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ORIGINAL ARTICLE

Seasonal morphological variability in an *in situ* Cyanobacteria monoculture: example from a persistent *Cylindrospermopsis* bloom in Lake Catemaco, Veracruz, Mexico

Owen LIND,[1*] Laura DÁVALOS-LIND,[1] Carlos LÓPEZ,[2] Martín LÓPEZ,[3] Juli DYBLE BRESSIE[4]

[1]Center for Reservoir and Aquatic Systems Research and Department of Biology, Baylor University, Waco, TX, 76798 USA; [2]Zooplankton Laboratory, Department of Biology, University of Zulia, Maracaibo, Venezuela; [3]Laboratory of Marine Pollution, Institute of Marine Sciences and Limnology, National Autonomous University of Mexico, Circuito Exterior S/N, Ciudad Universitaria, Coyoacán, 04510 México D.F., Mexico; [4]NOAA Northwest Fisheries Science Center, 2725 Montlake Blvd E., Seattle, WA 98112, USA
*Corresponding author: owen_lind@baylor.edu

ABSTRACT

The phrase cyanobacteria bloom *implies a transient condition in which one to few species dominates communities. In this paper we describe a condition in which the* bloom *is of multi-year duration consisting of different morphologies of a single cyanobacteria species. Lake Catemaco, Veracruz, México maintained a year-round massive (10^8 trichomes L^{-1}) population of potentially toxin-producing cyanobacteria,* Cylindrospermopsis spp. *The trichomes are present as straight and coiled morphotypes. The relative trichome morphology abundance varied with rainy (June-October) and dry seasons (November-May), but total trichome abundance did not vary. Coiled trichomes and heterocytes (occurring only on coiled trichomes) were significantly more abundant, both absolutely and relatively, during the dry season. Both coiled trichome and heterocyte mean volumes were significantly smaller during the rainy season than during the dry season. Biovolumes were largest in January when water temperature was 5°C cooler suggesting buoyancy as a morphology-determining factor. However, with a more than three-fold lower TIN concentration during the dry season, we hypothesized that the coiled morphotype became abundant primarily because it formed heterocytes, which the straight morphotype did not. Spatial trichome and heterocyte abundance differences were small among the 15 lake sites (average CV for all dates: 20%). However, there was a pattern of increased heterocyte and coiled trichome abundance from lake inflow, as a nitrogen source, to outflow during the rainy season. The total volume of heterocytes per litre of lake water increased progressively four-fold from a minimum early in the rainy season to a maximum at the end of the dry season. Morphological diversity, as seen in Lake Catemaco, can partially compensate for the lack of species diversity in determination of community structure.*

Key words: Cylindrospermopsis*; heterocyte; seasonality; Cyanobacteria bloom; trichome morphotypes.*

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INTRODUCTION

Diversity, a property of stable, well-functioning communities, provides resilience that responds to natural and anthropogenic perturbations (Cardinale *et al.*, 2012). Of the two diversity components, richness and evenness, the latter is more relevant to community function in the near-term (Hildebrand *et al.*, 2008). Near-monocultures, or blooms, especially represent the lack of evenness. Nevertheless, morphological diversity within near-monocultures may be able to provide functional diversity and community stability.

Harmful algal blooms (HABs) are a frequent consequence of nutrient-driven eutrophication. The public and aquatic professionals alike are concerned with HABs and their consequences (Backer, 2002; Hudnell, 2008). This is undoubtedly coupled to increased bloom frequency in temperate regions - a possible climate change consequence (Wiedner *et al.*, 2007; Briand *et al.*, 2004; Paerl and Huisman, 2008; Pearl *et al.*, 2011). *Cylindrospermopsis* spp. are tropical or sub-tropical potentially toxin-producing cyanobacteria with increasing temperate region occurrence (Fastner *et al.*, 2007; Jones and Sauter, 2005; Padisak, 1997). Considering this physiological tolerance, its documented temperate invasions, and the presence of human and animal health-endangering toxins, it is important to gather the basic ecological, physiological, and systematic data if management strategies to reduce its occurrence and dominance are to be effective. Lake Catemaco, México, which has high *Cylindrospermopsis* spp. abundances all year, provides an unusual opportunity for research of predictive value important to assessing this organism's temperate zone range expansion. One aspect of needed research is determining factors contributing to expansion and dominance of cyanobacteria populations under certain conditions or in particular ecosystems. The supply of growth-determining nutrients and their ratios have long been cited to determine cyanobacteria abundance (Smith, 1983; Downing *et al.*, 2001). A feature of some planktonic cyanobacteria is the formation of hete-

rocytes that provide an anaerobic environment enabling diatomic nitrogen fixation. With this capability, cyanobacteria can dominate the community when other phytoplankters are growth-limited by nitrogen deficiency. Factors other than nutrient concentrations and ratios have been suggested as promoting blooms. These include temperature, pH, carbon supply, intensity of herbivory, lack of turbulence and particularly, in larger species, the buoyancy provided by gas vacuoles causing *apparent blooms* on the surface (Urrutia-Cordero et al, 2015; Beaulieu *et al.*, 2014; Reynolds, 2006). Such an advantage, and the subsequent growth of cyanobacteria populations, has many ecosystem consequences. One of the more significant consequences is altered trophic dynamics with effects ranging from reduced light penetration (especially by buoyant species) reducing water column photosynthesis (Tilzer, 1987), to reduced, or pathway-altered, trophic transfer (Soranno *et al.*, 2014; Ferrao-Filho and Kozlowsky-Suzuki, 2011). *Cylindrospermopsis* spp. trichomes are dimorphic, as in Lake Catemaco (Fig. 1), capable of heterocyte formation, and, at times, of toxin production. In this paper we report the seasonal and spatial dissimilarity of *Cylindrospermopsis* trichome and heterocyte characteristics in Lake Catemaco and link these dissimilarities to seasonal nitrogen supply.

Our objectives are: i) to describe the morphologies and the relative abundance changes of different trichomes and trichome-associated heterocytes; ii) to use field data to associate the relative trichome abundance changes with tropical rainy and dry season environmental factors, especially nutrient availability; iii) to confirm the identity of *Cylindrospermopsis* spp. in Lake Catemaco using morphological and molecular approaches.

METHODS

The study lake

Lake Catemaco, Veracruz, México (18°24' N, 95°04' W; elevation: 334 m) (Fig. 2) is physically simple and described as a *simple, rounded shallow bowl (elliptic parabola) without complex features* (Komárková, 1998) (Tab. 1). Inflow is from two un-gauged perennial rivers (Rios Cosamaloapan and Margaritas) in the east-southeast corner (near sampling site 8) (Fig 2) with seasonal inflows from small drainages along the eastern shore (near sampling site 4). Outflow is diagonally across the lake at the northwest corner (near sampling site 5). A major factor affecting the lake's ecology is the pattern of intense rainfall from June through September, which accounts for approximately 80% of annual precipitation, followed by a period of almost no rainfall (Fig. 3). Lake elevation is maintained nearly constant (±0.5 m) by a small dam. The lake is classified (Carlson's Trophic State Index for chlorophyll, Secchi depth, and total phosphorus) as highly eutrophic or hypereutrophic (TSI_{chl}=73, TSI_{SD}=69, TSI_{TP}=96). The lake is polymictic and phyto- and bacterioplankton are more or less uniformly distributed vertically. Secchi visibility is consistently low (seasonal and spatial range from 0.5-0.6 m).

Previous studies have described Lake Catemaco's plankton (Torres-Orozco and Zanatta (1998), Torres-Orozco and Pérez-Rojas (2002), Komárková and Tavera (1996) and Tavera and Castillo (2000)). Komárková and Tavera (1996) reported two *Cylindrospermopsis* species - *C. philippinensis* (Taylor) Kom. (1984), and the more abundant newly described species, *C. catemaco* Kom-Legn and Tavera (1996) - for which this is the type locality (Komárková and Tavera, 1996, 2003). Based on our studies, described below, we question the appropriateness

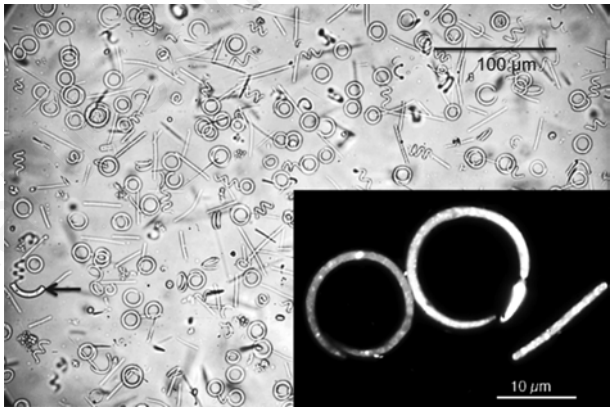

Fig. 1. Dimorphic *Cylindrospermopsis trichomes* from Lake Catemaco, Veracruz, México. Inset image is acridine orange stain preparation at high (100X objective) magnification showing straight and coiled morphs with and without a heterocyte. Larger image is Lugol's preserved at low (10X objective) showing near monoculture of the organism and constancy of trichome lengths. Arrow indicates an infrequent alternate species, *Aulacoseira granulata*.

Tab. 1. Physical properties of Lake Catemaco and catchment, Veracruz, México. From Pérez-Rojas and Torres-Orozco (1992) and Komárková (1998).

Lake	
Area	72.5 km^2
Vol	0.55 km^3
Z_{mean}	7.6 m
Z_{max}	21 m
Catchment area/lake area	3.3
Land use	
Agriculture and livestock	57%
Jungle	10%
Urban	2.5%

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of these identifications. For the objectives of this paper the correct identification of the Lake Catemaco species is less important than trichome morphology.

Sampling

We sampled 14 sites in May 2004 and 15 sites in January 2005, September 2005, and July 2006 (Fig. 2). Collections for phyto- and bacterioplankton were taken from the surface to 4.5 m with a rigid PVC integral tube (6.5 L capacity) sampler. The tube sample was emptied into a plastic container and mixed before sub-sampling. We consider that this integral sample adequately represented the water column of the well-mixed lake. Even at Site 1, the deepest (22 m), we found little vertical difference in any limnological property other than light. Mixed water sample temperature was taken immediately with a digital thermometer. One liter for nutrient analyses (except May 2004) and one liter for chlorophyll analysis were placed on ice in a darkened chest. Five milliliters were pipetted into 20 mL vials containing 5 mL of 4% formalin (2% final concentration) for bacterioplankton and *Cylindrospermopsis* microscopic analyses.

Laboratory

Genetic sequence analysis was conducted for both *Cylindrospermopsis* isolates established in culture (grown with and without nitrogen) and for environmental samples taken from Lake Catemaco to genetically characterize the *Cylindrospermopsis* and determine the degree of similarity among populations during different seasons and years. DNA was extracted from lake samples in September 2005, March 2007, June 2009, July 2009, October 2009 and January 2010, as described below. PCR amplification and sequencing of the *nifH* gene was performed for all dates and of the *cpcBA*-IGS region for all except July and October 2009. Samples were filtered (0.8 µm membranes (Supor, Pall Life Sciences) and frozen (-20°C) until DNA extraction as described previously (Dyble *et al.*, 2008).

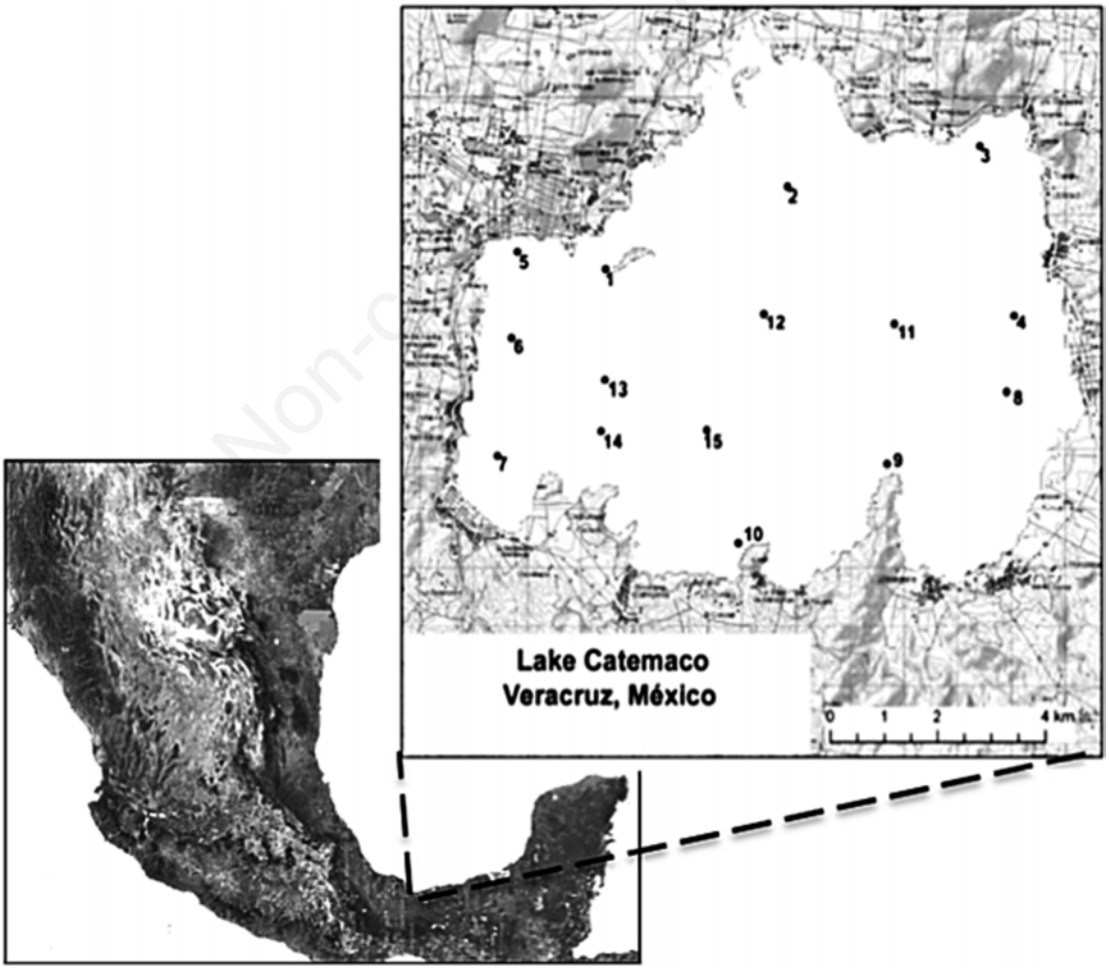

Fig. 2. Lake Catemaco location and sampling sites.

Briefly, cells were lysed by adding guanidine thiocyanate-based DNAzol (MRC, Cincinnati, OH, USA) to the tube containing the filter, heated (90°C) for 3-4 h and subjected to two rounds of bead beating (3 min each time using 150-200 µm glass beads). DNA was recovered by chloroform extraction and 70% ethanol precipitation and further purified using the DNeasy Plant kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. A negative control, with no DNA added, was run with every extraction set to ensure no carryover of DNA between samples.

Published primer sets and reaction conditions were used to PCR amplify each gene of interest using a C-1000 thermocycler (BioRad, Hercules, CA, USA), running negative controls with each set of PCR amplifications. The primers used were PCβR and PCαF for *cpcBA*-IGS (Neilan *et al.*, 1995), cyl2 and cyl4 for *rpoC1* (Wilson *et al.*, 2000), cyanonifF and cyanonifR for *nifH* (Olson *et al.*, 1998) and 16C and 23C ITS for ITS1-L (Neilan *et al.*, 1997). Amplification products were ligated into a pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA), transformed into *E. coli* INVαF' ultracompetent cells (Invitrogen). For each lake sample, five to six clones were sequenced in both forward and reverse directions with Big Dye terminators, following manufacturer's instructions (Applied Biosystems Inc., Foster City, CA, USA), using a 3100 Genetic Analyzer (Applied Biosystems Inc). Sequences were aligned manually and phylogenetic trees generated with the Neighbour-joining method and p-distance to compare sequence differences using MEGA4 software (Tamura *et al.*, 2007). These sequences were also compared with *Cylindrospermopsis* spp. and other closely related cyanobacterial sequences from GenBank to assess whether strains from Lake Catemaco were genetically similar to other *Cylindrospermopsis raciborskii* strains or whether they were genetically differentiated into separate species.

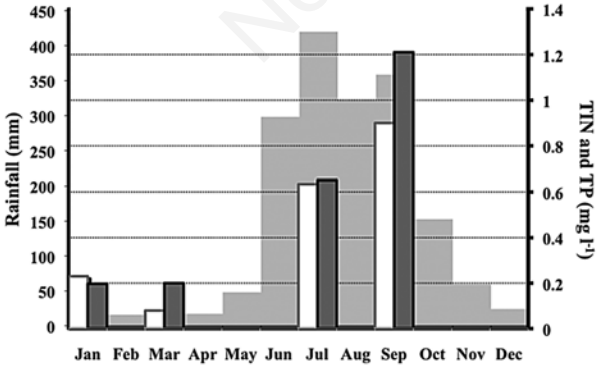

Fig. 3. Mean monthly precipitation (grey shading) showing the pronounced four-month rainy season and six month dry season. Diagonal bars show association of nutrient concentrations with seasons. Left white bar of each pair is total inorganic nitrogen (NO_3-N+NO_2-N+NH_3-N) and right bar is total phosphorus concentration. Rainfall data: thirty year Veracruz average (1951-80) (Comisión Nacional del Agua).

Samples for chlorophyll and chemical analyses were filtered (GF/F) soon after collection in a lakeside laboratory. Chlorophyll filters were frozen until 90% acetone extraction at Baylor University, Waco, TX. The extracts were clarified by centrifugation and chlorophyll *a* absorbance at 750, 665, 645, and 630 nm in the extract was measured on a Beckman 650 spectrophotometer. Chlorophyll *a* concentration was calculated according to the trichromatic spectrophotometric method (Lind, 1985). *C. raciborskii* were counted and measured by AODC epifluorescence microscopy (Nikon ES600) (Hobbie *et al.*, 1977) and digital images captured. This method was used because it permitted counting and measuring of both heterotrophic bacteria and *C. raciborskii* from the same slide preparation. A minimum of 70 random fields were photographed. *C. raciborskii* morphotypes were counted separately and coiled trichomes and heterocytes were measured by image analysis (Image-Pro-Plus). Three heterocyte dimension measurements were made: i) greatest diameter;, ii) distance from greatest diameter to apex of cone; and ii) distance from greatest diameter to base of spherical segment attached to remainder of trichome. Heterocyte volume was the sum of the cone volume and spherical segment volume. The trichome length and diameter of coiled trichomes were measured and volume calculated assuming cylindrical form. Data were examined for normality and, if appropriate, were log transformed. Differences in data among sites or among sampling seasons were tested by ANOVA using Tukey-Kramer HSD test at $P<0.05$ (SAS software JMP). The Lorenz Dissimilarity Index (LDI) (Damgaard and Weiner 2000), where LDI=0 indicates identical communities and LDI=1 indicates perfectly dissimilar communities, was used to describe heterogeneity among sites.

Nitrogen and phosphorus analyses were performed primarily in Mexico. Water for soluble nitrogen and total phosphorus analysis was transported in an insulated chest to the limnology laboratory at the Instituto de Ciencias del Mar y Limnología, UNAM, in México City. TP was determined by the PhosVer-3 Hach method following acid persulfate digestion (USEPA compliant method 8190), ammonia nitrogen by direct Nesslerization, and nitrate nitrogen by the cadmium reduction method (USEPA compliant method 8039) using Hach NitraVer 5.

RESULTS

Genetic similarity among nitrogen-determined phenotypes

Functional genes, *rpoC1* and *nifH* are the more conserved of the four genes assessed while the intergenic spacer regions *cpcBA*-IGS and ITS1-L are expected to

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have a higher capacity for strain differentiation due to mutation accumulation in non-coding regions. For the clones sequenced for *nifH* and *rpoC1,* the morphotypes were ≥99.7% similar at the nucleotide level, with most identical (Tab. 2). Clones sequenced for *cpcBA*-IGS had ≥99.6% similarity and ITS1-L clones had ≥ 99.1% similarity at the nucleotide level. Similarity at the amino acid level was ≥99% for all transcribed genes sequenced.

The *nifH* cultured strain sequence was in lake samples all six dates. For all dates, except June 2009 and January 2010, this culture sequence was the only *nifH* sequence isolated from the lake. For the other two dates, five additional strains were isolated from Lake Catemaco (three in June 2009 and two in January 2010) that were 99.7% similar to a cultured isolate and only in one of these five clones did the variable nucleotide result in amino acid change. The rest were silent polymorphisms not impacting gene expression. The samples also were analyzed by *cpcBA*-IGS and sequence similarity between samples from all dates and the culture sequence was 99.7-100%. The *Cylindrospermopsis* of Lake Catemaco composition was genetically very similar or identical from September 2005 to January 2010.

Phytoplankton mass (as chlorophyll a) and nutrients

Lake-wide plankton chlorophyll *a* concentrations were high and seasonally variable with a winter (dry season) minimum of 53 µg L^{-1} to almost 90 µg L^{-1} in July (rainy season) (Tab. 3). Spatial dissimilarity (Tab. 4) was small, but was greatest during the winter dry season

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Tab. 2. Percent similarity between *C. raciborskii* isolates from Lake Catemaco. Maximum and minimum values are given for each of the four genes sequenced, comparing both at the nucleotide and protein level. The total number of clones sequenced and the number sequenced from the +N and –N culture treatments are also provided.

	Max % Similarity		Min % Similarity		Total #		# clones
Gene	nt	aa	nt	aa	#bp	clones	+/- N
nifH	100	100	99.7	99.2	373	7	4/3
cpcBA-IGS	100	100	99.6	99.5	703	11	5/6
rpoC1	100	100	99.7	99	309	8	3/5
ITS1-L	99.8	n/a*	99.1	n/a*	547	6	4/2

*#bp, number of base pairs in sequence.*ITS1-L is a non-transcribed spacer region. nt, nucleotide. aa, protein.*

Tab. 3. Concentrations of chlorophyll *a* (µg L^{-1}), total phosphorus (mg L^{-1}), and total inorganic nitrogen (mg L^{-1}) by sampling site and month (no nutrient data for May). Upper integral water temperature in parentheses.

Site	May-04 (28°C)	Jan-05 (23°C)			Sep-05 (28°C)			Jul-06 (29°C)		
	Chl	Chl	TP	TIN	Chl	TP	TIN	Chl	TP	TIN
1	77.5	56.4	0.12	0.50	61.1	5.4	1.11	82.7	0.44	1.32
2	91.5	57.5	0.11	0.20	64.8	1.56	0.43	95.6	0.62	0.98
3	93.2	61.6	0.14	0.30	75	0.6	0.86	94.4	0.53	0.67
4	94.1	51.5	0.26	0.11	70.7	0.59	0.44	85.6	0.57	0.41
5	82.0	38.7	0.09	0.24	69.8	0.86	0.66	85.5	0.45	0.42
6	80.3	36.2	0.11	0.13	61.7	1.13	0.86	85.8	0.48	0.78
7	86.9	47.5	0.07	0.14	53.9	0.48	0.92	77.7	0.77	0.41
8	95.1	57.4	0.10	0.14	67.2	0.81	0.81	87.9	1.35	0.88
9	86.6	56.9	0.14	0.15	62.5	1.83	1.13	89.4	0.48	0.53
10	88.1	45.3	0.08	0.35	62.2	0.56	1.56	95.7	0.55	0.48
11	82.7	56.3	0.89	0.45	72.9	0.78	0.98	99.7	0.42	0.47
12	90.3	58.4	0.40	0.25	65.2	0.25	1.12	96.0	0.4	0.52
13	96.6	56.1	0.27	0.15	64.8	2.15	0.84	88.0	0.62	0.62
14	80.8	59.3	0.10	0.14	63.9	0.66	0.89	89.1	0.73	0.53
15	ND	56.2	0.07	0.18	71.1	0.51	0.88	87.5	1.39	0.51
Mean	88.3	53	0.2	0.23	65.8	1.21	0.9	89.4	0.65	0.63
CV (%)	6	14	108	53	8	105	31	7	48	40
LDI	0.03	0.06	0.35	0.21	0.03	0.29	0.07	0.03	0.17	0.15

CV, Coefficient of variation; LDI, Lorenz Dissimilarity Index, where 1.0=maximum dissimilarity; ND, not determined.

(among site LDI=0.06). Both TP and TIN concentrations were much greater during the rainy season (Fig. 3). For all dates, the TIN:TP ratio was approximately one or less. Nutrient (TP and TIN) concentrations were spatially and seasonally dissimilar (Tab. 4). TP had greater dissimilarity among stations than did TIN and, as for chlorophyll, both were greatest in January.

C. raciborskii morphotype and heterocyte abundances

Trichomes were dimorphic; *i.e.*, circular (including semi-circles) or straight (Fig. 1) (a helical morphology was infrequent) and present in different proportions at different times of the year. Total *C. raciborskii* trichome abundance was large and did not differ among dates (Tab. 5). Lake-wide mean total abundances ranged from 3.9×10^8 L^{-1} in the rainy season to 4.6×10^8 L^{-1} in the dry season with an annual mean of 4.4×10^8 L^{-1}. Site 4 had the greatest seasonal variation in total abundance with a range of from 3.0×10^8 L^1 in September to 6.9×10^8 L^{-1} in January. *C. raciborskii* dominated the phytoplankton at all sites and seasons (including numerous rainy and dry season samples from one to five selected sites from 2004 to 2009). Other phytoplankton were infrequent comprising approximately 2% (by number) of the phytoplankton (Mora Heredia, 2015) and consisted principally of the diatom, *Acaulosiera granulata curvata*, the green algae, *Pediastrum* sp., *Scenedesmus* sp., and *Synechococcus* sp. Coiled trichome abundance relative to total trichomes ranged from 18% at the start of the rainy season to 40% at the end of the dry season. Annual mean heterocyte abundance was 2.4×10^7 L^{-1} with a seasonal lake-wide range from rainy season low of 1.2×10^7 L^{-1} to a dry season high of 4.5×10^7 L^{-1} (Tab. 5). Heterocytes occurred only on coiled trichomes where their lake-wide average frequency ranged from 14% in the rainy season (July) to 25% at the end of the dry season (May) (annual mean=18%). Annually the volume of heterocytes per litre of lake water averaged 5.1 μm^3 L^{-1} with a lake-wide range from 2.4 to 9.7 μm^{-3} L^{-1} for the rainy and dry seasons respectively (Tab. 5). The lake mean total heterocyte volume per unit volume of lake water

Tab. 4. Spatial dissimilarity among sampling sites by season expressed as Lorenz Dissimilarity Index where zero is total similarity (equal amount at each site) and one is maximum dissimilarity (present at only one site).

Variable	May-04	Jan-05	Sep-05	Jul-06
Heterocyte number	0.07	0.15	0.15	0.25
Total trichomes	0.07	0.10	0.05	0.06
Straight trichomes	0.16	0.12	0.05	0.06
Coiled trichomes	0.10	0.11	0.08	0.01
Chlorophyll *a*	0.03	0.06	0.03	0.03
Total phosphorus	ND	0.36	0.29	0.17
Total inorganic nitrogen	ND	0.21	0.07	0.15
Secchi depth	0.04	0.06	0.03	0.05

ND, not determined.

Tab. 5. *C. raciborskii* mean ±SD total and coiled trichome (10^8 L^{-1}) and heterocyte (10^7 L^{-1}) abundances. Mean ±SD coiled trichome frequency as percent of total trichomes, mean ±SEM coiled trichome and heterocyte length, mean ±SEM coiled trichome and heterocyte volume (µm3), and mean ±SEM total heterocyte volume (10^7 μm^3 L^{-1}) by season in Lake Catemaco.

		Coiled trichomes		
Total trichomes	N	%	Len.	Vol.
		May (end of dry season)		
4.5 ± 0.9^{A}	1.8 ± 0.5^{A}	40±5.2	45.1 ± 0.8^{B}	62 ± 2.8^{B}
		Jan (early dry season)		
4.6 ± 1.1^{A}	1.4 ± 0.4^{B}	30±6.2	50.6 ± 1.7^{A}	85 ± 6.1^{A}
		Sept (end of rainy season)		
3.9 ± 0.6^{A}	1.1 ± 0.3^{BC}	28±6.6	41.3 ± 1.2^{BC}	51 ± 2.5^{C}
		Jul (early rainy season)		
4.5 ± 0.7^{A}	0.8 ± 0.2^{C}	18±3.7	39.6 ± 0.9^{C}	50 ± 2.8^{C}
Heterocytes				
	N	Len.	Vol.	Total Vol.
		May (end of dry season)		
	4.5 ± 0.8^{A}	4.1 ± 0.05^{A}	2.1 ± 0.1^{B}	9.7 ± 0.8^{A}
		Jan (early dry season)		
	2.0 ± 0.9^{B}	4.1 ± 0.08^{A}	2.6 ± 0.1^{A}	5.2 ± 1.0^{B}
		Sept (end of rainy season)		
	1.8 ± 0.9^{B}	3.9 ± 0.08^{A}	1.9 ± 0.1^{B}	3.2 ± 0.5^{B}
		Jul (early rainy season)		
	1.2 ± 0.7^{B}	3.7 ± 0.07^{B}	2.0 ± 0.1^{B}	2.4 ± 0.6^{B}

$^{A–C}$*Values with the same uppercase letter are not significantly different (ANOVA, P< 0.05).*

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was almost two-fold greater at the end of the dry season than early in the dry season and four-fold of that early in the rainy season.

C. raciborskii seasonal dissimilarity

While total trichome abundance did not vary significantly among the seasons, coiled trichome abundance did (Tab. 5). Coiled trichome and associated heterocyte abundance was greatest at the end of the dry season and least early in the rainy season. Total trichome spatial abundance dissimilarity among sites had LDI values ranging seasonally from only 0.06 in the rainy season to 0.10 in the winter dry season (Tab. 4). Abundance of coiled trichomes was more spatially dissimilar among seasons and sites with LDI ranging from 0.01 to 0.11. Heterocytes had greatest spatial dissimilarity early in the rainy season and least at the end of the dry season. Spatial dissimilarity formed a SE-NW pattern across Lake Catemaco during the rainy season when there were spatial relationships between coiled trichome and heterocyte abundances and inflowing waters from the east and southeast mountains (Fig. 4). During the dry season, when abundance was greater, there was a N-S pattern with greater abundances along the south shore. Water TP concentration was the only measured factor related to coiled trichome and heterocyte abundance during the rainy season (coiled trichomes=$1.9\times10^7\times TP+8.1\times10^7$, $R^2=0.43$, $P<0.001$; Heterocyte=$5.3\times10^6\times TP+1.1\times10^7$, $N=49$, $R^2=0.41$, $P<0.001$). There was no relationship of TP with the straight morphotype. In the dry season, there was no relationship of trichomes and heterocytes with either nutrient.

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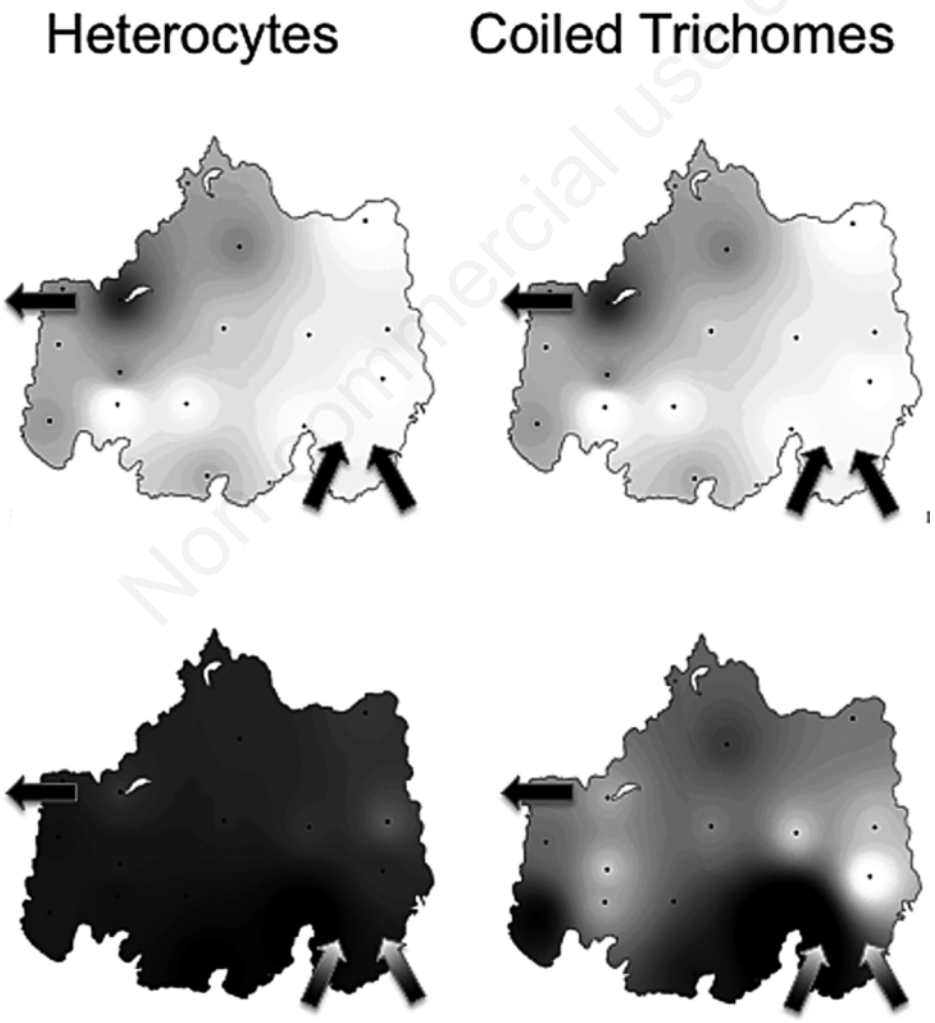

Fig. 4. Spatial patterns of coiled trichome and heterocyte abundance by season (upper, rainy; lower, dry). Shading intensity proportional to abundance. Arrows indicate outflow and principal sources of inflow. Note southeast to northwest (inflow to outflow) increase during the rainy season (upper panels) and the lack of an across-lake inflow to outflow pattern during the dry season (lower panels), but an increase in coiled trichomes and heterocytes at south shore sites.

Trichome and heterocyte size

We looked for variation among sample dates in coiled morphotype and heterocyte size attributes (Tab. 5). Average coiled trichome volume was significantly less during the rainy season. Maximum coiled trichome volume occurred in January, although because of the greater absolute and relative coiled trichome abundance in May, the total trichome volume present in the lake was greatest then. July coiled trichomes were shorter than dry season trichomes. Total heterocyte volume (μm^{-3} L^{-1}) appears to have increased progressively from the onset of rains to the end of the dry season (Fig. 5), but the only significant difference was the end of the dry season (May). The progressive increase in total heterocyte volume was a function of increased number of coiled trichomes and the percent with heterocytes - not to larger heterocytes.

DISCUSSION

Nitrogen, phenotypic plasticity, and taxonomy

Cell and trichome dimensions were factors used by Komárková and Tavera (1996, 2003) in erecting a new species of *Cylindrospermopsis*. *C. catemaco's* trichomes are shorter (10 to 45 µm), but overlapping, those of *C. philippinensis* (20 to 60 µm), while *C. catemaco's* cell length (4 to 10 µm) and width (0.8 to 1.3 µm) are smaller and non-overlapping those of *C. philippinensis* (10 to 18 µm long, 1.9 to 3.0 µm wide). *C. catemaco's* trichomes were illustrated as loose spirals or helices (Plate 1, Komárková and Tavera, 1996), and *C. philippinensis* as coils (Plates 3 and 7, Komárková and Tavera, 1996). The most abundant trichome in our studies was a straight trichome that Komárková and Tavera (1996) noted as *found very occasionally*. The straight and coiled trichome morphologies correspond with common morphologies of *C. raciborskii*. This confusion between the literature and our samples led us to turn to gene sequencing, the results of which strongly suggest that the two species reported for Lake Catemaco are actually very small morphotypes of *C. raciborskii*. Although the trichome and cell dimensions from our study (coiled=44.1±4.6 µm long, straight=22.2 ±13.2 µm long; coiled cell length and width=10.1±4.1×1.5±.03 µm; straight cell length and width=9.3±3.2×1.0±0.3 µm) were similar to those of Komárková and Tavera (1996). Genetic sequence data with morphological data are useful in species identification (Doers and Parker, 1988; Wilmotte and Golubic, 1991), and particularly so for cyanobacterial genera exhibiting phenotypic plasticity in response to variable environments. Despite the morphological variability of Lake Catemaco's population, four different genetic markers indicated that there was no genetic basis for different trichome morphotypes (Tab. 2).

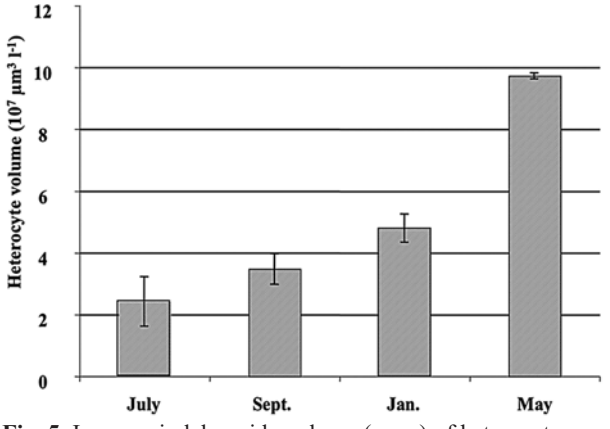

Fig. 5. Increase in lake-wide volume (mass) of heterocytes per litre of Lake Catemaco water from rainy (July and September) to dry season (January and May).

Unlike other cyanobacterial genera, *Cylindrospermopsis* sequences consistently cluster as a very discrete group based on multiple genetic markers, supporting its taxonomic distinctiveness. However, for the genus, there is considerable confusion about what defines a species, and the amount of strain level variability differs depending on the genetic region compared. Previous studies have shown that *C. raciborskii* originating from different parts of the world share nucleotide similarities of ≥99.1% for 16S rRNA, ≥98% for *rpoC1*, ≥97.5% for *nifH*, ≥94.5% for *cpcBA* intergenic spacer (*cpcBA*-IGS), and ≥91% for 16S-23S internal transcribed spacer region (ITS1) (Dyble *et al.*, 2002; Neilan *et al.*, 2003; Gugger *et al.*, 2005). Compared to global variability, genetic sequence analysis of Lake Catemaco samples during different years and seasons consistently show very high genetic similarity. Nucleotide similarity of at least 99.7% in conserved genes (*nifH*, *rpoC1*) and at least 99.1% in non-coding spacer regions (*cpcBA*-IGS and ITS1) is strong evidence that there are not multiple species represented by the strains sequenced. In particular, ITS1 has been used previously for differentiating between *Cylindrospermopsis* strains, and a 99% or greater sequence identity in this region has been used to confirm the presence of a single species (Gugger *et al.*, 2005; Alster *et al.*, 2010)

Cylindrospermopsis sequences from Lake Catemaco samples were compared with *Cylindrospermopsis* sequences in GenBank to further elucidate the taxonomic identity of these strains. There are few sequences in GenBank for *Cylindrospermopsis* species other than *C. raciborskii* and there are none attributed to *C. catemaco* or *C. philippinensis*. For the other *Cylindrospermopsis* species present in GenBank (*C. africana* and *C. curvispora*), sequences from each of these species are genetically identical to at least one *C. raciborskii* strain (and often more)

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for all genes with sequences available (*nifH*, *rpoC1*, *hetR*, ITS1 and 16S). While the Lake Catemaco isolates form a unique cluster (Fig. 6) for all four genes sequenced, these sequences do not have greater genetic distinctiveness than is found between strains of *C. raciborskii* from other regions of the world. This high genetic similarity between *C. africana, C. curvispora* and *C. raciborskii* calls into question the existence of any other species besides the cosmopolitan *C. raciborskii*. The hypothesis that there is only one *Cylindrospermopsis* species with environmentally-induced morphological variability should be investigated further, particularly in strains originating from Africa where a higher number of morphotypes identified as separate *Cylindrospermopsis* species have been reported. Sequencing these morphotypes in culture or monospecific in a bloom would be most helpful in testing this hypothesis. Knowing whether the morphological variability in *Cylindrospermopsis* is due to genetic or environmental causes impacts our understanding of its ecology and ultimately mediation of this harmful alga.

Trichome abundance and spatial variability

When comparing Lake Catemaco's *Cylindrospermopsis* trichome abundance and its ecological impact with *Cylindrospermopsis* of other lakes, one must consider the

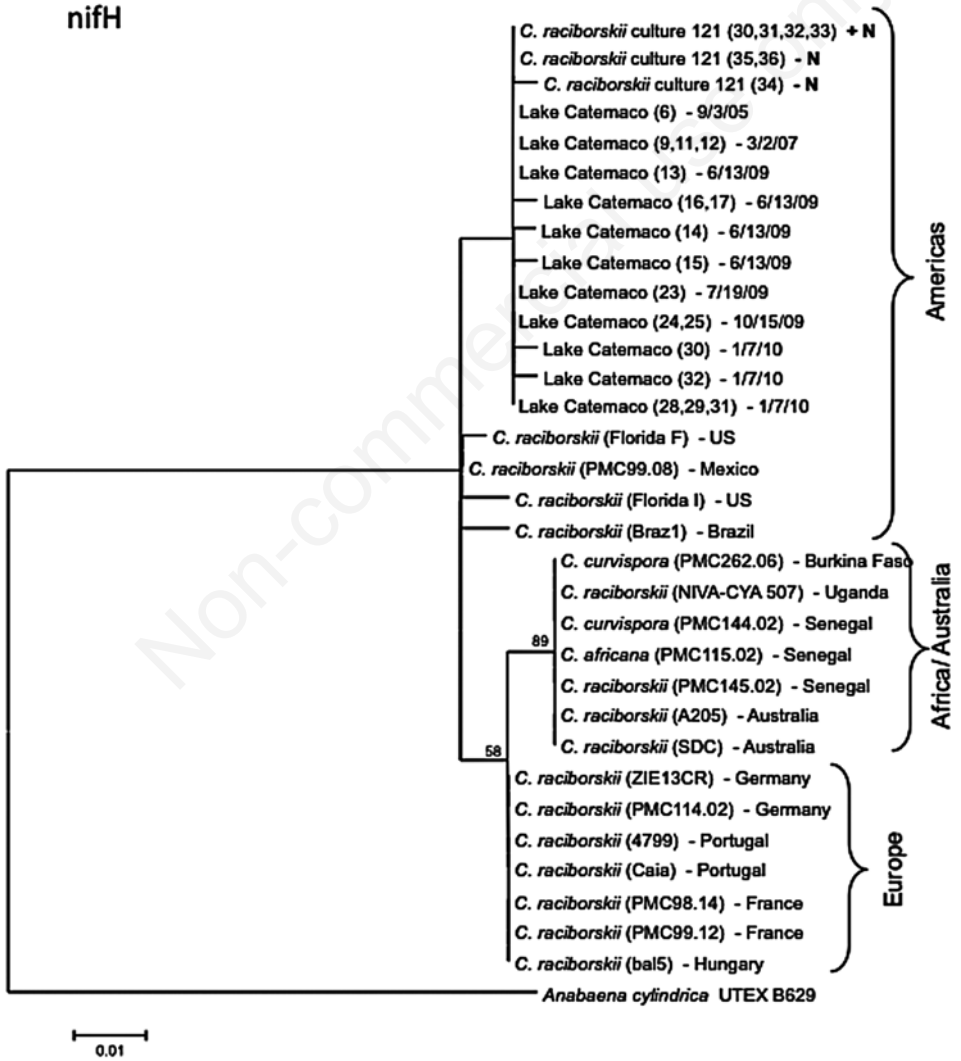

Fig. 6. Phylogenetic tree for *Cylindrospermopsis nifH* nucleotide sequences based on the neighbour-joining method and p-distances. Bootstrap values greater than 50% are given at each node. Names or numbers to identify strains are given in parentheses and multiple numbers indicates multiple strains had an identical sequence. Environmental samples from Lake Catemaco are shown with the date in which the sample was taken and it is noted in bold whether *Cylindrospermopsis* cultures isolated from Lake Catemaco were grown in media with or without nitrogen. All sequence data that is not from Lake Catemaco was taken from GenBank.

difference between populations having perennial *bloom-density* abundances and populations with periodic *boom or bust* dynamics. Lack of differences in our sequencing data related with date of collection indicates that a single strain is present in different morphotypes all year. Lack of a sequence of species at the producer level suggests a possible limitation of diversity at higher trophic levels. With *boom or bust* dynamics, the sequence of herbivores will change according to alternate feeding abilities and tolerance of inhibiting substances. Trophic structure is also altered by the collapse of blooms with oxygen depletion and different herbivore tolerances.

C. raciborskii's range is expanding perhaps due to global warming, (Briand *et al.*, 2004) into temperate regions contributing to the increasingly frequent harmful algal blooms (HABs) now attracting much attention and concern (Hudnell, 2008). Total trichome abundance in Lake Catemaco for any season was high compared to bloom (not perennial) abundances in other tropical and sub-tropical lakes or temperate region lakes (Tab. 6). Lake Catemaco's trichome abundance is little different than the combined *C. philippinensis* and *C. catemaco* reported for a 1993-1995 Lake Catemaco study (Tavera and Castillo, 2000). Their 1993-95 seasonal trichome abundance range was greater (1.4×10^8 L^{-1} to 4.6×10^8 L^{-1}) than our seasonal range (Tab. 5). The greater range in 1993-95 perhaps was driven by the great abundance that followed a record-breaking 1993 rainy season.

The *Cylindrospermopsis* high abundance gives the lake an apparent color. Komárková and Tavera (2003) described the lake seen in 1993-95 as *permanently slightly blue-green color*. We never made this observation, but always found the water, viewed in the lake or in sample bottles, to be pale golden and due to suspended trichomes (Tab. 5) because the color was removed by filtration (GF/F). Lake Catemaco is only moderate size, thus the inflow of the one principal river plus several lesser seasonal streams on the east and southeast shore are possible nutrient sources potentially affecting the distribution of phytoplankton (Fig 4). Nevertheless, the abundance of trichomes was unexpectedly uniform across the lake. We found small spatial dissimilarity in trichome abundance only during the rainy season associated with inflow and outflow. The weak spatial concentration pattern of nitrogen of the rainy season (Fig. 4) may be due to the riverine nitrogen loading being supplemented by rainfall with electrically fixed nitrate nitrogen on the lake surface and by cyanobacterial fixation in the lake.

Lake Catemaco's catchment is small (catchment area/lake area=3). Although partly adjacent to the Los Tuxtlas Biosphere Reserve along the north shore, the lake is impacted by agriculture. Coiled trichome and heterocyte abundance decreased from outflow to inflow in the rainy season suggesting a lessening dependency on nitrogen fixation in the presence of inflowing and rain waters. The TIN concentrations, though high, were low relative to TP, which favours cyanobacteria (Smith, 1983). The high lake water TP concentration (mean=580 µg L^{-1}) is characteristic of lakes, as Catemaco, situated in basins with phosphorus-rich volcanic ash soils (Andisols) (Chorover 2002). Lake Chapala, a similar latitude, volcanic region Mexican lake, has TP concentrations exceeding 1000 µg L^{-1} (Lind *et al.*, 1992).

Seasonal variability

Unlike the small spatial dissimilarities, seasonal differences were large. There were insignificant differences in total trichome abundances, but significant differences for trichome morphotype abundances, heterocyte abundances, and trichome and heterocyte sizes (Tab. 5). The relative abundance of coiled morphotype and, to a lesser extent, of heterocytes increased progressively from the start of the rainy season through the dry season. Relative *C. raciborskii* morphotype abundance varies greatly among lakes. Jones and Sauter (2005) reported a subdominant abundance of the coiled morphotype in temperate USA lakes, while the coiled morphotype reached 98% of a multi-month bloom in a Brazilian reservoir (Bouvy *et*

Tab. 6. *Cylindrospermopsis* spp. abundances for selected temperate and tropical lakes.

Lake or river	Trichomes (L^{-1})	Reference	Notes
Catemaco	4.4×10^8	This study	Mean all seasons and sites
Catemaco	2.2×10^8	Tavera and Castillo (2000)	Two species. 1993-95 mean
Fitzroy River	1.2×10^8	Bormans *et al.* (2005)	Maximum value. Reservoir, Aust
Lemon Lake, USA	6.5×10^7	Jones and Sauter (2005)	Greatest of 19 lakes
Soloman Dam	5.6×10^6	Saker *et al.* (1999)	Maximum (from Fig. 3)[#]. Aust
Fitzroy River	2.13×10^6	Fabbro and Duivenvoorden (1996)	From cell counts°. Reservoir, Aust
Newmans Lake, USA	1.2×10^5	Chapman and Schelske (1997)	
Lake Waahi, New Zealand	2.3×10^4	Wood and Stirling (2003)	From cell counts[§]

[#]Estimate based on coiled morph. cell number=23; °estimated average of 15 cells per trichome from figure; [§]estimate based on using 15 cell value from Australia.

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al. 1999). Numerous studies have reported only straight morphotypes (Briand *et al.*, 2004; Chonudomkul *et al.*, 2004; Wood and Stirling, 2003). As we found for Lake Catemaco's population, comparison of genomic sequences of the morphotypes of *C. raciborskii* has not produced evidence of a genetic basis for determining morphotype (Shafik *et al.* ,2003; Saker *et al.*, 1999). Nevertheless, environmental factors are implicated either directly or indirectly through gene activation. Field collections of Nostocace placed in a laboratory culture greatly change morphology (Zapomelova *et al.*, 2008). But, until our study, evidence of a specific environmental factor (TIN and TIN:TP) in support of field observations of a *Cylindrospermopsis* spp. morphotype determining factor was lacking. For the year-round *bloom* in Lake Catemaco the coiled morphotype relative abundance varied seasonally. Although never dominant, the lake-wide coiled average was 40% of total trichomes at the peak of the dry season decreasing quickly to a low of 18% early in the rainy season. These proportions are low compared with the Brazilian reservoir or an Australian reservoir (80%) (Saker *et al.*, 1999).

Because of the association of heterocytes with coiled morphotype, relative heterocyte frequency had a similar seasonal pattern (Table 5). Heterocyte frequency on the coiled morphotype varied from 25% in the dry season to 15% in the rainy season. Unlike number, heterocyte volume varied less seasonally with only winter size being significantly larger. We suggest that this winter difference was a consequence of greater cold water density affecting the form resistance and thus settling of trichomes with the thick-walled heterocyte and coiled morphology (Padisak *et al.*, 2003; Booker and Walsby, 1979). Coiled trichome volumes and heterocyte volumes each were significantly greater in January than any other month and significantly smaller in both rainy season months. Heterocyte volume increased with increase in coiled trichome volume presumably increasing nitrogen fixation capacity per heterocyte-bearing trichome. The increase was mostly by greater heterocyte diameter.

Determination of morphotype abundances

These morphologies occur in other lakes (Bouvy *et al.*, 1999; Neilan *et al.*, 2003; Saker and Neilan, 2001; Padisak, 2003), and maintain their identity through generations in culture with no transitional forms (Saker and Neilan, 2001). The circular trichomes may be either complete and partially overlapping circles or semi-circles. In culture the circular form is favored by low light (Saker *et al.*, 1999). The straight trichome in Lake Catemaco is usually shorter than reported for *C. raciborskii* in continuous culture (Shafik *et al.*, 2003), batch culture (Saker *et al.*, 1999) or coastal lagoon (Komarkova *et al.* 1999. In culture (Lind unpublished) the Lake Catemaco straight trichomes elongate similarly. Such morphological trichome variation of the well-studied *C. raciborskii* is almost all environmentally and not genetically determined. (Neilan *et al.*, 2003; Chonudomkul *et al.*, 2004; Saker and Neilan, 2001).

Several hypotheses have been offered to explain the relative abundance of different morphotypes including bottom-up controls such as light intensity, temperature, and nutrients (Saker *et al.*, 1999). Different shape-determined buoyancies may be another mechanism (see above). Top-down determination by differential grazing resistance of morphotypes also may produce different population proportions (Fabbro *et al.*, 2001; Hawkins and Lampert, 1989). In preliminary laboratory experiments, we found *Ceriodaphnia dubia* had a preference for the coiled Lake Catemaco morphotype (Lind, unpublished). Rotifers and copepods are important Lake Catemaco zooplankton and small Cladocera (*Bosmina longirostris* and *Diaphanosoma brachyurum*) are not abundant (< 0.5 ind L^{-1}). Bouvy *et al.* (2001) reported rotifer and copepod peaks coinciding with a multi-month *Cylindrospermopsis raciborskii* bloom (98% coiled morphotype). This was true for Lake Catemaco. May had the greatest absolute and relative coiled morphotype abundance and the greatest abundance of rotifers and Calanoid copepods (3.1 ind L^{-1}, approximately 5X that of September) (Tab. 7), which suggests that there was no selective zooplankton grazing on the coiled form. The rotifer, *Brachionus angularis*, was reported to ingest straight morphotypes (Fabbro and Duiv-

Tab. 7. Mean lake-wide zooplankton abundances for Lake Catemaco (org. L^{-1}).

Rotifera	May-04	Jan-05	Sep-05
Brachionus angularis	1.9		0.9
B. havanaensis	3.1	0.14	8.5
Epiphanes macrurus	3.8	0.52	2.7
Keratella cochlearis	7.2	0.15	0.1
Tricocherca spp	0.3	0.08	0.1
Asplanchna brightwelli			
Polyarthra vulgaris	0.3	0.05	
Filinia longiseta	0.2		
Hexarthra sp	1.7		0.2
Conochilus spp	1.2	0.3	
Cladocera	**May-04**	**Jan-05**	**Sep-05**
Bosmina cf. *tubicen*	0.16	0.1	<0.1
Diaphanosoma fluviatile	0.11	<0.1	<0.1
Copepoda	**May-04**	**Jan-05**	**Sep-05**
Nauplii	19.8	0.1	1.5
Calanoid[#]	3.12	1.9	0.6
Cyclpoid copepodites	3.8	0.4	0.5
Cyclopoid adults	0.9	0.5	0.1

[#]*Includes copepodites and adults.*

envoorden (1996), but we found no relationship between either straight or coiled morphotype abundance and the abundance of *B. angularis* or *B. havanensis* - both common in Lake Catemaco.

Although both buoyancy and selective grazing may be factors, we propose nitrogen as the morphotype-determining factor. An important feature of the population in Lake Catemaco is that heterocytes occur only on coiled morphotypes. The positive role of environmental nitrogen deficiency for inducing heterocyte formation in *Cylindrospermopsis* is well known (Saker and Neilan, 2001) and heterocyte abundance has been used to infer nitrogen-fixation rates in other cyanobacteria (Findlay *et al.*, 1994). Laboratory algal growth assays have confirmed that Lake Catemaco's phytoplankton production is nitrogen limited (Davalos-Lind, unpublished). This is consistent with the low ambient TIN:TP ratio. The greatest TIN:TP ratio was 1.2 in January and less than one other months. Seasonal differences in N supply should translate into heterocyte abundance differences and thus coiled morphotype abundance differences. Atmospheric nitrogen in rainfall, both as run-off from the catchment and directly to the lake surface, also can be an important source. In Lake Chapala, another nitrogen-limited Mexican lake of similar latitude, Limón *et al.* (1990) found that direct rainfall on the lake surface was the principal source of TIN during the rainy season that caused major increases in algal biomass. Direct rainfall may be relatively important for Lake Catemaco with such a small catchment. Atmospheric rainfall nitrogen content is unknown for Lake Catemaco; however data from the National Atmospheric Deposition Program (2000) in the United States permits an estimate. Using Puerto Rico as a similar site (population density, climate, *etc*.). Lake Catemaco should directly receive on the order of 14,000 kg N y^{-1}. Using this figure, we calculate that atmospheric fixed nitrogen contributes approximately 10% of the lake's nitrogen budget from wet deposition. Thus terrestrial rainfall runoff from the catchment and nitrogen fixation within the lake must be the major sources. Regardless of the source, the rainy season TIN concentration was more than triple that of the early dry season (Tab. 3). We note that this great rainy *versus* dry season difference was not reported in 1993 when Komárková and Tavera (2003) reported much lower nitrogen concentrations and little seasonal difference (rainy=0.190 mg N L^{-1}, dry=0.187 mg N L^{-1}).

Our proposed linkage between nitrogen and absolute and relative abundance of different morphotypes requires laboratory or *in situ* mesocosm testing using different concentrations of nitrogen and at different temperatures simulating the range seen in Lake Catemaco. Additional study should attempt to discern the form; *i.e*., nitrate or ammonia, responsible.

Possible ecosystem consequences of abundance and variable morphotypes

The fact that heterocytes occurred only on the coiled morphotype possibly has multiple ecosystem consequences. For example, seasonal and spatial differences in relative morphotype abundance may be important in determining the lake's trophic structure, or a consequence of trophic structure, or both. Rotifers and copepods are relatively more abundant than Cladocera in *C. raciborskii* dominated lakes. This was true for Lake Catemaco and for Ingazeira Reservoir, Brazil where Bouvy *et al.* (2001) described a *C. raciborskii* bloom-modulated trophic cascade where copepods broke trichomes and so made fragments available to smaller plankton. In that reservoir, rotifers and copepods increased in parallel with *C. raciborskii*. We do not know if such a bloom trophic response is an appropriate model for Lake Catemaco with its perennial high density.

Not only is the quantity of food available to a particular zooplankton determined by morphotype (Saker, 1999), but also the quality due to different cellular stoichiometries or toxins (Mitra and Flynn, 2005) or both. Saker and Griffiths (2000) found, in isolates of *C. raciborskii*, the highest concentration of the toxin, cylindrospermopsin, in straight trichomes. However, Saker *et al.* (1999) previously found no difference in the cylindrospermopsin concentration for straight or coiled morphotypes. Fabbro *et al.* (2001) suggest that toxin production is a function of increased nutrient supply and/or growth rate. The production of cylindrospermopsin requires nitrogen and should be limited if nitrogen is scarce - suggesting greater toxin production by Lake Catemaco's heterocyte-bearing coiled trichomes.

Little research has been done on the question of different trichome stoichiometries and presence of heterocytes. The placement of heterocytes only on the coiled morphotype may have stoichiometric consequences– their nitrogen content might be increased relative to the straight morphotype favoring those zooplankton capable of ingesting them.

CONCLUSIONS

C. raciborskii possesses features giving it broad tolerances to a range of light, temperature, and nutrient conditions (Dufour *et al.*, 2006) enabling the spread of an organism that threatens both ecosystem and human health. Understanding constraints on the growth dynamics of different global populations is essential. This study demonstrated that perennial blooms with their associated consequences are a possibility. The unusual restriction of heterocytes to a single morphotype is a factor requiring further study. As ambient nitrogen supply changes so also will heterocyte frequency change and with it morphotype.

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Multiple factors relating to the toxin-producing aspect of this organism's biology require exploration. At this time we have two toxin analyses from Lake Catemaco that show toxin accumulation by economically important fish and snails (Berry and Lind, 2012; Berry *et al.*, 2012). The possible linkage of toxin production with nitrogen supplies, heterocytes, and thus coiled *versus* other morphotypes needs experimental examination.

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