

3.5. Analysis of microbial community, toxins and environmental parameters

To potentially identify and distinguish relationships between toxins, environmental parameters and the microbial community, we compared the station similarity based on the distribution of the microbial community as defined by the presence of their 16S rDNA

Table 2
Cyanobacterial abundance (total) and microcystin concentration and chemical congeners. ND – not determined. Microcystin concentrations ($\mu g\,g^{-1}$ dry weight) are given ± of analytical replicates ($n=2$).

	Cyanobacteria (cells mL^{-1})	*Microcystis* 16s rDNA	*mcyA*	Microcystin ($\mu g\,kg^{-1}$)	Toxin types (%)			
					-LR	-YR	-RR	-LA
1	5544.7 ± 427.8	+	–	44.3 ± 7.2	41	2	50	7
4B	5774.0 ± 1058.4	ND	ND	15.2 ± 2.7	40	3	41	16
7	1968.7 ± 110.8	+	–	1.4 ± 0.0	3	–	–	97
10	9274.7 ± 1652.0	+	–	39.1 ± 9.8	42	3	45	9
14	4216.7 ± 2243.8	+	–	8.8 ± 0.9	6	–	–	94
16	16,196.3 ± 3797.7	+	+	176.9 ± 37.8	32	2	65	1
19	3050.7 ± 537.3	+	+	5.2 ± 1.2	32	–	27	40
21	2419.3 ± 201.2	+	–	13.1 ± 1.0	61	2	14	23
24	858.7 ± 248.1	+	–	<1.4	11	–	–	89
27	888.7 ± 444.3	ND	ND	16.1 ± 1.9	20	–	28	52
30	663.3 ± 51.6	ND	ND	4.1 ± 0.2	18	–	26	56
30B	1577.7 ± 108.7	ND	ND	53.4 ± 3.5	41	3	56	–
30C	5368.0 ± 2550.1	+	–	5.5 ± 1.9	30	2	31	37
CH	17,549.7 ± 765.1	+	+	631.3 ± 27.1	23	2	75	–

sequences (UniFrac similarity dendogram, Fig. 6A) with station similarity as derived from the quantitative distribution of the environmental parameters listed in Table 1 using the cluster analyses function in Primer-E (Fig. 6B). The results demonstrated that the two stations with the highest measured toxin concentra-

tions (Stations 16 and Channel) clustered together in both treatments. Outside of these two sites (and station 30C), the reconstruction of relationships between different stations based on environmental parameters and eubacterial communities differed from each other.
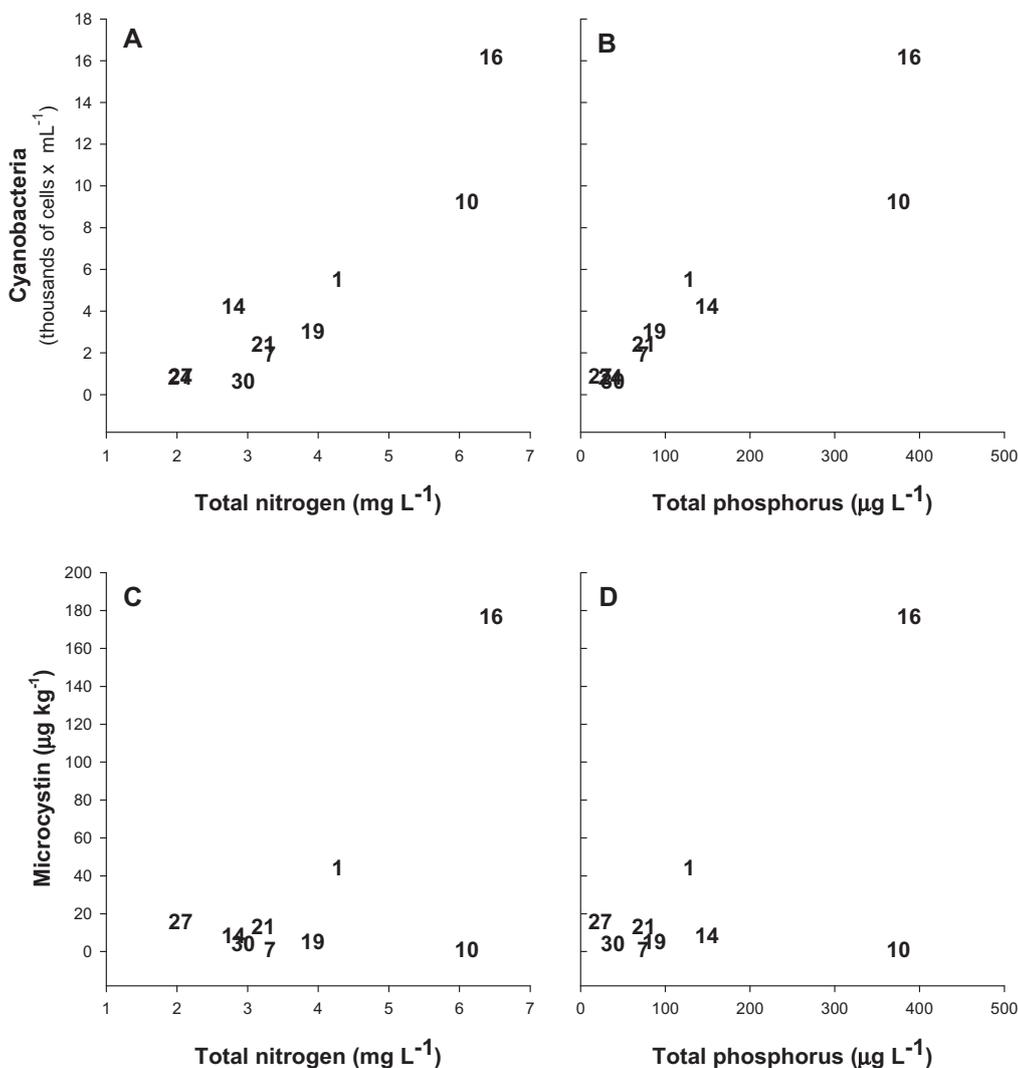
Fig. 2. The relationships between cyanobacterial abundance (cells mL^{-1}) and toxin concentrations (microcystin LR equivalents, $\mu g\,kg^{-1}$ dry weight) relative to total nitrogen or phosphorus concentrations. Numbers represent stations within the lake. The results show a strong relationship between cyanobacteria and total nitrogen concentration (A; R^2 = 0.829) as well as total phosphorus concentration (B; R^2 = 0.888). The relationships between microcystin concentrations and total nitrogen (C; R^2 = 0.381) and total phosphorus (D; R^2 = 0.367) were weaker.
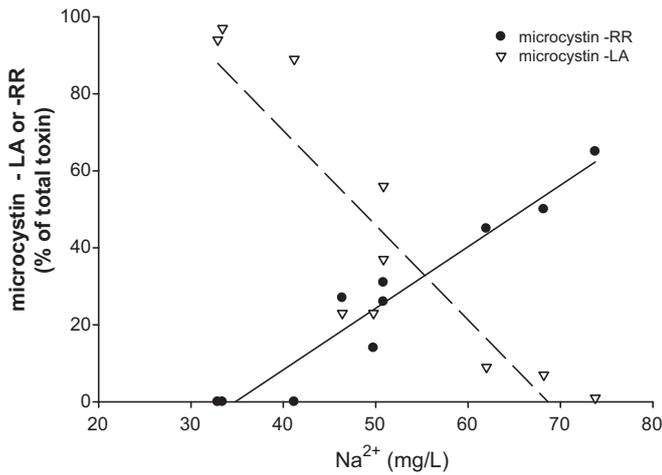
Fig. 3. The relationship between the presence of microcystin-RR and microcystin-LA and sodium ion concentrations in Taihu. As the concentration of sodium increases, the microcystin congeners switch from -LA ($p = 0.0005$) to -RR ($p < 0.0001$) in samples.

4. Discussion

The global proliferation of toxic cyanobacterial species within freshwater ecosystems is an increasing concern for scientists and environmental managers. In the current study, we surveyed a large, regionally important lake that serves as a major industrial and potable water reservoir for millions of consumers to examine how environmental factors and the endemic eubacterial community are related to the presence of hepatotoxic microcystins – likely the most widespread and problematic of the toxic cyanobacterial secondary metabolites (Carmichael, 2008). While questions

Table 3
Richness and diversity estimation (Chao1, Shannon's diversity (H'), and evenness (E)) and sequence coverage for 16S rDNA amplicons libraries from pyrosequencing analysis. As shown operational taxonomic units (OTUs) were determined at the 97% level.

Stn	Reads	OTUs	CHAO 1	H'	E
1	1280	268	499.4468	4.16	0.74
7	2304	352	746.02	3.89	0.66
10	1887	379	704.44	4.75	0.80
14	2183	480	973.7808	5.17	0.84
16	2534	429	879.7377	4.73	0.78
19	1355	251	393.3448	3.76	0.68
21	1359	309	643.4667	4.68	0.81
24	1943	375	982.1163	4.05	0.74
30C	805	175	343.8889	4.14	0.80
CH	1329	274	540.8837	4.65	0.83

concerning whether phosphorus and/or nitrogen are major drivers of algal biomass is an area of current discussion (Howarth and Paerl, 2008; Schindler et al., 2008; Scott and McCarthy, 2010), it is important to recognize that nutrient inputs driving high algal biomass are often biochemically transformed by the co-occurring bacterial community, and that this processing of materials can shape the resulting algal community. Moreover, given many algal blooms are driven by anthropogenic nutrient loads from agricultural and urban centers, we investigated whether potential pathogens and indicators of human activity from these sources to Taihu tracked either photosynthetic biomass or the dominant cyanobacterial toxin. Our results indicate that proliferation of *Microcystis* in Taihu is likely linked to loads of anthropogenically generated nitrogen and phosphorus compounds, and that there is relationship between high concentrations of microcystin and the diversity of the endemic eubacterial community.
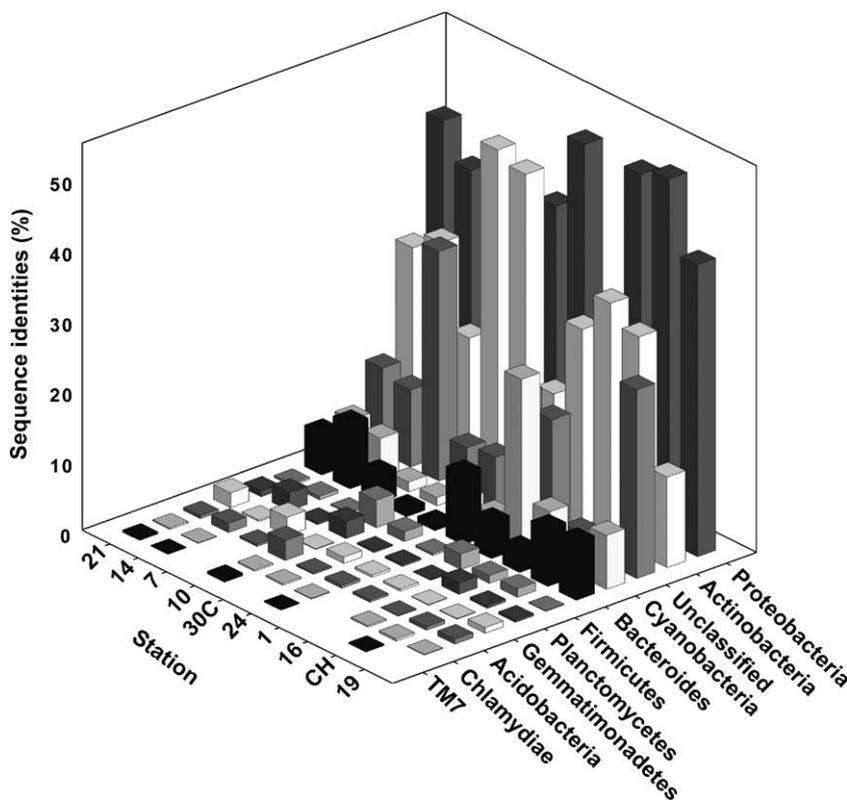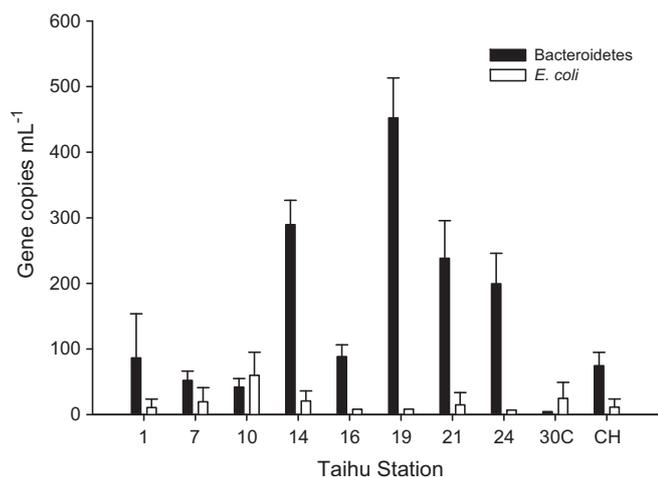

Fig. 4. The distribution of eubacterial clones across ten stations sampled as part of this study. Identities were determined using the classifier set at the Ribosomal Database Project. Unclassified sequences were denoted as such and binned together for this component.

Fig. 5. Quantification of total *Bacteroidetes* (ABAC) and *E. coli* copies mL^{-1} in samples collected from the Taihu survey during May 2009. PCR inhibition was not detected in any samples.

4.1. The relationship between environmental conditions, cyanobacterial abundance and microcystin

The seasonal proliferation of cyanobacteria in Taihu and its expansion in recent years is well documented (Duan et al., 2009; Guo, 2007; Qin et al., 2010; Ye et al., 2009b). The hypereutrophic nature of Taihu is seasonally dependent on inputs of both nitrogen and phosphorus to the system (Xu et al., 2010). While previous studies have focused on temporal changes at a location close to the Taihu Lake Laboratory Ecosystem Research Station at Meiliang Bay, here we examined the naturally occurring gradient (from north to south) in biomass, cyanobacterial proliferation and toxin production to identify those environmental parameters associated with bloom events. Our results provide an independent confirmation that both total nitrogen and total phosphorus concentrations are strongly related to cyanobacterial biomass in this system. Previous studies (Xu et al., 2010) have demonstrated that there is a seasonal pattern in nutrient limitation, with phosphorus supply controlling biomass in winter and spring, and nitrogen in summer and fall.

During this survey, two stations (16 and the Channel site) had high concentrations of total microcystin (in μg toxin per gram dry weight). Using conservative estimates of total planktonic chlorophyll a (Table 1) and the concentration of chlorophyll in the biomass used for toxin analyses, the microcystin concentrations at the most toxic sites (Channel – 6.3 μg L^{-1}; Station 16–1.8 μg L^{-1}) were above the established World Health Organization limits on drinking water (1 μg L^{-1}). While higher concentrations of microcystin have been observed in large lakes around the world (Carmichael, 2008) as well as previously in Taihu later in the season (Shen et al., 2003), these concentrations early in the season remain a concern because the lake serves as a direct source of drinking water on a year-round basis (Qin et al., 2010).

Other common cyanobacterial toxins, including anatoxin-a, cylindrospermopsin and BMAA were not observed in these samples. This is a reflection of both the relatively rare occurrence of these toxins outside of specific locations (Boyer, 2008) and the dominance of *Microcystis* spp. in the phytoplankton community of Taihu.

While no relationship between surface temperature and toxin concentrations was found in this study, the surface temperatures at the two most toxic stations were ∼4 °C warmer than the low toxicity stations (Fig. S3) with the exception of station 14. Previous observations have suggested blooms in Taihu occur during the warmest periods of the year (Ye et al., 2009b) and prior studies have suggested that increases in surface temperatures of freshwater systems may lead to increased proliferation of toxic cyanobacterial populations and/or increases in toxin production (e.g., Paerl and Huisman, 2008). Lake Erie surveys have suggested that toxic *Microcystis* cells and toxin concentrations are also higher in warmer months (Rinta-Kanto et al., 2009a). Controlled laboratory studies have further demonstrated that growth rates of *Microcystis* spp. appear to peak at 26–28 °C (Konopka and Brock, 1978; van der Westhuizen and Eloff, 1985), although this relationship likely varies depending on the cyanobacterial strains that are present. In mesocosms located in smaller lake systems of New York (USA), Davis and colleagues (Davis et al., 2009) demonstrated that shifts in temperature elevate the number of genes (*mcyD* genes per sample), an observation consistent with the observation of Kim et al. (2005) where *mcyB* transcripts were higher at 25 °C than at either 20 °C or 30 °C. Combined, these and other studies suggest that changes in cellular growth, gene transcription,
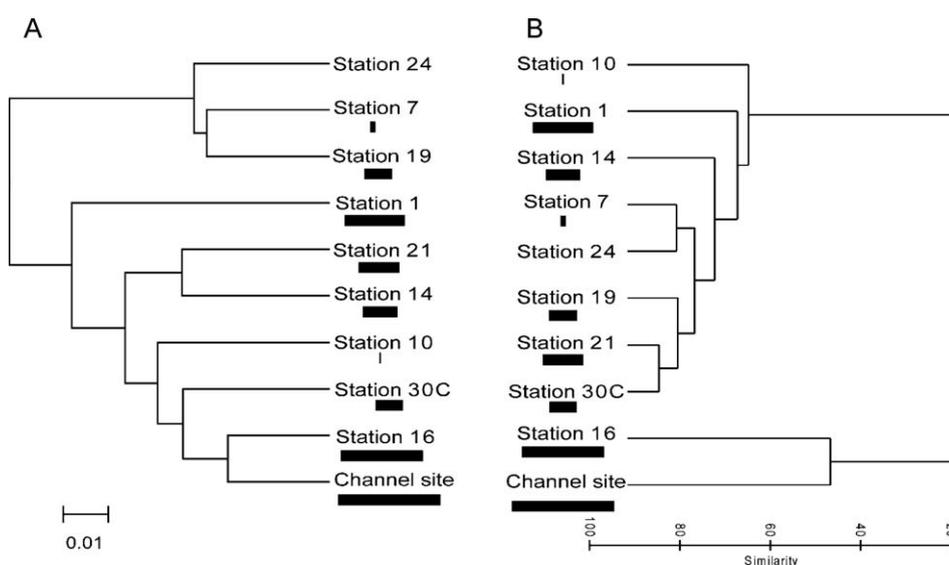
Fig. 6. Similarity matrices for stations in Taihu. (A) Eubacterial diversity. The scale bar shows the distance between clusters in UniFrac units: a distance of 0 means that two environments are identical and a distance of 1 means that two environments contain mutually exclusive lineages (B). Water column chemistry and environmental parameters as determined by a similarity analysis (percentage) in Primer-E. Each sample is denoted by its corresponding station number and accompanied by a horizontal bar proportional to the concentration of microcystin below the label.

and the production of toxin by *Microcystis* populations may be responding to a temperature threshold. While it should be noted that our data demonstrate that this relationship is not absolute (since there was little toxicity at station 14), they suggest that surface temperatures maybe one of several combined environmental queues. Future shifts in climatic conditions (including warming) may result in even more sudden and dramatic increases in toxic cyanobacterial blooms than previously predicted.

One surprising relationship we observed in this study was with the distribution of toxin types (specifically microcystin-RR and -LA) with respect to sodium concentrations (Fig. 3). A strong inverse relationship between two microcystin congeners was observed, where microcystin-RR increased in contribution to the total toxin pool with Na concentration, while microcystin-LA significantly decreased. No effects were seen on the other microcystin variants, and no noteworthy relationships existed with other cations. Currently, we do not have an explanation for this relationship. It may arise from a differential effect of sodium on the two different polyketide synthase or polypeptide synthetases responsible for the biosynthesis of microcystin-LA and -RR. It may however, also indicate the presence of strains of *Microcystis* with differential toxin patterns and sensitivities to sodium. Finally, it is also possible that sodium may be acting as a tracer of terrestrial or anthropogenic inputs. As we continue to explore this relationship, it is worth noting here as it may arise (and be overlooked) in other data sets.

4.2. The relationship between eubacterial diversity, microcystin and environmental conditions

By employing pyrosequencing to resolve 16S rDNA amplicon sequences, we have dramatically increased the resolution as well as spatial coverage of the eubacterial community. Wu et al. (2007) were amongst the first to examine the phylogenetic composition of heterotrophs in Taihu. Their study, conducted using classic Sanger-style sequencing, shed some light on the diversity of members of the community, but suffered from an under sampling of sequence diversity ($\sim$330 clones). Their efforts suggested significant coverage (60–84%) of the community with 30–50 OTUs present. Although we sequenced shorter reads, we detected a larger number of OTUs. However, in spite of this increased coverage, rarefaction analyses suggested we under-sampled community diversity (Fig. S2).

It is also interesting that our observations on richness are much higher than previously reported (Wu et al., 2007). This was likely due to increased coverage of rarer sequence types achieved using pyrosequencing (Fig. S1). The organisms responsible for these rarer OTUs remain unidentified. It is possible they are populations that were present at low density or were dormant during sampling. Even though this study generated a large number of amplicons sequences, this represented only $\sim$0.0002% of the population estimated to be present in a milliliter of water for each station. To this end, sequences appearing just once in our libraries may appear many more times in nature. Conversely these sequences may represent evolutionary "dead ends" or sequences persisting in recently generated cells that are not functional but that were captured during our survey. Continued advances in high-through-put sequencing, leading to increased coverage of individual amplicon libraries, will shed additional light on this question.

Given the above caveats, it was interesting to note that the stations which grouped together based on environmental conditions and toxicity also grouped together based on the UniFrac distance. No other relationship between eubacterial diversity, environmental conditions (that we measured) or toxin concentrations were observed. Further, the relationships between stations were independent of the presence of cyanobacterial sequences within libraries (data not shown). While the relationship between environmental conditions and the microbial community is generally easy to explain, the relationship between microbial community richness and the concentration of the toxin remains less obvious. We can hypothesize that the presence of the toxin may allow certain bacterial types to degrade the toxin as a potential carbon source (Jones et al., 1994). That said, it may also be that these variables are responding to different environmental queues and simply co-varied during our observations.

Within this data set, we also examined for the presence of bacteria arising from fecal sources. Bacteroidetes concentrations in this study were similar to those reported in rivers from Japan (up to 10^4 copies/100 mL) (Savichtcheva et al., 2007), and slightly lower that the concentrations detected by direct PCR (10^3 to 10^4 copies mL^{-1}) in a creek in Tennessee (Bell et al., 2009). However the concentrations detected in this study were more than 100 fold lower than those measured in fecally contaminated surface waters in Bangladesh ($>1 \times 10^5$ genes mL^{-1}) (Knappett et al., 2010), which has a very dense human population and poor sanitation. The correlation between *E. coli* concentrations and ammonium, phosphate and nitrite concentrations in the water samples suggest an anthropogenic origin for both *E. coli* and nutrients. However, the presence of *E. coli* and other pathogens could not be tied to recent human fecal contamination in the current study using our quantitative molecular probes. This apparent discrepancy may arise from decay rates of fecal *Bacteroides* and *E. coli* in surface water with *Bacteroides* removal being considerably faster than *E. coli* (Bell et al., 2009; Okabe and Shimazu, 2007) or may reflect a "nonhuman" anthropogenic source of both nutrients and bacteria. Taihu has a large poultry industry (Guo et al., 2004) and future source tracking may help identify the contribution of these industries to the lake ecology: indeed the strong relationship between these microbes and ammonium concentrations is consistent with wastes from the poultry industry. Overall, while it remains apparent that anthropogenic nutrients are the likely drivers of the massive blooms seen in Taihu, human biological pollution appears to be of lower concern.

The documented global increase in frequency and persistence of blooms of potentially toxic freshwater cyanobacteria continues to alarm researchers and environmental managers. In the case of Taihu, the annual reoccurrence, duration (up to 8 months per year) and toxicity levels of these events suggest that factors initiating and regulating these events are complex. Our results confirm previous studies in Taihu and many lakes worldwide, demonstrating that both nitrogen and phosphorus inputs play a role in microbial community biomass production and structure. They also demonstrate that the toxicity of the community is not closely coupled to key factors leading to bloom formation. Future research, undoubtedly complicated by changes in climate and water use (Paerl and Huisman, 2008), need to address these interactive issues before ecosystem scale models can be used as effective and reliable management tools.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2010.10.001.

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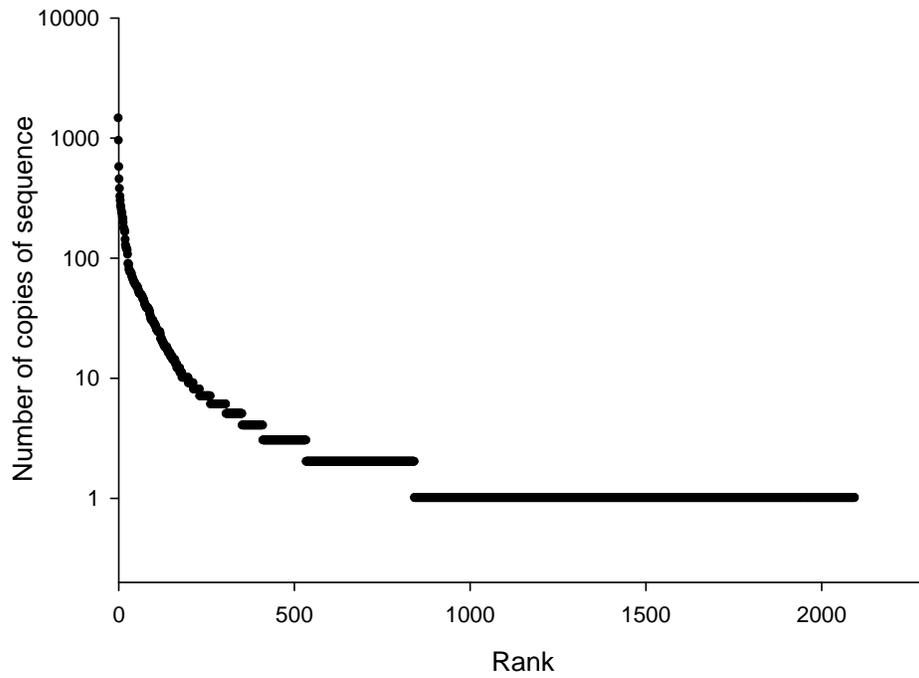
Supplemental Fig. 1 Rank abundance curve for sequences collected across all libraries. In total 843 OTUs were identified 2 or more times across all libraries while 1253 sequences were unique.
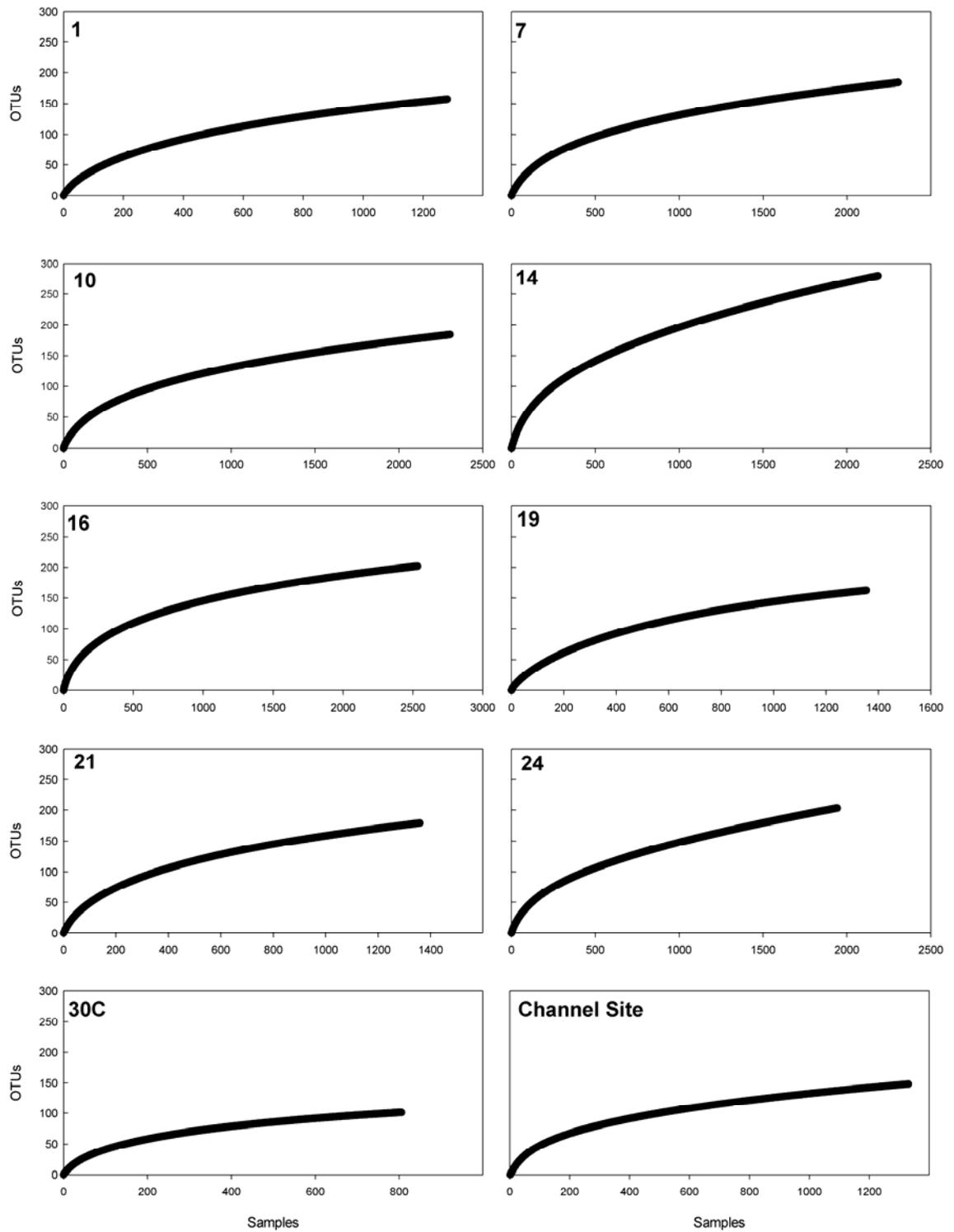
Supplemental Fig. 2 Rarefaction curves (based on 97% identity) for sequences collected at stations in Taihu.
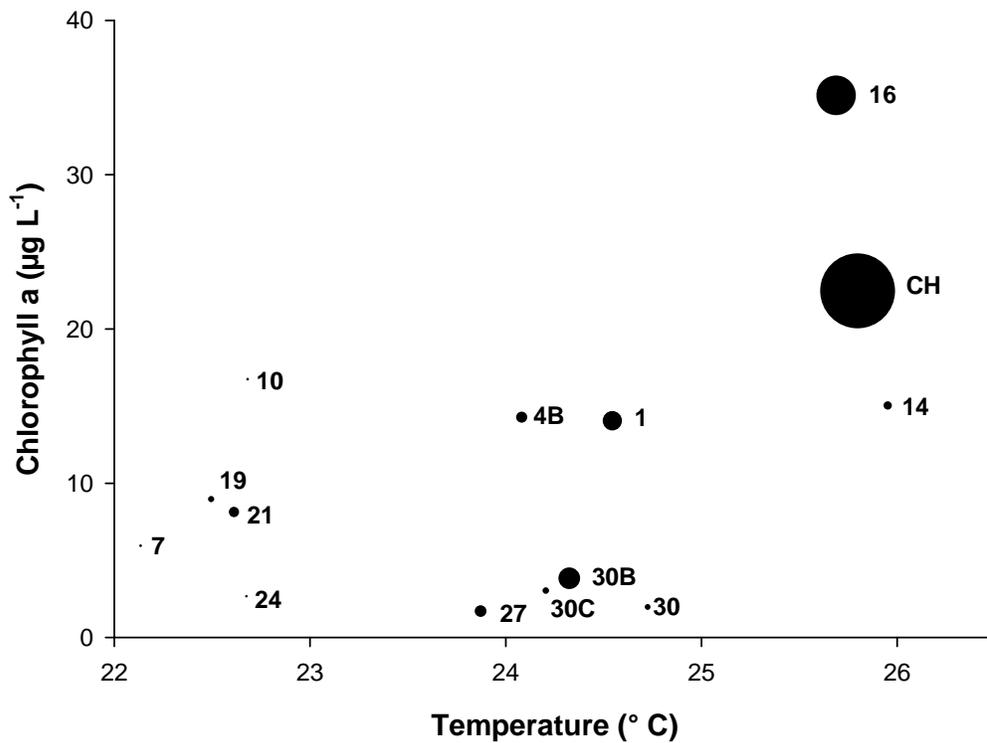
Supplemental Fig. 3 Bubble plot showing temperature vs chlorophyll concentrations for stations. Bubble size is proportional to toxicity (µg/kg dry weight microcystin) and each bubble is annotated with its corresponding station number.