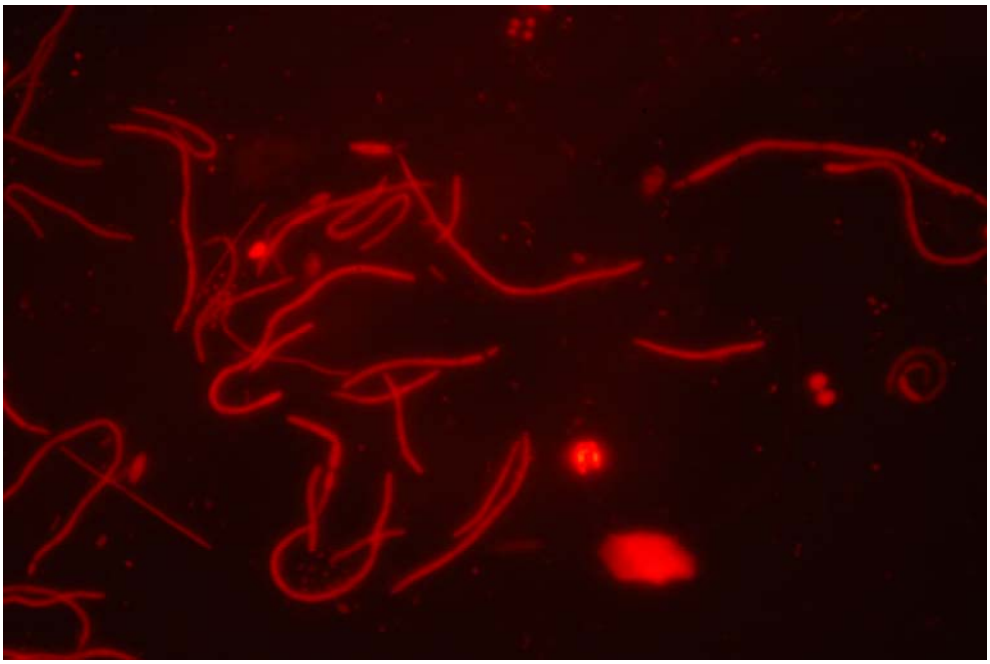
Distribution and Abundance of *Cylindrospermopsis raciborskii* in Indiana Lakes and Reservoirs



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EXECUTIVE SUMMARY

An invasive, toxin-producing cyanobacterium named *Cylindrospermopsis raciborskii* (Cylindro) was first discovered during routine sampling in Indiana on August 15, 2001 in Ball Lake, Steuben County. This Ball Lake sample contained 320,000 Cylindro cells per ml. Due to decisive State action, a health advisory was issued for Ball Lake and additional samples were collected for toxin analysis. Toxins were present at very low concentrations but heavy rains had flushed the lake prior to this additional sampling. A Toxic Algae Task Force of statewide experts was convened to assess the situation. The task force endorsed a study to assess the distribution and abundance of *Cylindrospermopsis raciborskii* in Indiana. This document is the final report of that study.

Lakes included within this study do not represent all public lakes in Indiana. Water samples for Cylindro analysis were collected from lakes during July and August of 2002 and 2003, during routine lake assessments conducted under the Indiana Clean Lakes Program. Targeted samples were collected during routine Indiana Clean Lakes Program sampling in 2004 and by trained citizen volunteers who participate in the Volunteer Lake Monitoring Program. Targeted sampling was restricted to August and September, 2004 when lake conditions indicative of a Cylindro bloom (reduced transparency and dark green subsurface water color) were detected. In all, 182 lakes were sampled. All were public lakes except for four private lakes sampled by volunteers.

We detected Cylindro in 21 samples from 19 lakes. All three samples collected from Lake Lemon, a reservoir in Monroe County, were positive with Cylindro densities ranging from 357,592 to 246,641 cells/ml, the highest densities detected during the study. Glen Flint Lake, a reservoir in Putnam County, was the only other lake with densities exceeding 100,000 cells/ml. Overall, 14 impoundments, 4 natural lakes, and 1 quarry were positive for Cylindro. Fifteen of the positive samples were collected in August while only 6 were collected in July. This reinforces the notion that Cylindro, a formerly tropical species, experiences its highest growth in temperate zones in the warmest water of late summer.

Lakes with detectable Cylindro tended to be shallow with lower Secchi disk transparency, higher epilimnetic total phosphorus, higher chlorophyll *a*. All of these differences were statistically significant.

Because this study was limited in scope (we did not sample for toxins, we did not sample all lakes in Indiana, and we sampled most lakes only once), care should be taken in interpreting the results, particularly the non-detect results. Until more is known about the seasonal growth dynamics and toxin production of this species, continued monitoring is recommended, especially in late-season when symptoms characteristic of a *Cylindrospermopsis raciborskii* bloom are visually apparent.

1.0 Introduction

Among the usual visitors to Indiana lakes during the summer of 2001 was a new and unwanted one – a blue-green alga called *Cylindrospermopsis raciborskii*. This species, which had never before been identified in Indiana, was first identified by Dr. Ann St. Amand from samples collected on August 15, 2001 from Ball Lake, a 75-acre natural lake in Steuben County. The Ball Lake sample contained 320,000 cells per ml. Its presence has since been confirmed independently in several Indiana reservoirs. The occurrence *C. raciborskii* in Indiana caused quite a stir because these organisms are known to produce potent toxins.

Indiana state government responded rapidly to this potential threat. A Public Health Advisory was issued for Ball Lake on August 21, 2001. Water samples from Ball Lake were collected on August 22, 2001 by representatives from the Indiana Department of Environmental Management (IDEM) and the Indiana Department of Natural Resources (DNR) and were driven to the laboratory of Dr. Wayne Carmichael at Wright State University in Ohio for toxin analysis. Toxins were detected but at very low amounts. There were few visible signs of a *C. raciborskii* bloom when the toxin samples were collected. On August 17, 2001, an extended rain storm began dumping several inches of rain over Ball Lake. This likely washed out the *C. raciborskii* cells and/or diminished optimal growing conditions for this species so that by the August 22 sampling, very little evidence of the bloom remained.

A task force of representatives from the DNR, IDEM, Indiana Department of Health, U.S. Army Corps of Engineers, Indianapolis Water Company, Indiana Lakes Management Society, Indiana University, and Purdue University was convened on 9/26/01 and has met periodically since then to address policy and health concerns related specifically to *C. raciborskii*.. However, without information on the distribution and abundance of this species in Indiana, state policies and mitigation measures could not be developed.

The current project was undertaken to determine the distribution and abundance of *C. raciborskii* in Indiana lakes and reservoirs, with the understanding that the results from this study would provide important and necessary input into potential policymaking regarding this and other toxin-producing blue-green algae.

2.0 Literature Review

2.1 Harmful Algal Blooms (HAB): Background and Distribution

Algal blooms that adversely affect environmental, plant, or animal health are referred to as *harmful algal blooms (HABs)* (Backer 2002). HABs occur in freshwater, marine, and estuary systems and are quickly becoming a public health issue. HABs are primarily associated with surface scums from blue-green algae or cyanobacteria that occur in nutrient enriched freshwater lakes and reservoirs (Figure 2-1). HABs often have negative environmental impacts including reduced light levels and reduced dissolved oxygen concentrations that may lead to fish kills, and may even cause shifts in plankton populations. Cyanobacteria are unique to other bacteria because they are capable of photosynthesis, have heterocysts that fix nitrogen from the atmosphere, and gas vesicles that help them regulate their buoyancy, allowing them to move throughout the water column. Some cyanobacteria like *C. raciborskii* can also produce toxins. These characteristics often give cyanobacteria a competitive advantage over other groups of algae.

Figure 2-1. A lake surface algal scum.

There are about 150 known genera of cyanobacteria, 40 of which are known to be toxic (Saker et al. 1999a). The primary toxin-producing cyanobacteria include *Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis, Nodularia, and Planktothrix (Oscillatoria).* There are two major groups of toxins which are named for their effects on animals: neurotoxins and hepatotoxins (liver toxins). Neurotoxins (anatoxin-a, anatoxin-a(s), saxitoxins, and neosaxitoxin) interfere with nerve communication and hepatotoxins (microcystins, cylindrospermopsin, and nodularins) block protein synthesis, promote chromosome breakage, and may even cause tumors. *C. raciborskii* produces cylindrospermopsin (CYN), saxitoxins, anatoxin-a, and paralytic shellfish poisons (PSPs).

C. raciborskii is unique to cyanobacteria for two reasons. First, it typically does not form surface blooms. Maximum densities of *C. raciborskii* cells occur at 2-3m below the surface (Saker and Griffiths 2001). Therefore, *C. raciborskii* blooms are hard to detect. *C. raciborskii* blooms in Indiana have been described as exhibiting dense green, 'foggy-looking' water just below the surface. In Wooster Lake, Kansas it was reported as a thick surface scum that disintegrated and turned the water a greenish-yellow color (Padisák 1997 [Prescott and Andrews 1955]). Second, *C. raciborskii* does not produce the volatile organic compounds such as geosmin and MIB that cause taste and odor problems that are commonly associated with algal blooms (Chiswell et al. 1997).

Animals are more prone to algal poisonings because they are not deterred by foul tastes, odors, or surface scums. Human exposure usually occurs through direct contact or accidental uptake via swallowing or aspiring cells. Additionally, untreated drinking water may also increase human exposure risks. There is also evidence that toxins may bioaccumulate in freshwater crustaceans (Saker and Eaglesham 1999) and shellfish (Saker et al. 2004).

Freshwater cyanobacterial blooms have been reported in over 45 countries and in every continent (Table 2-1). Within the US, 27 states have reported blooms (Codd et al. 2005) but this number is probably higher. Cyanobacteria possess the ability to produce toxins, which makes them a major public health threat. There have been multiple reports of cyanobacterial toxins affecting farm animals such as cattle (Saker et al. 1999a, Hawkins et al. 1997) and even humans. One of the worst cases of cyanobacterial poisoning occurred in Brazil in 1996, when a dialysis clinic treated its patients with water infected with microcystins and caused the death of 76 people (Carmichael et al. 2001). Most recently, *Aphanizomenon flos-aquae* and its toxin anatoxin-a were implicated in the death of a Wisconsin teenager in the summer of 2003 (Behm 2003).

Table 2-1. Countries with Reported Cyanobacterial Blooms

Europe	Belgium, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Norway, Poland, Portugal, Russia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Ukraine, United Kingdom
Americas	Argentina, Bermuda, Brasil, Canada, Chile, Mexico, USA (at least 27 states), Venezuela
Middle East and Asia	Bangladesh, India, Israel, Japan, Jordan, Malaysia, Nepal, Peoples Republic of China, Philippines, Saudi Arabia, Sri Lanka, South Korea, Thailand, Turkey, Vietnam
Australia	Australia (New South Wales, Queensland, South Australia, Tasmania, Victoria, Western Australia), New Caledonia, New Zealand
Africa	Botswana, Egypt, Ethiopia, Kenya, Morocco, South Africa, Zimbabwe
Marine	Baltic Sea, Caribbean Sea, Atlantic, Indian and Pacific Ocean
Antarctica	McMurdo Ice Shelf

(Metcalf and Codd 2004)

C. raciborskii is perhaps best known for its role in the Palm Island Mystery Disease (Hawkins et al. 1985). In November 1979, 149 people (mostly children) became ill with symptoms of hepato-enteritis, vomiting, constipation (Griffiths and Saker 2003; McGregor and

Fabbro 2000 [Falconer 1996]) kidney malfunction, and diarrhea (Hawkins et al. 1997). Originally thought to be attributed to the consumption of unripe mangoes, medical officers noticed that the outbreak occurred three days after the major water supply was treated with copper sulfate to control an algal bloom. An epidemiological study of the incident later confirmed the linkage between the outbreak and the water supply (Bourke et al. 1983). The copper sulfate had caused *C. raciborskii* cells to die and lyse, causing the release of toxins in to the water.

Initially classified as a tropical species, *C. raciborskii* was first identified in India in 1913 (St. Amand 2002a [Desikachary 1959]). It has since been identified throughout the world. *C. raciborskii* was first identified in the United States in 1955, in Wooster Lake, Kansas. However, at the time it was recorded as a new species of *Anabaenopsis* (Padisák 1997 [Prescott and Andrews 1995]). It was first reported in Minnesota in the 1960s [Hill 1970] and in Texas in the 1980s (Padisák 1997 [Lind 1984]). It was also found in archived slides in 1982 from Madison, Wisconsin (Richard Lathrop, pers. comm.). Researchers believe *C. raciborskii* may have arrived in Florida almost 35 years ago (Chapman and Schelske 1997). *C. raciborskii* was first identified in Indiana in 2001 at Ball Lake (Steuben County) by Ann St. Amand (Jones 2001).

C. raciborskii, until recently, has always been thought of as a tropical species due to its affinity for warm water temperatures (25-30°C), but its tolerance to a wide variety of temperatures may have facilitated its immigration to more temperate regions. It has been suggested that its range may have expanded by transport of akinetes via migrating birds or imports of tropical fish (Padisák 1997).

2.2 Morphology

C. raciborskii is extremely small when compared to other algae. Filaments, also known as trichomes, are typically 2-3 µm wide (St. Amand 2002a) and are highly variable in length, ranging from 10 – 120 µm long (Briand et al. 2002). Individual cells are often difficult to distinguish because they are rarely constricted at the cell walls. Cell length ranges from 3 – 10 µm long (Table 2-2). Differences in cell size is a well known feature common of natural *C. raciborskii* populations (Hawkins et al. 2001). Due to the small size of *C. raciborskii*, they are often missed in typical tow net plankton samples because they can easily pass through the mesh.

Table 2-2. Varying World Wide Morphologies of *C. raciborskii*

Country	Morphs Found	Cell Width	Cell Length	Author
Japan	Straight, coiled	1.9-2.3 µm	3.0-18.5 µm	Chonudomkul et al. 2004
Australia	Straight, coiled, sigmoid	2-2.3 µm 2.5-3 µm 1.5-1.8 µm	4.5 – 6 µm 6-7 µm 5-7 µm	McGregor and Fabbro 2000
France	Straight	1.5 µm	11.5 µm	Briand et al. 2002
USA, Indiana	Straight, Coiled	_____	7.8 µm 7.3 µm	This study

C. raciborskii is difficult to identify because of the many similarities between morphotypes, other species of *Cylindrospermopsis*, and other cyanobacteria that closely resemble *C. raciborskii*. *C. raciborskii* has been formally misidentified as *Anabaenopsis, Raphidiopsis,* and *Cylindrospermum* (Hawkins et al. 1997). The development of the conspicuous terminal heterocyst of *C. raciborskii* led to the eventual break from *Anabaenopsis*, which has a heterocyst in the center, causing the filament to break in two. Until recently, keys have not included *C. raciborskii* as a distinct species.

Morphotype variability is common in many cyanobacteria, but especially so with *C. raciborskii* (McGregor and Fabbro 2000 [Hamar 1997; Horecka and Komarek 1979; Hindak 1988; Komarek and Kling 1991; Komarkova et al. 1979]). There are two main morphotypes: straight and curly (McGregor and Fabbro 2000; Saker et al. 1999b) (Figure 2). Within each morph, there is a great deal of variability in the size and number of cells per trichome. The straight morphotype tends to be larger and produce more toxins per cell. They often grow without a heterocyst and rarely have akinetes (St. Amand 2002a).

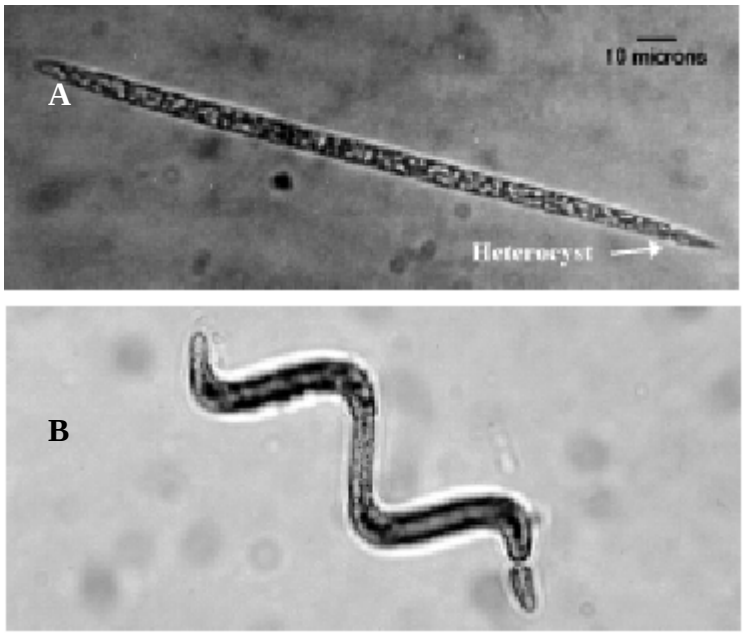


Figure 2-2. Morphological Forms of *C. raciborskii:* Straight Morph (A) and Curled Morph (B). Images courtesy of Ann St. Amand.

Variations within and between morphs have been attributed to both genetic and environmental variability (Hawkins et al. 1997; Neilan et al. 2003). Despite having two distinct morphs, genetic analyses have proven that they belong to the same species. Genetic analysis has also been proven to be an accurate way to identify *C. raciborskii* (Wilson et al. 2000).

Plasticity in morphotype and trichome size has been attributed to varying environmental conditions, especially to bloom size, in cultured studies. Coiled morphs seem to grow faster than straight morphs in high temperatures and lower light intensities (Saker et al. 1999b). Given

different nitrogen sources (n-depleted, nitrate, ammonium ion) Saker and Neilan (2001) found significant differences in morphology of trichomes (i.e. lack of heterocysts, tapering, and longer trichomes with enriched ammonium). Chiswell et al. (1997) also found heterocyst production was dependent on environmental conditions.

Hawkins et al. (2001) found that in cultures, trichome lengths decreased as cell density increased. He believes that this may be an adaptation to reduce entanglement or allow trichomes to move more freely throughout the water column to better capture light. This may explain why *C. raciborskii* doesn't form surface blooms (entanglement causes blooms of *Anabaena* and *Microcystis*) (Hawkins et al. 2001).

Morphotype variability has also been reported to vary with the age of the bloom. Padisák (2003) found that there are multiple morphological changes that *C. raciborskii* undergoes over an annual lifecycle. The primary filaments (those derived directly from akinetes) are mostly the straight morphotype with cell walls that are often impossible to detect, fine granulation, and no gas-vacuoles. Similarly, Saker and Griffiths (2001) observed that the straight morphotype was more numerous overall, but coiled morphotypes increased during or immediately after blooms of straight morphs.

2.3 Growth

C. raciborskii can grow under a wide variety of environmental conditions making it difficult to predict its occurrence or proliferation (Briand et al. 2002). Conditions favoring its growth (estimated by cultures) include warm surface waters, seasonal warming of hypolimnetic waters (>23°C), low light conditions (Briand et al. 2004); highly stable thermally-stratified water columns (McGregor and Fabbro 2000 [Dokulil and Mayer 1996]), and environmental consistency (McGregor and Fabbro 2000).

As a function of its primary distribution in tropical ecotypes, most of what is known about conditions favoring *C. raciborskii* growth is based upon tropical environments. However, with its expanding distribution into temperate climates such as Indiana, different trends seem to be emerging. For example, while *C. raciborskii* typically occurs in deep (>50m), stratified lakes in tropical regions, it seems that they prefer shallow waters in temperate zones (i.e. <10m) (Padisák 1997). Additionally, annual *C. raciborskii* populations in temperate systems appear to be highly variable and extremely dependent on temperature and climate. In Hungary, *C. raciborskii* build up large populations only in exceptionally warm years while in cooler years, only single filaments appear, if at all, in late summer (Padisák 1997). In Minnesota, Hill [1970] saw a large population one year and then couldn't find any two years later (Padisák 1997).

2.3.1 Temperature

The major factor influencing *C. raciborskii* populations seems to be temperature. While it can survive perennially in tropical areas (Briand et al. 2004 [Bouvey et al. 1999]) it seems to be limited to warm summer months in temperate regions (Briand et al. 2002; Saker et al. 2003). *C. raciborskii* tends to favor surface water temperatures over 25°C (Saker et al. 1999b; Saker and Griffiths 2001; McGregor and Fabbro 2000 [Fabbro and Duivenvoorden 1996]; Hawkins et al. 1997 [Vinogradska, 1974]) and seasonal warming of hypolimnetic waters (>23°C) (McGregor and Fabbro 2000 [Gorzo 1987; Fabbro and Duivenvoorden 1996]; Padisák 1997). In cultures,

the optimum temperature seems to be 30°C with a range of 'sub-optimum' temperatures from 25-35°C. (Briand et al. 2004; Shafik et al. 2001 [Saker and Griffiths 2000]). This seems to be a very wide range and may suggest how they can spread into northern waters. However, Briand et al (2004) believe this is not evidence of a general temperature-specific adaptation. Briand predicts the migration north is a combination of increasing water temperatures of lakes and the wide temperature tolerance. Akinetes, Briand says, are the key to northern expansion. They can survive the cold winters and only germinate when water temperature or sediments reach 22-23°C (Padisák 1997) while other heterocystic blue-greens can germinate from 18-25°C [Gorzo 1987]. This may explain why *C. raciborskii* prefer shallow systems in the temperate regions. Akinetes were rarely observed in tropical Australian strands (Saker and Griffiths 2001) but were present in high concentrations in temperate strands (Saker et al. 2003).

2.3.2 Light

C. raciborskii is not prone to effects of self-shading like most cyanobacteria. *C. raciborskii* has a high shade tolerance (Briand et al. 2002 [Padisák and Reynolds 1998]) and their blooms can persist without sudden collapses (Padisák 1997). They have been reported to survive at 0.5 μmol photons/ m^2s^2 in shallow turbid Brazilian lakes (Bouvey et al. 2000; Briand et al. 2004 [Bouvey et al. 1999]). In Lake Balaton, *C. raciborskii* had a calculated low light tolerance of 26 μmol photons/ m^2s^2 which is similar to other low light adapted species (Shafik et al. 2001). While *C. raciborskii* only needs low light levels, it can tolerate high light levels, up to 500 μmol photons/ m^2s^2) (Briand et al. 2004). Optimal irradiance has been reported to be 80 μmol photons/ m^2s^2 (Shafik et al. 2001), but optimal growth in other studies show the highest growth rates to be more temperature dependent, occurring at 30°C at sub-optimal irradiance (30μmol photons/ m^2s^2).

Briand et al. (2002) reported that during *C. raciborskii* blooms in Europe, Secchi disk transparency depths decreased to 0.068 m, which is slightly lower than in Brazilian reservoirs with *C. raciborskii* blooms. Secchi disk readings of 30 cm are common in *C. raciborskii* blooms (Padisák 1997). In Florida, the mean Secchi depth for blooms was less than 1.0 m (Chapman and Schelske 1997).

2.3.3 Water column stability

There are many contradictory reports on the dependence of *C. raciborskii* on water column stability. In Brazil *C. raciborskii* dominated in shallow, well-mixed systems (Saker and Griffiths 2001 [Bouvey et al. 1999]). Similarly, in Florida *C. raciborskii* dominated 97% of biovolume in Lake Griffin which is regulated by a flow structure. In contrast *C. raciborskii* only constituted 0-50% of total cell count in Lake Jesup, FL, a system without a flow structure and having water levels that vary up to seven feet in a season (Dobberfuhl 2003). Saker and Griffiths (2001) observed that a stable water column favored *C. raciborskii* growth in Lake Julius, Australia. Similar conditions have been reported in Lake Victoria [Komarek and Kling 1991], Kariba Reservoir [Ramberg 1987], North Pine Dam [Harris and Baxter 1996], and Lake Valencia [Lewis 1986]. *C. raciborskii* may also prefer well mixed systems with reduced mixed-to-euphotic depth ratios (Z_m:Z_{eu}). *C. raciborskii* populations in Australia were the largest when the Z_m:Z_{eu} ratio ≈1. Additionally, when the ratio increased to greater than 3, *C. raciborskii* was either completely excluded or existed only in low numbers (Saker and Griffiths 2001).

2.3.4 Nutrients

C. raciborskii may even be less dependent on nutrients than other cyanobacteria (Bouvey et al. 2000). *C. raciborskii* blooms in late season, when the temperatures are the warmest and most of the nutrients have been used by other cyanobacteria. Additionally, their low light tolerance and preference to stratified systems may permit them to utilize suspended nutrients that have escaped from the hypolimnion during stratification.

Phosphorous

C. raciborskii has a high affinity and storage capacity for phosphorous relative to other cyanobacteria (Istvánovics et al. 2000; Shafik et al. 2001). Phosphorous uptake was measured at four times greater than other cyanobacteria, while phosphorous affinity has been measured at an order of magnitude greater (Istvánovics et al. 2000). These adaptations permit *C. raciborskii* to grow in low levels of phosphate (Branco et al. 1994; Briand et al. 2002 [Presing et al. 1996]) and may give *C. raciborskii* a competitive advantage. Istvánovics et al. (2000) suggests that *C. raciborskii* is a phosphorous opportunist that has adapted to temperate conditions. He gives four main reasons for this. First, they bloom last and are used to low nutrient conditions (blooms grew <1-2 μg-P/l). Second, in unstable, shallow systems slight sediment re-suspensions may generate enough phosphorous for *C. raciborskii* to use (10 μg-P/L for 15 minutes is enough to sustain a bloom). Third, akinetes germinate when water gets warm, typically only in late summer. Fourth, low light allows *C. raciborskii* to survive below the surface.

However, in high phosphorous levels *C. raciborskii* looses its advantage In Florida, Dobberfuhl (2003) found negative correlations between *C. raciborskii* and dissolved TP. *C. raciborskii* declined at dissolved TP >0.02mg/L while *Microcystis* attained maximum density at 0.08 mg/L. Similarly, blooms in Hungary occurred when SRP was below detectable levels. In this system, however, the sediments were rich in P- suggesting internal loading may support blooms (Padisák 1997).

Chlorophyll-*a*

Several studies claim that chl-*a* is not an effective measurement to monitor *C. raciborskii* populations because they have a relatively low chl-*a* content (McGregor and Fabbro 2000 [Dokulil and Mayer 1996]; Saker and Griffiths 2001). However, Dobberfuhl (2003) suggests measuring chl-*a* may be a valuable tool to estimate bloom densities, especially if examining chl-*a*/TP ratios. In blooms without *C. raciborskii*, he found a low ratio (>0.5). However, chl-*a*/TP ratios increased to almost 2 as *C. raciborskii* populations increased. Chiswell et al. (1997) also saw correlations between *C. raciborskii* growth and chl-*a*.

Nitrogen

Like most cyanobacteria, *C. raciborskii* have heterocysts that allow them to utilize atmospheric nitrogen through a process known as nitrogen fixation. Typically, this adaptation allows cyanobacteria to survive in low nitrogen systems. However, *C. raciborskii* does not seem to be highly dependent on N-fixation. Studies have found that *C. raciborskii* had few heterocysts in reservoirs with low nitrate concentrations. This is often attributed to *C. raciborskii's* preference for ammonium as its nitrogen source (Briand et al. 2002 [Bouvey et al. 1999; Presing et al. 1996]). In cultures, they grow faster with nitrate or ammonium than without a fixed

nitrogen source (Griffiths and Saker 2003 [Saker 2000]; Hawkins et al. 2001; Saker and Neilan 2001; Shafik et al. 2001).

2.3.5 Salinity, conductivity, and pH

Similar to the other growth characteristics of *C. raciborskii,* there are conflicting reports of this species' tolerance to salinity. It has been recorded twice in the North Caspian Sea at salinities of 1.5-2% (Padisák 1997). Similar reports of *C. raciborskii* thriving in waters with conductivities above 4000 μS/cm in Lake Alexandria (Padisák 1997 [Baker 1996]) and over 2000 μS/cm in Venezuela (Padisák 1997 [Lewis 1986]). However, in North Carolina, Moisander et al. (2002) found salinity concentration of 2g/L NaCl slowed *C. raciborskii* growth. Magnesium may also be a limiting factor in *C. raciborskii* populations (Dobberfuhl 2003). Similar to most cyanobacteria, *C. raciborskii* does not occur in acidic waters. *C. raciborskii* typically occurs in lakes with a pH of 8.0 to 8.7 (Padisák 1997).

2.3.6 Rainfall

In semi-arid tropical regions, Saker and Griffiths (2001) found that *C. raciborskii* (at 0.5 m) displayed seasonal patterns with maximum abundance occurring during the summer. Overall in Australia, most blooms occur in years of low rainfall and increased draw-down of the water column after wet seasons. Others have found *C. raciborskii* dominate after rainy periods when nutrients have been diluted (Branco and Senna 1994). In Ball Lake, Indiana, the *C. raciborskii* population fell from 320,000 cells/mL to below 3,000 cells/mL (Ken Wagner 2001, unpublished data) in one week. Some speculate that the population crashed due to a storm and plummeting temperatures that occurred prior to the second sampling.

2.3.7 Competition

Low nutrient and light requirements should make *C. raciborskii* an excellent competitor near the edge of other cyanobacterial ranges. Similarly, it is speculated that *C. raciborskii* may lose this competitive advantage during periods of high nutrient conditions. However, in nutrient rich systems in Florida, *C. raciborskii* is thriving, dominating up to 97% of the cell volume. *C. raciborskii* may even be causing decreases in species diversity and richness (Dobberfuhl 2003). Reports of cyanobacterial populations gradually shifting from a dominance of *Microcystis* and *Aphanizomenon* to *C. raciborskii* (Saker and Griffiths 2001 [Finlayson et al. 1984; Boland 1993 - Australia], [Padisák 1991 – Lake Balaton, Hungary]; Chapman and Schelske 1997 – FL) give further evidence to Padisák's (1997) description of *C. raciborskii*'s as an 'expanding' species.

2.3.8 Predation

C. raciborskii seem to be somewhat resistant to zooplankton predation (Branco and Senna 1994). *Paramecium cf. caudatum* has proven to be a successful grazer of toxin-producing *C. raciborskii* in the lab (Fabbro et al. 2001). While *C. raciborskii's* toxin cylindrospermopsin (CYN) is not lethal to daphnids, *C. raciborskii* avoid daphnid predation because their small trichomes clog filters thus slowing filtering rates (Padisák 1997; Hawkins and Lampert 1989). As a result, *C. raciborskii* has been reported to promote decreasing *Daphnid* body sizes (Hawkins and Lampert 1989; Nogueira et al. 2004). However, Nogueira et al. (2004) found that daphnids present in *C. raciborskii* blooms experienced high mortality and low fecundity.

Some have reported that rotifers are dominant only during and immediately after *C. raciborskii* blooms (Griffiths and Saker 2003 [Hawkins 1988]). In Brazil, rotifers and copepods may actually be able to cut up and shorten filaments into edible sizes for other zooplankton. Rotifers and copepods increased with *C. raciborskii* blooms and then decreased after the bloom while cladocerans increased after the bloom. The Shannon index revealed an increase in zooplankton diversity (especially with rotifers) when *C. raciborskii* was present (Bouvy et al. 2001).

2.4 Toxicology

C. raciborskii produces several toxins: Cylindrospermopsin (CYN), saxitoxins (also known as Paralytic Shellfish Poison), anatoxin-a (Chorous and Bartram 1999) (Table 2-3). Cylindrospermopsin is *C. raciborskii's* primary toxin (St. Amand 2002a). It is hepatotoxic, causing liver and kidney damage in mouse bioassays (Ohtani et al. 1992; Falconer et al. 1999; Hawkins et al. 1985, 1997). Preliminary evidence suggests that CYN may also be carcinogenic (Falconer and Humpage 2001; McGregor and Fabbro 2000 [Falconer 1996]; Shen et al. 2002; Humpage et al. 2000). Anatoxin-a and saxitoxins are both neurotoxins that affect communication between nerves (Chorous and Bartram 1999).

There is mounting evidence that *C. raciborskii* and its toxins are affecting both human and wildlife populations. As mentioned earlier, 149 people in Palm Island, Australia were hospitalized with symptoms of hepato-enteritis after consuming contaminated drinking water. *C. raciborskii* has also been implicated in cattle mortality (Hawkins et al. 1997). There are also reports that CYN can accumulate in crayfish (Saker and Eaglesham 1999), mussels (Saker et al. 2004), and daphnids (Nogueira et al. 2004), suggesting it may bioaccumulate.

Cylindrospermopsin toxicity is lower than other cyanobacterial toxins. Pure CYN in mouse bioassays has a 24 hour LD_{50} of 2100 μg/kg and 200 μg /kg at 5-6 days (Ohtani et al. 1992). *C. raciborskii's* other toxins are much more toxic. Saxitoxin has an LD_{50} of 10-30 μg /kg while anatoxin-a(s) has an LD_{50} of 20 μg /kg (Chorous and Bartman 1999). When compared with other cyanotoxins, even the highest recorded concentrations of CYN are relatively low. Record high CYN concentrations of 589 μg/L (Saker and Eaglesham 1999) are an order of magnitude smaller than record anatoxin-a concentrations (3,300μg/L for anatoxin a (Griffiths and Saker 2003 [Henriksen et al. 1997]) and two orders of magnitude smaller than maximum the concentration for microcystin-LR (25,000 μg/L (Griffiths and Saker 2003 [Chorus et al. 1998]). Padisák (1997) claims this is because *Microcystins* and *Anabaena* attain higher cell concentrations when they form surface scums, atypical of *C. raciborskii*.

The mechanism by which *C. raciborskii* produces CYN appears to vary with and between strands, morphotype, and environmental conditions. Different morphs with different toxin capacities are not unique to *C. raciborskii*. *Microcystis aeruginosa* has been documented to produce multiple strains with different toxin production capacities (Kurmayer et al. 2002). While the Australian strand of *C. raciborskii*, thought to be an analogue of strands in the USA, works by producing CYN and inhibiting protein synthesis, Brazilian strands typically have neurotoxic effects in mice, similar to effects of saxitoxins (Bernard et al. 2003). Others found that blooms with curled morphs produced a lower [CYN] to #cells ratio; which may be due to environmental strains that influence the curled morphotype (McGregor and Fabbro 2000).

Table 2-3

Toxin	LD50 (i.p. mouse) of pure toxin	Organisms	Acute Effect	Mechanism of Action	Signs and Symptoms of Intoxication	Therapy
Anatoxin-a	200-250 µg/kg	*A. flos aquae, A. lemmermanii, Anabaena, Planktothrix, Aphanizomenon, Cylindrospermum*	Neurotoxicity	Blocks post-synaptix depolarization, mimics acetycholine	Progression of muscle fasciculations, decreased movements, abdominal breathing, cyanosis, convulsions, death	None known, respiratory support may give time for detoxification
Anatoxin-a(s)	20 µg/kg	*A. flos aquae*	Neurotoxicity	Anticholinesterase • (s) is for salivation	Hypersalivation, muciod nasal discharge, tremors, diarrhea, paresis, death from respiratory paralysis	Not thoroughly investigated
Saxitoxin, neosaxitoxin	10-30 µg/kg	*Anabaena, A. flos aquae, Lyngbya, Cylindrospermopsis,*	Neurotoxicity	blocks sodium channels • most commonly know for dinoflagellate marine Red tides • also in Paralytic Shellfish Poisoning (PSP)	Incoordination, recumbency, death by respiratory failure, numbness of lips and then extending to the face and extremities, motor weakness, paralysis	Activated charcoal, artificial respiration
Cylindrospermopsin	2100 µg/kg-d, 200 µg/kg/5-6 d	*Cylindrospermopsis, Aphanizomenon, Umzakia*	Hepatoxicity	Inhibition of protein synthesis, cumulative toxicity affecting kidneys, intestines, and lungs. The liver is the main organ affected. Also geotoxic.	Huddling, anorexia, slight diarrhea, gasping respiration, enlarged liver, malaise, vomiting, headache	Not well investigated
Microcystins	45-1000µg/kg	*Microcystis, Anabaena, Planktothrix, Nostoc, Hepalosiphon*	Hepatoxicity	Alterations of actin microfilaments, destruction of parenchymal cells, lethal hemorrhage or hepatic insufficiency, inhibition of protein phosphatases, tumor-promoting activity, liver hemorrhage	Weakness, reluctance to move, anorexia, mental derangement, photosensitization	Powdered charcoal, cholestryamine, therapeutic support
Nodularins	30-50 µg/kg	*Nodularia spumigena*	Hepatoxicity	Inhibition of protein phosphatases, tumor promoting activity, liver hemorrhage	Skin and eye irritation with dermal contact	

Created from Backer (2002); Chorus and Bartram (1999); Chorus et al. (2000); Haider et al. (2003); Metcalf and Codd (2004)

However, in an Australian bloom, Saker et al. (1999b) found two morphs, curly and straight, but no significant difference in CYN content.

CYN production may also be related to environmental pressures. CYN production was found to be greater in cultures lacking a fixed nitrogen source compared to those with a supply of ammonium (Ohtani et al. 1992). Parallel toxin concentration relationships with nitrogen limited conditions occur in *Aphanizomenon flos-aquae* (Shaw et al. 1999b [Rapala et al. 1993]). Researchers found that microcystin toxin production rates decreased as the cell division rates decreased due to nitrate-limitation in the culture. They explain this by claiming microcystin production is controlled by environmental effects on cell division, not through metabolic pathways (Orr and Jones 1998). Researchers found negative correlations between temperature and cellular CYN concentrations in cultures (Griffiths and Saker 2003 [Saker and Griffiths 2000]). Toxin production is likely a function of stress, and grazing pressures may influence CYN production in *C. raciborskii* (Fabbro et al. 2001). Grazing pressure has been documented to increase microcystin production in *Microcystis aeruginosa* (Jang et al. 2003).

While *C. raciborskii* growth has been positively correlated with ammonium concentrations (Hawkins et al. 2001; Saker and Neilan 2001; Shafik et al. 2001), CYN production is negatively correlated with ammonium concentrations. Cultured *C. raciborskii* produced more CYN in the absence of N-fixed source and less with ammonium (Saker and Neilan 2001; Griffiths and Saker 2003 [Saker et al. 1999; Saker 2000]; Hawkins et al. 2001; Li et al. 2001). Ammonium, as a by-product of zooplankton metabolism, has also been shown to promote decreasing CYN production in cultures (Saker and Neilan 2001).

CYN production is highly variable. *C. raciborskii* does not appear to consistently produce toxins. Correlations between CYN concentration and *C. raciborskii* biomass are weak (Griffiths and Saker 2003). While some researchers have recorded CYN concentrations up to 589 μg/L with a population of $32.5x10^6$ cells/mL there are reported instances of large *C. raciborskii* populations, up to $300x10^3$ cells/mL that were free of detectable CYN (Saker and Eaglesham 1999). However, researchers have found that typically only *C. raciborskii* concentrations over $15x10^3$ cells/mL have detectable CYN concentrations (McGregor and Fabbro 2000). Shaw et al (1999) found concentrations of 0.1 μg/l or greater were always associated with *C. raciborskii* populations over $16x10^3$ cells/mL (Griffiths and Saker 2003). In addition to environmental pressures influencing CYN production, genetic variability may also explain varying CYN concentrations. There are multiple isolates of *C. raciborskii*. Researchers have found that they all exhibit a variety of CYN content, varying up to several orders of magnitude (Griffiths and Saker 2003 [Saker 2000]). Saker and Neilan (2001) identified genetic differences as the main source of CYN variations between seven *C. raciborskii* isolates.

The age of the bloom also seems to influence CYN production. Cultures have shown that in the growth phase, 80-90% of CYN is intracellular. As cultures reached the exponential growth phase, intracellular CYN concentrations grew twice as fast as biomass. In the post exponential phases, extracellular CYN accounted up to 50% of the total toxin (Saker and Griffiths 2003 [Saker and Griffiths 2000]; Hawkins et al. 2001). This may be due to lysis of aging cells, or that older cells are more 'leaky.'

C. raciborskii is not the only cyanobacteria to produce CYN. CYN is produced by *Anabaena bergii, Aphanizomenon ovalisporum, Raphidiopsis, Umezakia natans,* and *C. raciborskii* (Griffiths and Saker 2003). *C. raciborskii* and *Aphanizomenon ovalisporum* were both present in the Palm Island poisoning (McGregor and Fabbro 2000 [McGregor and Everding 1998]). To confirm *C. raciborskii* was at fault, Hawkins undertook a 4 year study on the ecology of the reservoir. He found and cultured two varieties of *Anabaena circinalis* and previously unreported *C. raciborskii*. Toxicity studies proved that only *C. raciborskii* was highly toxic (Hawkins 1985). In contrast, Shaw et al. (1999) found that in a different lake in Queensland, Australia, CYN concentrations ranged from 4μg/L to 120μg/L in samples dominated by *Anabaena ovalisporum*.

Methodology to confirm toxicity of cyanobacterial toxins is complex and very expensive. Current tests include chemical assays, mouse bioassays, and protein synthesis assays. Each of these tests require high concentrations of cells and toxin, both of which vary according to environmental conditions, and can take up to a week to obtain results (Fergusson and Saint 2003). As observed in Ball Lake, *C. raciborskii* blooms can change dramatically in a single week. The most accurate method to confirm toxicity is based upon molecular classification which involves amplification, cloning, sequencing, and phylogenic reconstruction based on the entire 16s rRNA gene. However, it is a very time consuming and complicated process. Castiglioni et al. (2004) have developed a universal microassay based upon a litigation detection reaction (LDR) and polymorphism of the 16s rRNA gene to target 19 different cyanobacteria. This test is sensitive up to 1 fmol (femto = 10^{-15}) of the PCR (Polymerase Chain Reaction) amplified 16s rRNA gene. Fergusson and Saint (2003) are developing a PCR test that is 'rapid, reliable, and economical.' Their multiplex PCR assay works in less than one day by identifying both CYN and the potential of *C. raciborskii* to produce CYN by identifying enzymes involved in secondary metabolite synthesis. In the future, researchers hope to develop handheld devices for the detection of toxic algae (Metflies et al. 2004).

2.5 Toxin treatments

While many factors control or influence toxin concentrations, one of the most critical components to effective toxin removal is whether the toxin is intracellular or extracellular. Intracellular toxins are those that are trapped within the bacterial cell. They do not pose a human health risk unless they are released from the cell into the water column, where they become extracellular. Typically, cyanobacterial cells release their toxins only when they are stressed from unfavorable environmental conditions or when the cells lyse and dye. The CYN can remain in water column up to 6 weeks after *C. raciborskii* blooms drop below detectable levels (McGregor and Fabbro 2000). Therefore, depending on cyanobacterial identification to guide treatment regimes is ineffective. Drinking water treatment facilities must be able to deal with both forms of toxins.

2.5.1 Intracellular Toxin Removal

Slow Sand and Membrane Filtration

Slow sand filtration is low cost, widely used option for small scale water treatment systems. It works by mechanically removing particles as they move through a sand bed which develops a biofilm that allows for biological degradation of dissolved substances. The benefit of

slow sand filtration is the gentle removal of cells that minimizes the release of extracellular toxins. However, blocking caused by overloading can limit the effectiveness of sand filtration (Chorus and Bartram 1999).

Similarly, membrane filtration is the physical removal of materials via a semi-permeable membrane. While there is some evidence that membrane filtration may damage a small portion of cells, no significant increases of toxins have been observed (Chorus and Bartram 1999 [Chow et al. 1997]). Filtration alone cannot be expected to remove extracellular toxins. It is best used in combination with other treatments that will remove extracellular toxins (Griffiths and Saker 2003).

Coagulation and Dissolved Air Flotation

Coagulation works by using chemicals such as aluminum or ferric chloride to aggregate smaller particles into larger particles which can be removed by sedimentation, filtration, or flotation. It is effective at removing intact cyanobacterial cells, but not at removing toxins. The effectiveness is also dose dependent, and there are reports of high doses of alum promoting cell lysis (Chorus and Bartram 1999; Haider et al. 2003).

Dissolved Air Flotation (DAF) involves introducing air bubbles following a flocculation stage which causes the floc to float to the surface. DAF may be more effective than sedimentation because floating sludge tends to be removed more frequently than in settling tanks where the build up of algae may die and lyse. Periods of high turbidity however interrupt this process (Chorus and Bartram 1999).

2.5.2 Extracellular Toxin Removal

Chlorination

Chlorination is perhaps the most common treatment for removal of dissolved algal toxins in North America (Svrcek and Smith 2004). It typically involves exposing the water to variable doses of chlorine for thirty minutes at an elevated pH (Chorus and Bartram 1999). Chlorine is effective at removing microcystin, nodularin, saxitoxins, and cylindrospermopsin at varying pH ranges, but not anatoxin-a. However, little has been done to characterize the chlorination by-products which may also be toxic (Svrcek and Smith 2004).

Chlorination would be best if used at the end of the water treatment process and in combination with filtration or coagulation. It can cause cell lysis and the release of toxins. If organic materials, including cyanobacterial cells, are removed in the primary treatments, then occupational exposure to toxic levels of chlorine can be reduced (Chorus and Bartram 1999).

Ozonation

Ozone is one of the most powerful oxidizing agents and is extremely effective at rapidly destroying microcystins, nodularins, and anatoxin-a. Because it is a complex process, it is considered an advanced treatment process (Chorus and Bartram 1999; Haider et al. 2003; Svrcek and Smith 2004). Dissolved organic carbon and alkalinity have been shown to reduce the efficacy of ozone treatments. Because ozonation causes the lysis and release of toxins, it is

recommended that ozonation also be used in combination with primary treatments (Hoeger et al. 2002; Svrcek and Smith 2004).

Photochemical degradation

Ultra violet (UV) light is widely used to remove organic compounds in water supplies and is especially effective at degrading CYN and microcystin (Haider et al. 2003). CYN is known to rapidly degrade when exposed to sunlight in the presence of plant pigments (Chiswell et al. 1999). However, destruction of most algal toxins requires a dosage of UV that is several magnitudes higher than what is practical in a water treatment plant (Svrcek and Smith 2004).

Activated Carbon

The ineffectiveness of conventional treatments at removing soluble organic material has led to the use of activated carbon in Europe and North America (Chorus and Bartram 1999). Activated carbon is used to absorb soluble organic materials such as cyanobacterial toxins. There are two main forms of activated carbon. Powdered activated carbon (PAC) is used for targeted treatments while granular activated carbon (GAC) can be used continuously. Activated carbon has been proven effective to treat microcystins and saxitoxins (Svrcek and Smith 2004), but tests have shown that chlorine is more effective than PAC at removing CYN (Griffiths and Saker 2003 [Gray 1996]). It must be cautioned that after a treatment, the toxins still remain adsorbed to the activated carbon until they are biodegraded or chemically destroyed (Svrcek and Smith 2004).

2.6 Risk, Exposure, and Guidelines

Due to the unpredictable nature of cyanobacterial toxin production, managing human health risks in water is a difficult task. Human health risks are both a function of cyanobacterial toxicology and exposure. Reducing human exposure to cyanotoxins can be accomplished through preventing the occurrence of cyanobacterial blooms (by using best management practices) or by utilizing preventative measures (such as drinking water or bathing restrictions and water treatment methods listed above).

The seriousness of an exposure to cyanotoxins is dependent on bloom intensity (cells per unit volume), toxicity of the bloom (type of toxin and its concentration at exposure), and the route of exposure. There are three main routes of exposure to cyanotoxins: direct contact of exposed body parts, accidental swallowing, and inhalation of water. Intake through ingestion or aspiration, both documented to cause animal and human illnesses, represent high risks of cyanotoxin exposure in water sports. Culture experiments indicate that repeated exposure to microcystins can cause cumulative liver damage. Direct contact, while not well documented, can result in dermal reactions such as skin rashes (Chorus and Bartram 1999).

The “Tolerable Daily Intake” (TDI) is the amount of a harmful substance that can be consumed over a lifetime without causing significant adverse health effects. To minimize human health risks from cyanobacteria, the World Health Organization (WHO) has developed guidelines for drinking water quality. These guideline values are calculated by estimating the TDI for an individual based upon body size, daily water intake, and the proportion of the daily intake of the contaminant that is ingested from drinking water (Chorus and Bartram 1999).

The WHO has adapted a provisional guideline of 1µg/L for microcystin-LR. Due to insufficient data on other cyanobacterial toxins, this is the only WHO developed guideline (Chorus and Bartram 1999). However, countries such as Australia are leading the way to developing provisional guidelines for Anatoxin-a, saxitoxin, and CYN. While there are no formal guidelines in the USA, individual states are developing their own guidelines. Worldwide provisional drinking water guidelines are as follows:

Anatoxin-a
- Australia: 3 µg/L suggested

Saxitoxin
- Australia: 3 µg /L suggested
- 80 1µg/ 100g shellfish (used in North America to close shellfish growing areas)

Cylindrospermopsin
- Australia: 1-13 µg /L
- NOAL 1ug/L (Humpage and Falconer 2003)

Microcystin LR
- 1 µg/L (Falconer et al. 1999)
  * WHO guideline
- 1 µg/g (1ppm) for microcystins in Oregon (Oregon Health Division and Oregon Dept. of Agriculture)

(Adapted from Chorus and Bartram 1999)

While the WHO did not develop specific guidelines for all cyanobacterial toxins, they did develop "Safe Practice Guidelines" for health impairments in recreational waters. These guidelines are based upon incremental levels of risks based upon cyanobacterial cell counts. The WHO guidelines are in summarized in Table 2-4. In concert with these guidelines, lake managers should also include algal identifications, cell count estimates, chlorophyll-*a* concentrations, nutrient assessments, Secchi disk transparency measurements, and toxicity tests to quantify and predict cyanotoxin risks.

There are seven countries that are moving towards establishing MAC (maximum acceptable concentrations) for microcystins (Svrcek and Smith 2004). In an effort to better understand the risks of cyanotoxins in the USA, the Environmental Protection Agency (EPA) has included nine microorganisms, including freshwater algae and their toxins, in the 1998 Contaminant Candidate List (CCL) as part of the Safe Drinking Water Act (SDWA). The CCL is a list of contaminants, which lack critical information to make regulatory decisions. While the CCL does not specify which toxins will be studied, the Office of Groundwater and Drinking Water is currently reviewing toxicological, epidemiological, and occurrence studies and drinking water treatment literature (Jim Sinclair 2005 pers. comm.).

Table 2-4. World Health Organization Cyanobacterial "Safe Practice Guidelines"

GUIDANCE LEVEL	GUIDANCE DERIVATION	HEALTH RISKS	RECOMMENDED ACTION
1. High risk of adverse health effects			
Cyanobacterial scum formation in bathing areas, 10,000,000 cells/ml or 5000 µg chlorophyll-*a*/L	*Inference from oral animal lethal poisonings *Actual human illness case histories	*Potential for acute poisoning *Potential for long-term illness with some cyanobacterial species *Short term adverse health outcomes, e.g. skin irritations, gastrointestinal illness	*Immediate action to prevent contact with scums *possible prohibition of swimming and other water-contact activities *Public health follow-up investigation
2. Moderate probability of adverse health effects			
100,000 cells cyanobacteria/ml or 50 µg chlorophyll-*a*/L with dominance of cyanobacteria	provisional drinking-water guideline for microcystin-LR, and data concerning other cyanotoxins	*Potential for long-term illness with some cyanobacterial species *Short term adverse health outcomes, e.g. skin irritations, gastrointestinal illness	*Watch for scums *Restrict bathing and further investigate hazard *Post on-site risk health advisories *Inform health authorities
3. Relatively Mild and/or low probabilities of adverse health effects			
20,000 cells cyanobacteria /ml or 10 µg chlorophyll -*a*/L with dominance of cyanobacteria	human bathing epidemiological study	*Short term adverse health outcomes, e.g. skin irritations gastrointestinal illness, probably low frequency	*Post on-site risk health advisories *Inform health authorities

(Adapted from Chorus et al. 2000)

3.0 Methods

3.1 Sampling locations and schedule

A two-pronged sampling approach was used to collect water samples for the detection of *C. raciborskii.* This approach used both non-targeted and targeted sampling. During June, July, and August of 2002 and 2003, we collected samples of *C. raciborskii* as part of our routine lake assessment protocol with the Indiana Clean Lakes Program (CLP). Approximately 160 lakes throughout northern Indiana were included in this non-targeted sampling. In 2004, samples were collected using a second approach targeting *C. raciborskii* bloom conditions by trained citizen volunteers who participate in the Volunteer Lake Monitoring Program. We also sampled lakes with symptoms of *C. raciborskii* during routine CLP sampling in August of 2004. Lakes included in targeted sampling were located in various counties in Indiana (Figure 3-1).

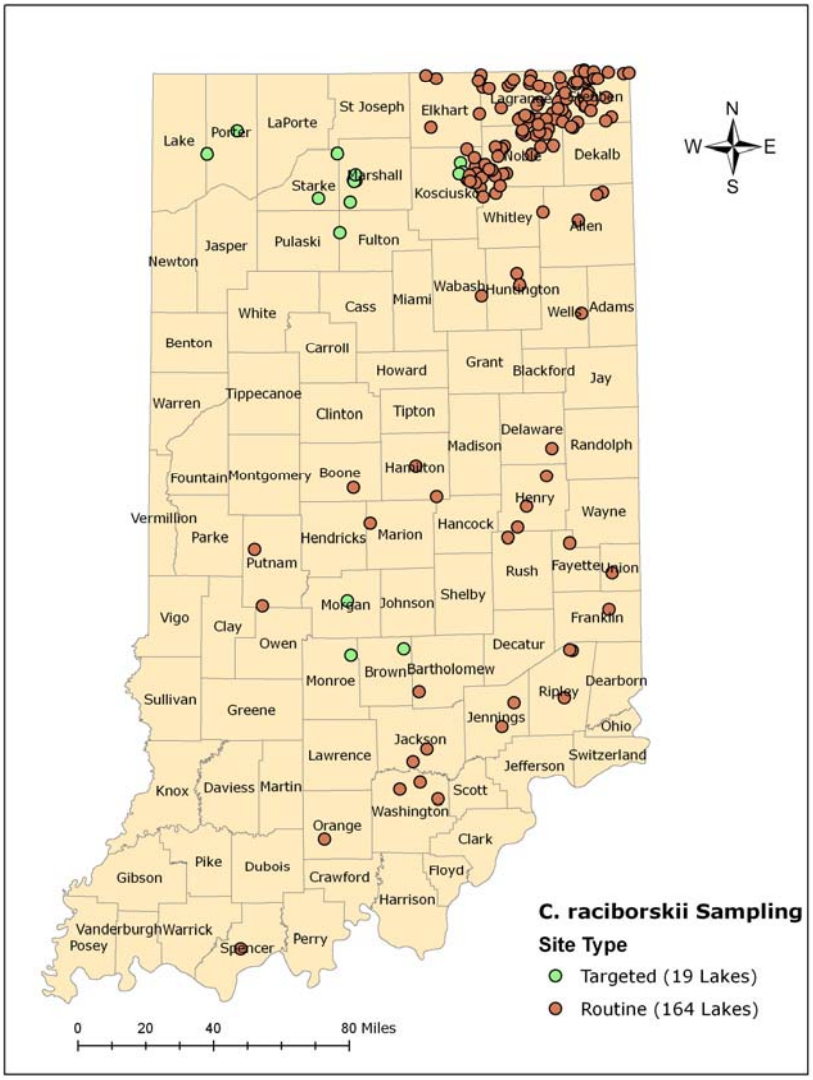


Figure 3-1. Indiana lakes sampled for *C. raciborskii.*

3.2 Field Sampling

3.2.1 Clean Lakes Program

All *C. raciborskii* samples were obtained using a two meter long, 1" I.D. integrated PVC pipe sampler at the lake's maximum depth. Water from the two meter water column layer was mixed before adding a portion to bottles for preservation. Algal samples were preserved using Glutaraldehyde (final concentration of 0.25-0.50%) and stored in a dark refrigerator (St. Amand 2002b). In addition to the collection *C. raciborskii* samples, water samples for each lake were analyzed for nutrients (chlorophyll *a*, TKN, nitrate-nitrogen, ammonia-nitrogen, soluble reactive phosphorus, and total phosphorus) and physical and chemical parameters (Secchi disk transparency, dissolved oxygen, pH, alkalinity, and temperature) as part of the regular CLP protocol.

3.2.2 Volunteer Lake Monitoring

In the late summer of 2004, volunteer monitors at thirty-nine lakes were asked to collect samples if their lake experienced a decrease in water transparency along with the appearance of 'green' or 'dark' water below the surface without a surface bloom. All samples were collected using an integrated sampler and shipped on the day of collection to the CLP lab in Bloomington, IN using express delivery. Once in the lab, samples were preserved using Glutaraldehyde (final concentration of 0.25-0.50%) and stored in a dark refrigerator.

3.3 Lab Analysis

3.3.1 Slide Preparation

Four permanent algal sample mounts were produced for each lake using the HPMA (2-hydroxypropyl methacrylate) method (St. Amand 2002b). Samples were filtered through mixed ester nitrocellulose filters (0.45 um, 25 mm, plain) placed on a three station Millipore Filtration Tower. The first algal mount of each lake was used to obtain a relative estimate of the filtration volume necessary to have 20 – 30 cells in each 200 x field of view. The final three algal mounts were made using a constant sample volume between 5 – 75 mL. After the samples filtered through the towers, the ester nitrocellulose filters were removed and placed face down on glass cover slips (25 mm X 25 mm, #1.5). One- two drops of HPMA resin, obtained from PhycoTech Consulting, were then added to cover the back of the filter. After residing in a drying oven for 12–24 hours, the cover slips were then attached to glass slides after an additional application of the HPMA resin. Slides were then placed in the drying oven and allowed to polymerize for 48 hours.

3.3.2 Counting and Algal Identification

C. raciborskii cells were identified and enumerated using a Nikon Optiphot-2 epifluorescent microscope equipped with a Chroma Technology Corp. Phycoerythrin R and B long pass filter assembly (excitation at 545 nm and emission at 590 nm). Identification of *C. raciborskii* was based upon training, a CD, and images from Ann St. Amand. Morphotype, trichome length, and cell sizes were recorded.

Counting consisted of scanning 15 fields at 400x per slide under epifluorescence and phase contrast. *C. raciborskii* concentrations were recorded in both natural units (NU/mL) and number of cells (# cells/mL). The formulas used were:

NU/mL = (tower area * NU count) / (number fields * field area * filter volume)

cells/mL = (NU/mL * average size of NU) / (length of cell)

3.4 Data processing

All results were entered into a MS Access database. Relationships between environmental parameters (total Kjeldahl nitrogen (TKN), nitrate-nitrogen, ammonia-nitrogen, soluble reactive phosphorous (SRP), total phosphorous (TP), Secchi disk transparency, dissolved oxygen (DO), pH, alkalinity, temperature) and 'detect' and 'nondetect' samples were examined in MS Excel and SigmaPlot.

4.0 Results

4.1 Phytoplankton Assemblages

During the three year study period, *C. raciborskii* was detected throughout the state of Indiana. Of 182 lakes sampled, 19 had detectable *C. raciborskii.* Lake Lemon was sampled on two dates and from two sites on one date so a total of 184 samples were analyzed. Both the straight and curled morphotypes were observed. A third coiled morph was also present in a small number of lakes (Figure 4-1). Mean trichome length for the straight form was 50 ± 34µm (mean ± standard deviation) (n = 17). Trichomes of the curled form were smaller than the straight morphotype with a mean trichome length of 27 ± 18µm (n = 11). In both forms, drop-shaped heterocysts were often observed at the terminal ends of the trichomes and akinetes were rarely seen. There was little or no constriction at the crosswalls of the vegetative cells making cell size difficult to decipher. Cell lengths between forms were very similar. The straight form had an average cell length of 7.76 ± 1.82µm (n = 17) and the curled form had an average cell length of 7.27 ± 1.56µm (n = 11).

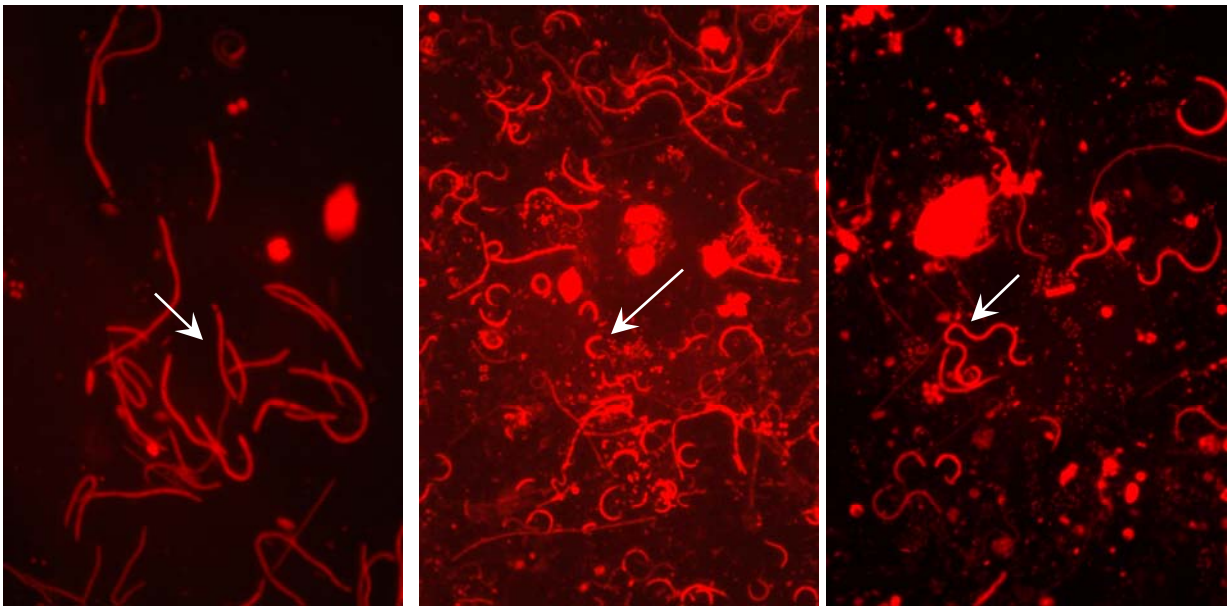


Figure 4-1. Morphotypes of *C. raciborskii* detected in Indiana imaged using epi-fluorescence, which colors blue-green algal cells bright red. Straight morph (A), coiled morph (B) and a coiled morph (C).

Overall the straight form was more common, occurring in 19 of 20 samples (17 of 18 lakes) with detected *C. raciborskii* populations. Only one sample had only the curled morph while nine samples had only the straight morph. In six of the ten samples containing both morphs, the straight form was most prevalent. The curled form was only detected in eleven samples. Concentrations of straight cells were also higher than curled cells (T test, p = 0.3510, DF = 38) (Figure 4-2).

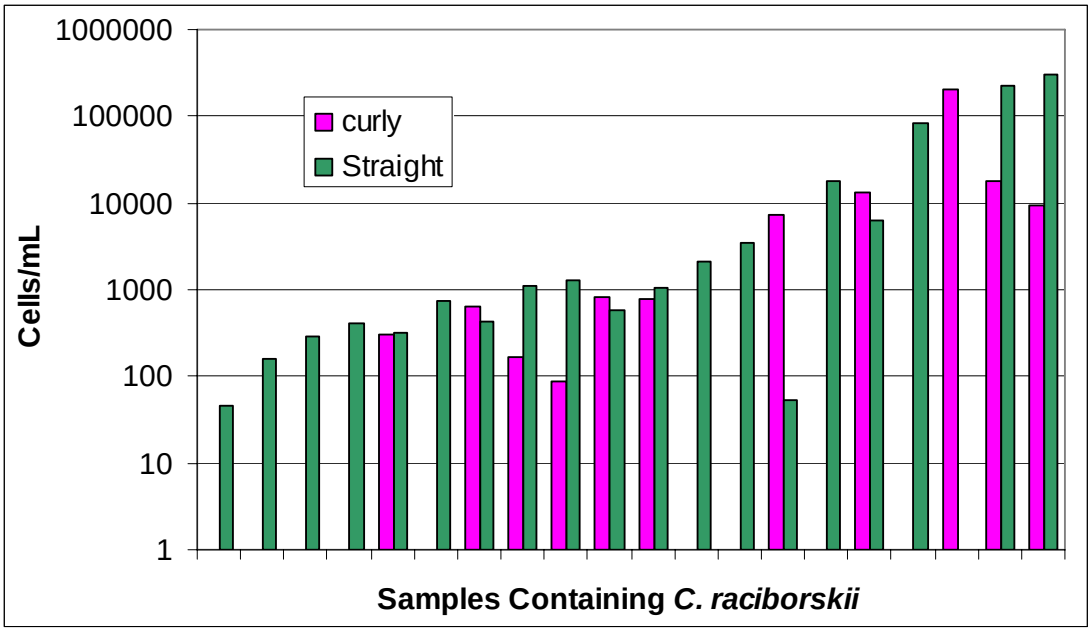


Figure 4-2. Occurrence of straight and curly morphs in Indiana Lakes.

4.2 Spatial Analysis

C. raciborskii was detected in sixteen counties throughout the state of Indiana during our study period (Table 4.1). The complete list of sampled lakes is included in Appendix A. Five of the detected lakes are currently being used as public drinking water supplies. While the routine sampling was primarily confined to the northeastern corner of the state, detected *C. raciborskii* populations appear focused in central Indiana (Figure 4-3). Five lakes within the Upper White River Watershed (HUC 05120201) comprising Owen, Morgan, Johnson, Hendricks, Marion, Hancock, Boone, Hamilton, Tipton, Madison, Delaware, Randolph, and Henry counties had *C. raciborskii* detected (Figure 4-4).

Table 4-1. Distribution and Abundance of *C. raciborskii* Detected in Indiana Lakes and Reservoirs.

Lake Name	County	Date	cells/mL	Lake Name	County	Date	cells/mL
Lemon (Riddle Pt)	Monroe	7/7/2002	357,592	Kunkle	Wells	8/20/2002	1,389
Lemon (Reed Pt)	Monroe	8/18/2004	315,632	Eagle Creek	Marion	8/14/2002	1,364
Lemon (Reed Pt)	Monroe	7/7/2002	246,642	Chrisney	Spencer	7/15/2002	1,250
Glen Flint	Putnam	8/7/2002	204,251	Bruce	Fulton	8/16/2004	1,072
Prairie Creek	Delaware	8/20/2002	84,257	Koontz	Starke	8/17/2004	747
Morse Reservoir	Hamilton	8/14/2002	19,640	Brush Creek	Jennings	7/16/2002	624
Ole Swimming Hole	Morgan	8/16/2004	17,894	Starve Hollow	Jackson	7/16/2002	416
Cagles Mill	Putnam	8/7/2002	7,292	Bass	Starke	8/16/2004	289
Bischoff	Ripley	7/22/2002	3,445	Clare	Huntington	8/19/2002	163
Cedarville	Allen	8/13/2002	2,131	Irish	Kosciusko	8/18/2003	64
Geist Reservoir	Marion	8/14/2002	1,861	* 5 of the 'Detect' Lakes are Public Water Supplies			

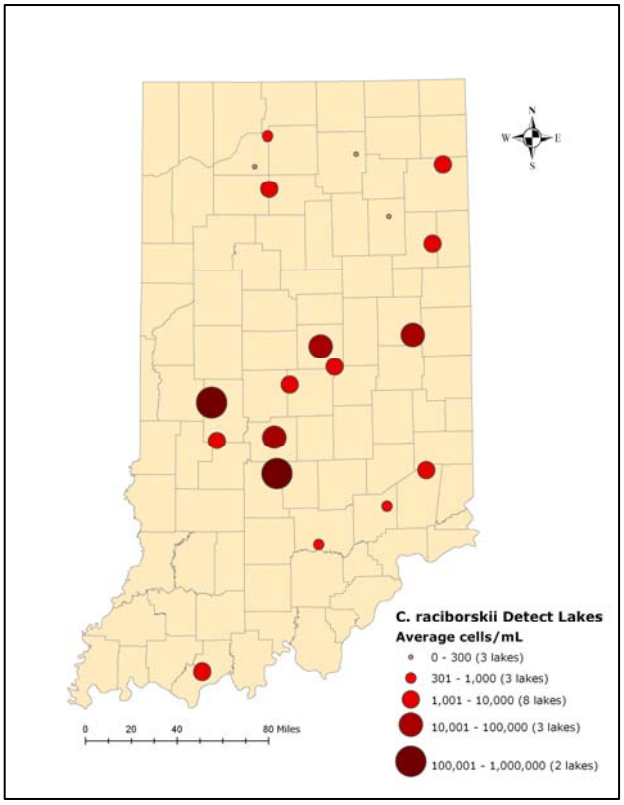


Figure 4-3. Distribution and abundance of *C. raciborskii* in Indiana.

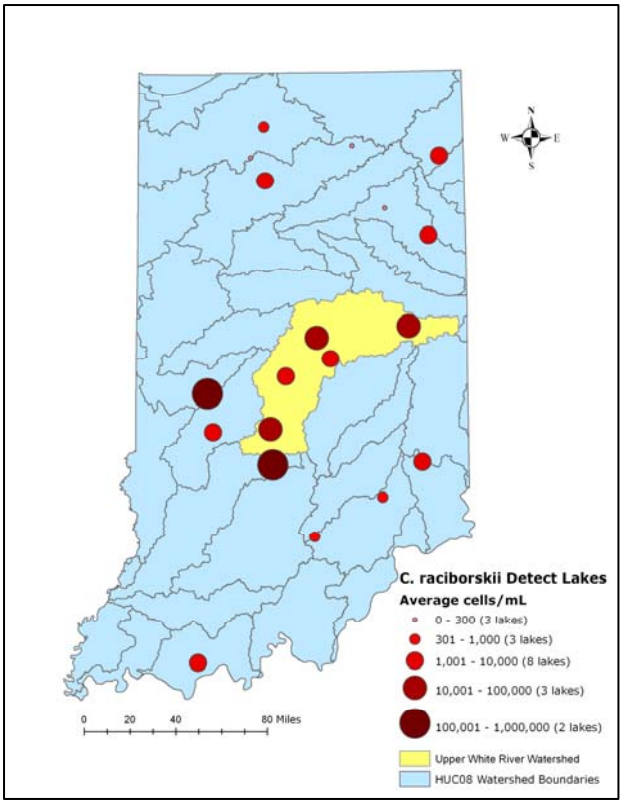


Figure 4-4. Distribution and abundance of *C. raciborskii* in watersheds (8 Digit HUC).

4.3 Lake Characteristics

To attempt to identify characteristics that differentiate the 'detect' lakes from the 'non-detect' lakes, we looked at multiple chemical, biological, physical, and morphological features of each lake. Lakes with detected *C. raciborskii* populations had significantly shallower maximum depths (8.7 m) than non-detect lakes (12.5 m, $p = 0.03$, d.f. = 182) (Figure 4-5). Similarly, 'detect' lakes had lower Secchi disk transparency (mean Secchi depth 0.71 m vs. 2.26 m, $p = 0.00001$, d.f. = 175) (Figure 4-6). 'Detect' lakes also had higher concentrations of epilimnetic total phosphorous (mean TP = 0.081 mg/L vs. 0.037 mg/L, $p = 0.000005$, d.f. = 177) (Figure 4-7) and chlorophyll-*a* (mean chl-a = 17.58 µg/L vs. 7.40 µg/L, $p = 0.00008$, d.f. = 165) (Figure 4-8). There were no statistically significant correlations between surface temperature, SRP, nitrogen, pH, or zooplankton abundance. The detect lakes had a significantly higher temperature at maximum depth (18.2 ^{o}C) than did the non-detect lakes (11.8 ^{o}C) (Figure 4-9). Statistical T-test results are summarized in Table 4-2.

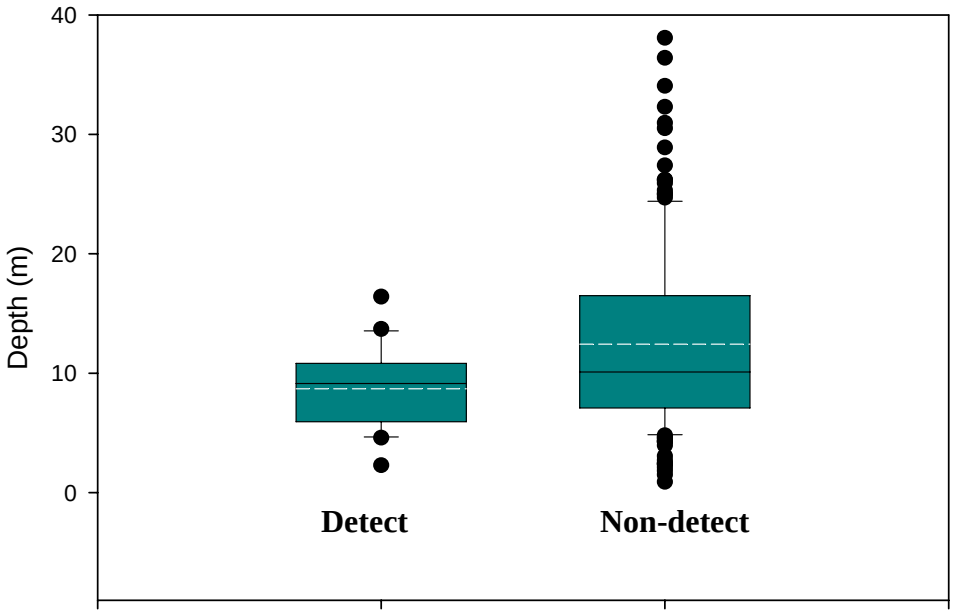


Figure 4-5. 'Box and Whisker' plot of mean maximum depth in detect and non-detect lakes. The median value is shown by the solid dark line; the mean in the dashed white line. The top of the box represents the 75 percentile of the sample distribution while the bottom of the box is the 25 percentile. The 90th and 10th percentiles are indicated by the lines and all outliers are shown as filled circles.

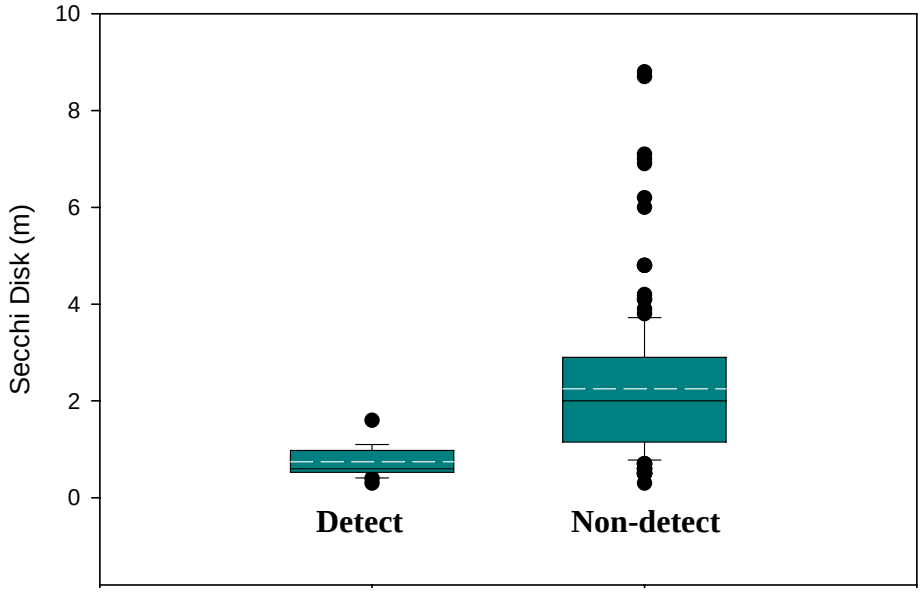


Figure 4-6. Mean Secchi disk transparency depths in detect and non-detect lakes.

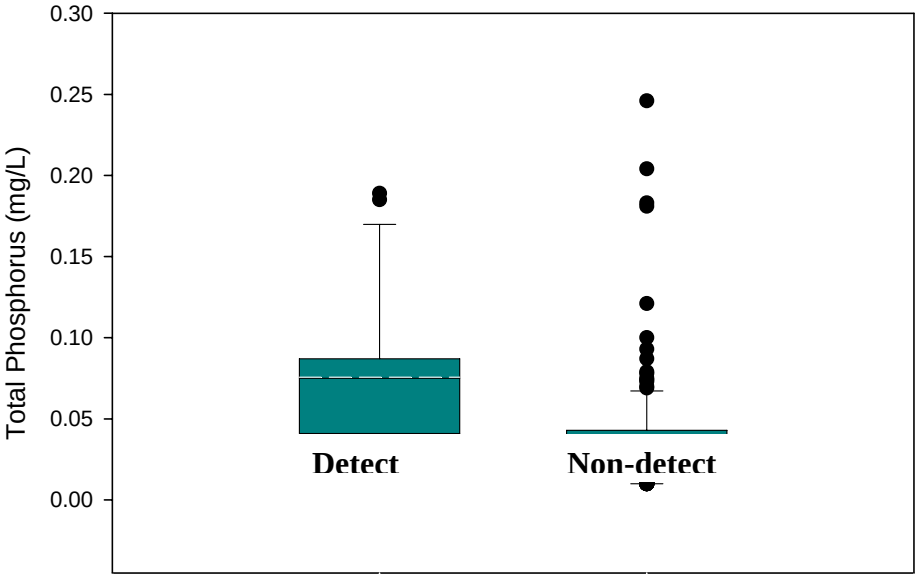


Figure 4-7. TP concentrations in detect and non-detect lakes.

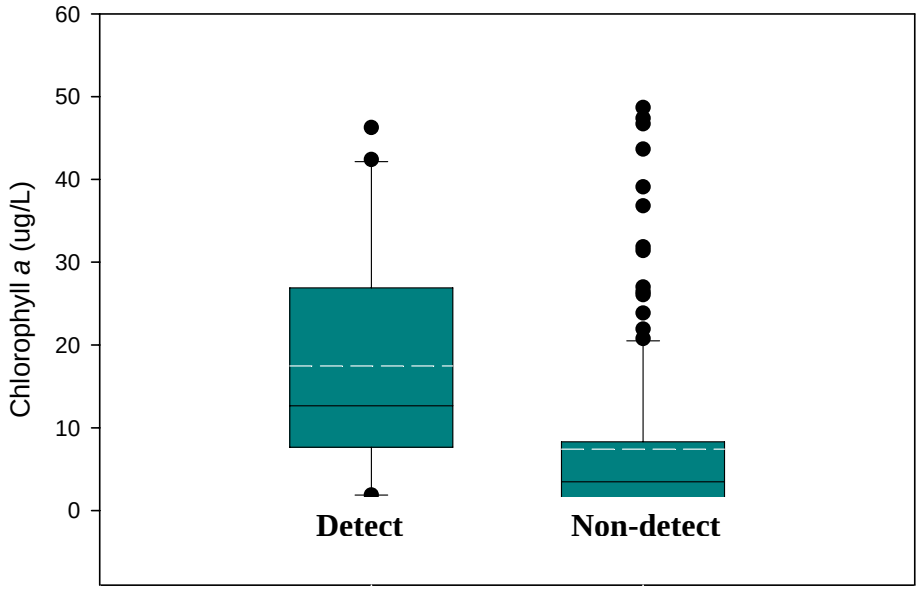


Figure 4-8. Chlorophyll *a* concentrations in detect and non-detect lakes.

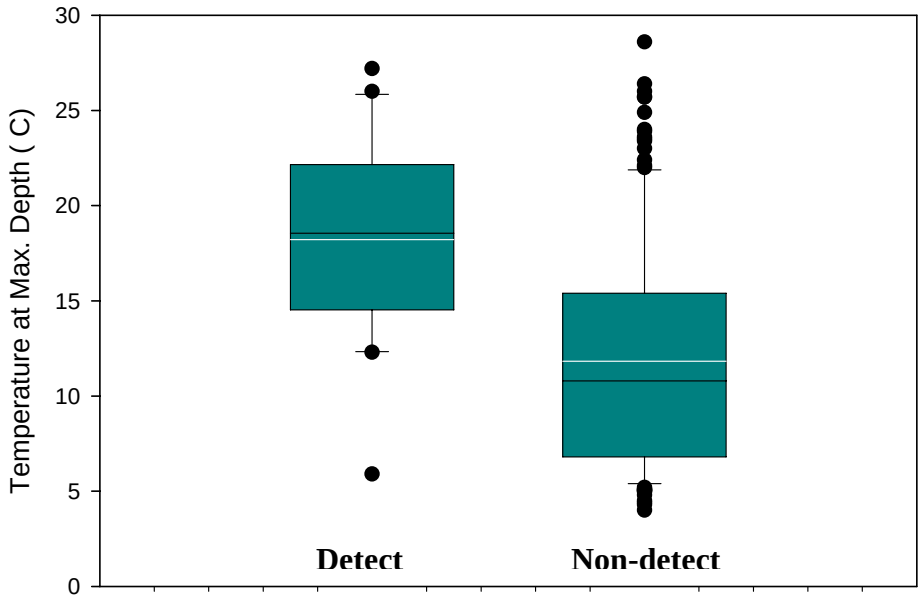
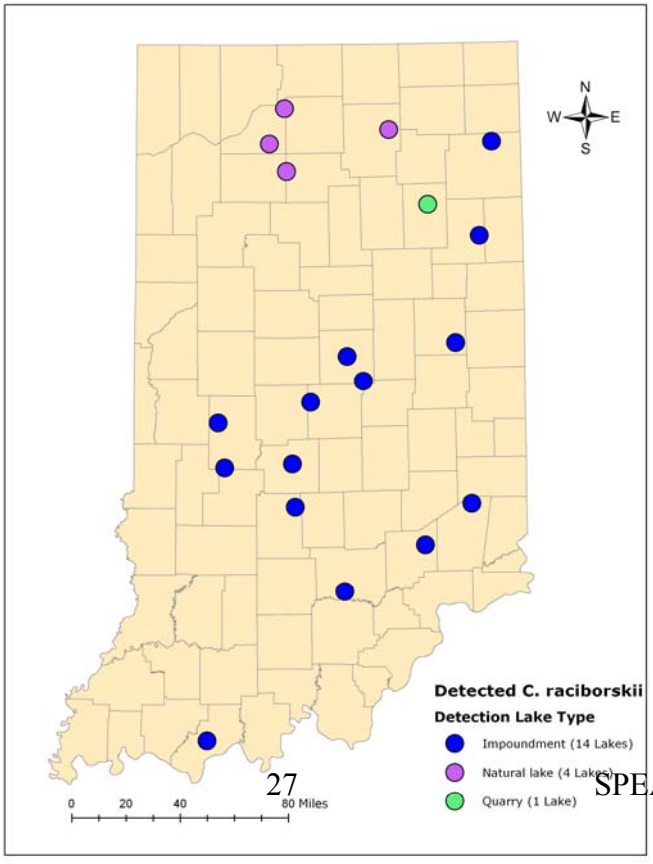


Figure 4-9. Water temperature one-meter off the bottom at the maximum lake depth in detect and non-detect lakes.

TABLE 4-2. Results of T-Tests

PARAMETER	PROB.	DEGREES OF FREEDOM	MEAN, DETECT LAKES	MEAN, NON-DETECT LAKES
Max. Depth (m)	0.03	182	8.7	12.5
Secchi depth (m)	0.00001	175	0.706	2.26
Epilimnetic Total Phos. (mg/L)	0.000005	177	0.081	0.037
Epilimnetic Chlorophyll *a* (µg/L)	0.00008	165	17.58	7.40
Temp @ Max. Depth (^{o}C)	0.000008	173	18.2	11.8
Epilimnetic NH_4 (mg/L)	0.420	173	0.031	0.065

C. raciborskii in Indiana was more abundant in impoundments than in natural lakes. Populations were detected in 35% of the impoundments sampled (n = 40) and only 2.9% of the sampled natural lakes (n = 139). Of the detected lakes, 73.7% (n = 19) were impoundments. *C. raciborskii* was also detected a quarry, Lake Clare (Figure 4-10).



Carlson's Trophic State Index (TSI) (Carlson 1977) is often used to quantify the productivity of lakes. When comparing detect to non-detect lakes we observed that most of the detect lakes were above the eutrophic threshold for total phosphorous (TP) and for Secchi disk transparency depth (Figures 4-11 and 4-12).

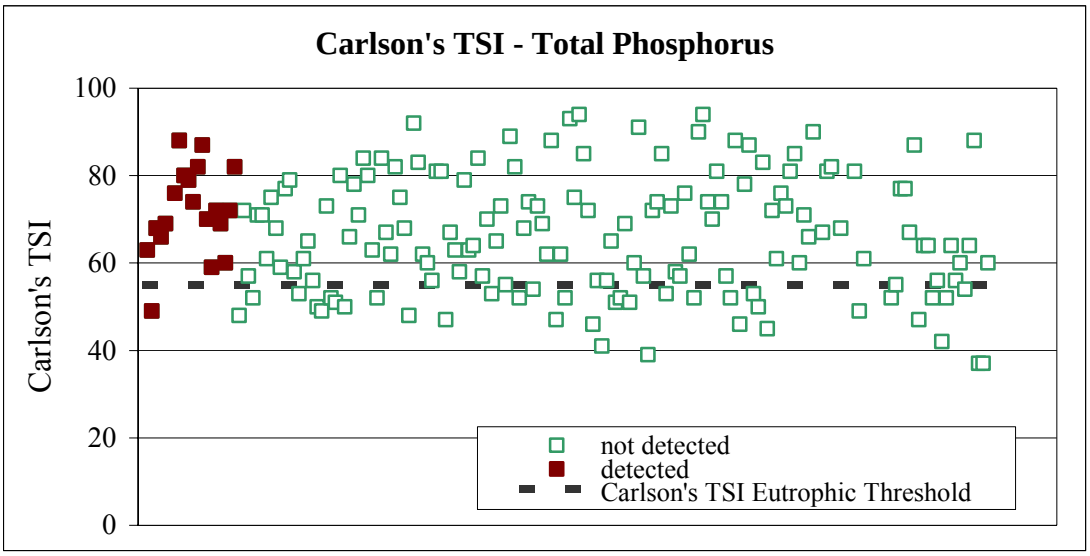


Figure 4-11. Carlson's Trophic State Index for total phosphorous in detect and non-detect lakes in Indiana.

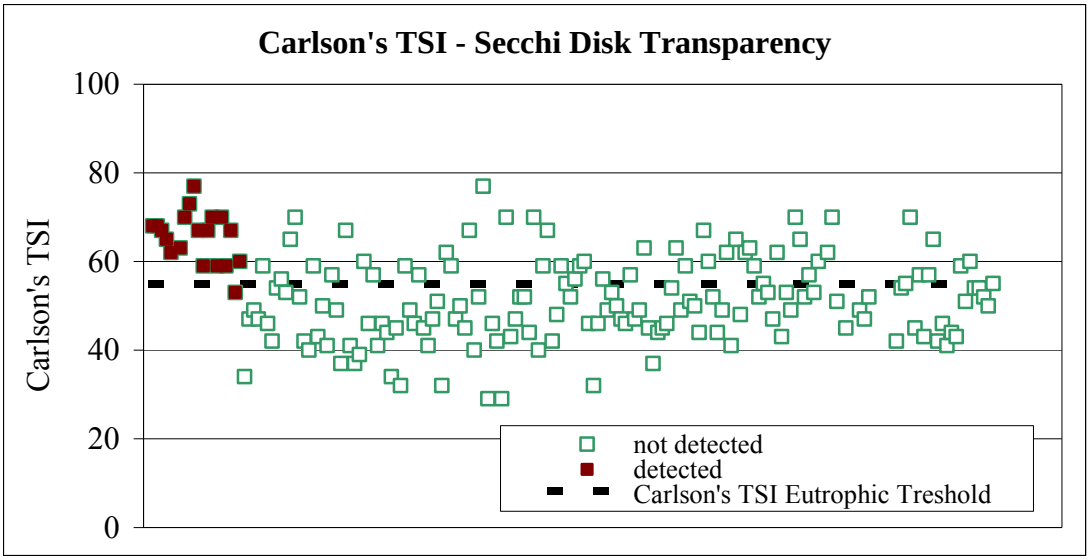


Figure 4-12. Carlson's trophic state index for Secchi disk transparency depth in detect and non-detect lakes in Indiana.

4.5 Targeted v. Non-targeted Sampling

A total of 181 lakes were sampled as part of this study. Five lakes were sampled more than once. Twenty one lakes were sampled in 2004 as part of the targeted sampling and 167 lakes were sampled during routine, non-targeted sampling in 2002 and 2003. Targeted lakes had a higher *C. raciborskii* detection rate (23.8%, n = 21) than non-targeted lakes (9.0%, n = 167) (Figure 4-13).

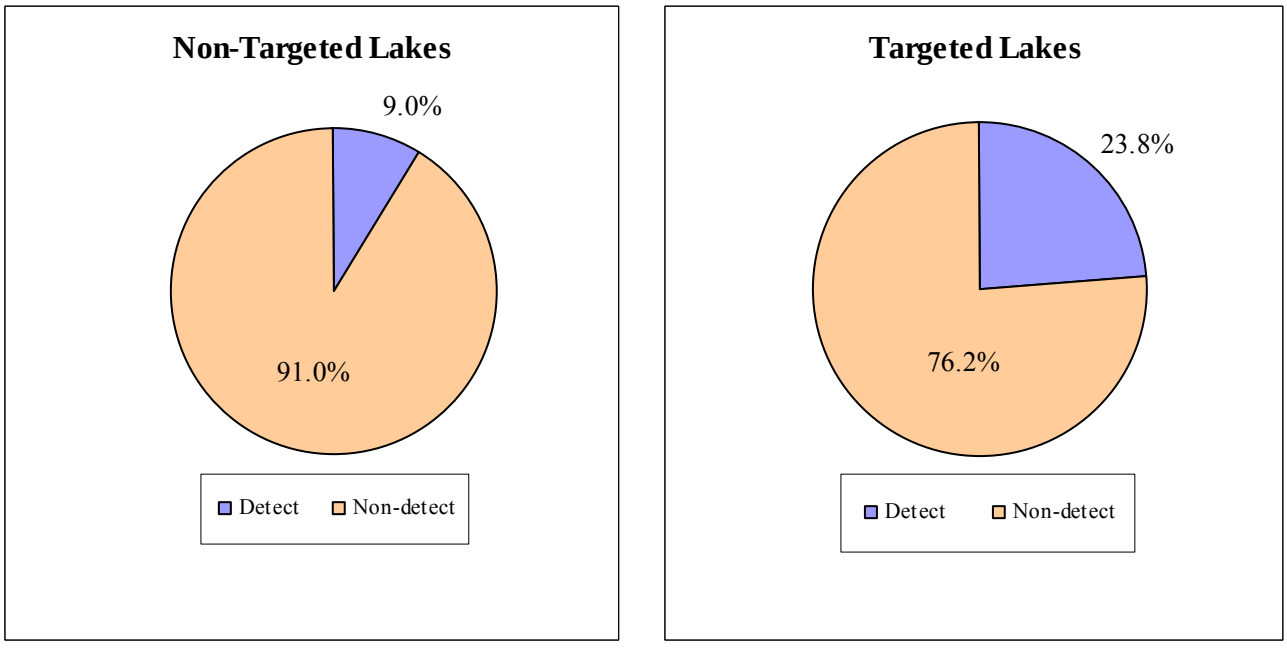


Figure 4-13. Detection of *C. raciborskii* in non-targeted and targeted lakes.

The targeted sampling utilized both trained volunteer monitors and CLP employees. Sample bottles were sent to volunteer monitors on 39 lakes in the summer of 2004. Eleven volunteer samples were collected and *C. raciborskii* was detected in one of them, Ole Swimming Hole (Figure 4-14). A survey conducted to determine why the remaining 28 lakes were untested yielded the following results: 23 volunteers did not see the described symptoms, 2 didn't have time to participate in the sampling program, and 3 volunteers did not respond to our survey.

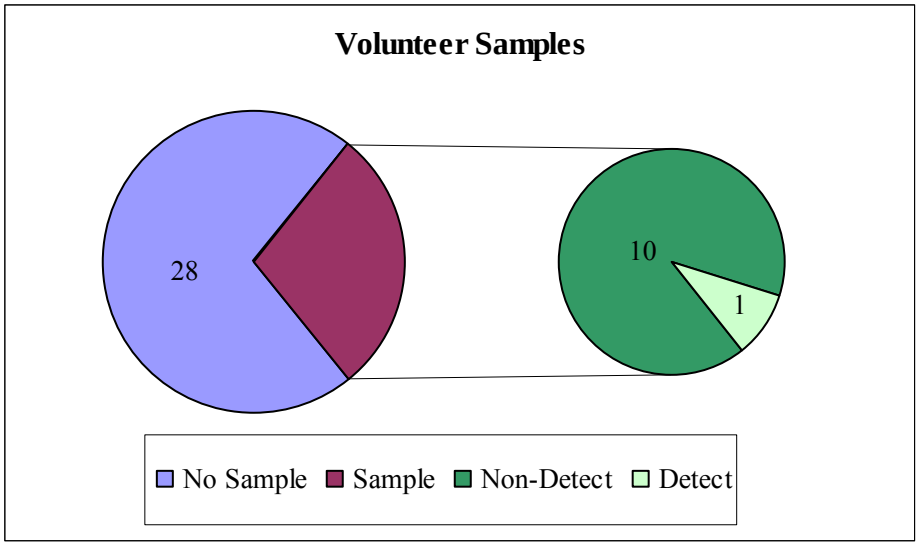


Figure 4-14. Volunteer *C. raciborskii* sampling.

5.0 Discussion

5.1 Distribution of C. raciborskii in Indiana

In addition to lakes and reservoirs sampled during this study, and the initial detection at Ball Lake, *C. raciborskii* has been confirmed in a number of other Indiana reservoirs. The Army Corps of Engineers has detected *C. raciborskii* in Monroe Reservoir, Salmonie Reservoir, Brookville Reservoir, and Patoka Reservoir (Figure 5-1). The Indianapolis Water Company, now Veolia Water Company, has detected it in Morse Reservoir, Geist Reservoir, and Eagle Creek Reservoir. Our study expands the detected distribution of *C. raciborskii* to eleven more reservoirs, four natural lakes, and one quarry (Lake Clare).

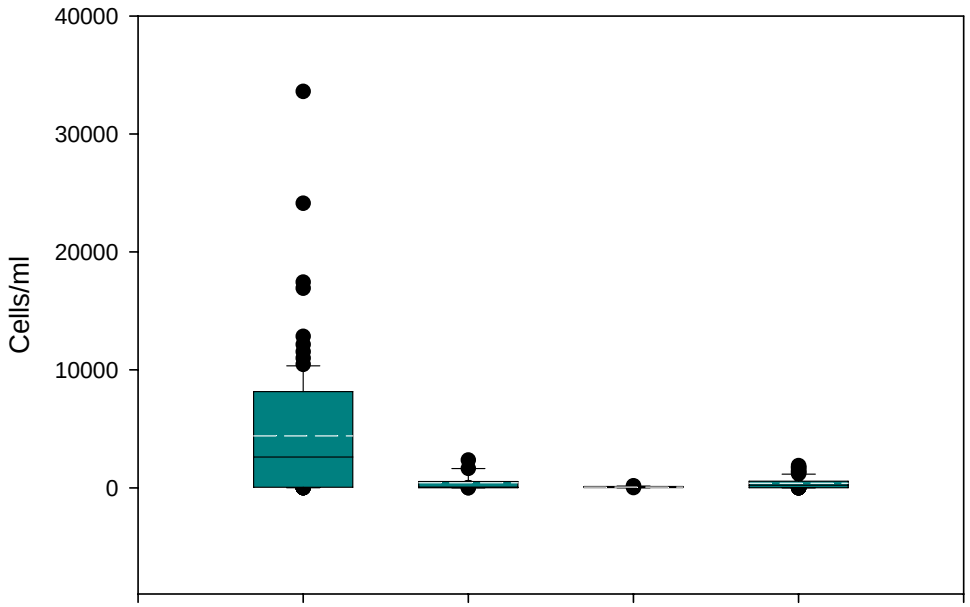


Figure 5-1. *Cylindrospermopsis raciborskii* densities in COE reservoirs. Monroe (2001-02); Salamonie (2002); Brookville (2002); Patoka (2001-02).

The present study was not randomized nor did it include all lakes and reservoirs in Indiana. Therefore, care should be taken to not misinterpret our results and conclude that these are the only lakes in Indiana that contain *C. raciborskii*. In addition, many of the lakes we included in this study were sampled in July. Our own results show that only 6 of 21 samples testing positive for *C. raciborskii* were sampled in July (Figure 5-2). Evidence from other researchers (Briand et al. 2002; Saker et al. 2003) suggests that this species is most likely to bloom during the warm, late-summer months of August and September. It is conceivable that we could have had more detections of *C. raciborskii* by conducting more of our sampling during August and September.

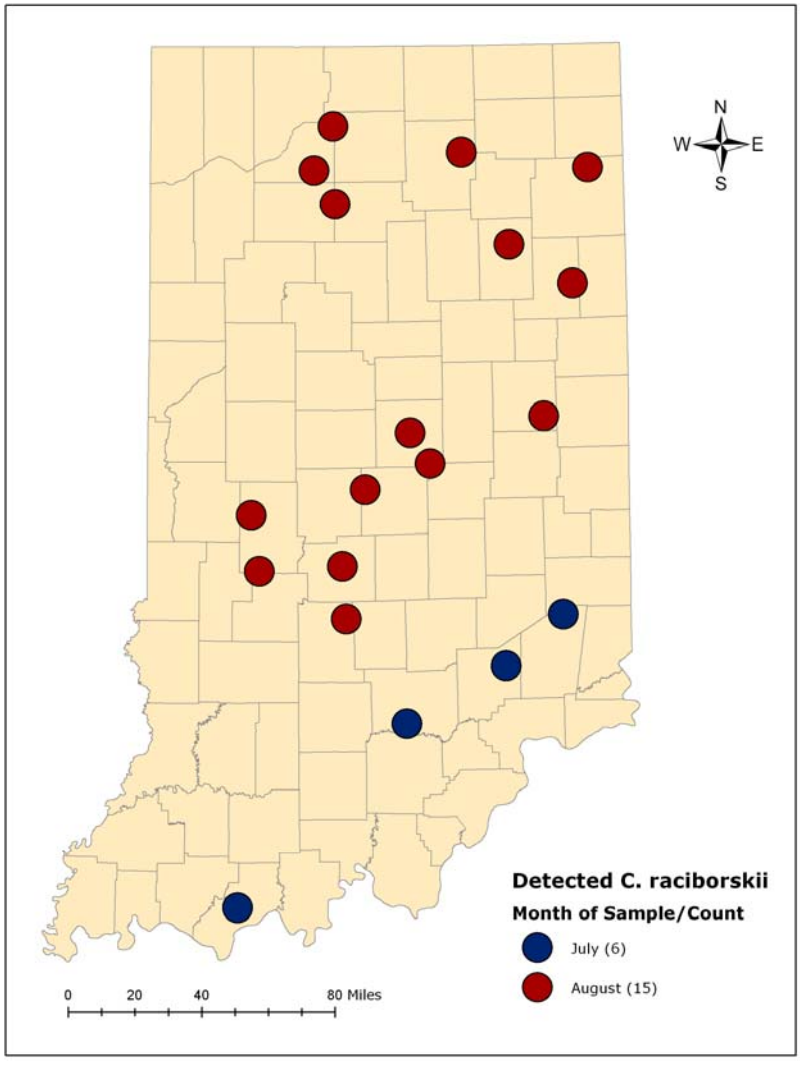


Figure 5-2. Detection of *C. raciborskii* by month.

We detected the most *C. raciborskii* in 2004, some in 2002, and only one in 2003 (Figure 5-3). Why the discrepancy? We suggest two reasons for this: meteorological events and sampling routine. Padisák (1997) suggests that in temperate regions, *C. raciborskii* is sensitive to changes in temperature and wind-induced physical disturbances. The U.S. Drought Monitor shows Indiana experienced a drought in 2002, but 2003 and 2004 were 'normal'. The summer of 2002 was 'hot and dry,' while February 2003 was the snowiest on record. Overall, 2003 was the 10th 'wettest' year on record. Large storms on Labor Day, the Fourth of July, flooding on Mothers Day, a record low temperature in June, and the wettest September on record may have combined to keep water temperatures cool, lakes stirred up, and *C. raciborskii* populations down in 2003. In 2004, May was hotter and wetter than normal, July was wetter and colder than normal, and August was cold and wet (http://www.crh.noaa.gov/ind/cli.php - National Weather Service Forecast Service, Indianapolis, Indiana).

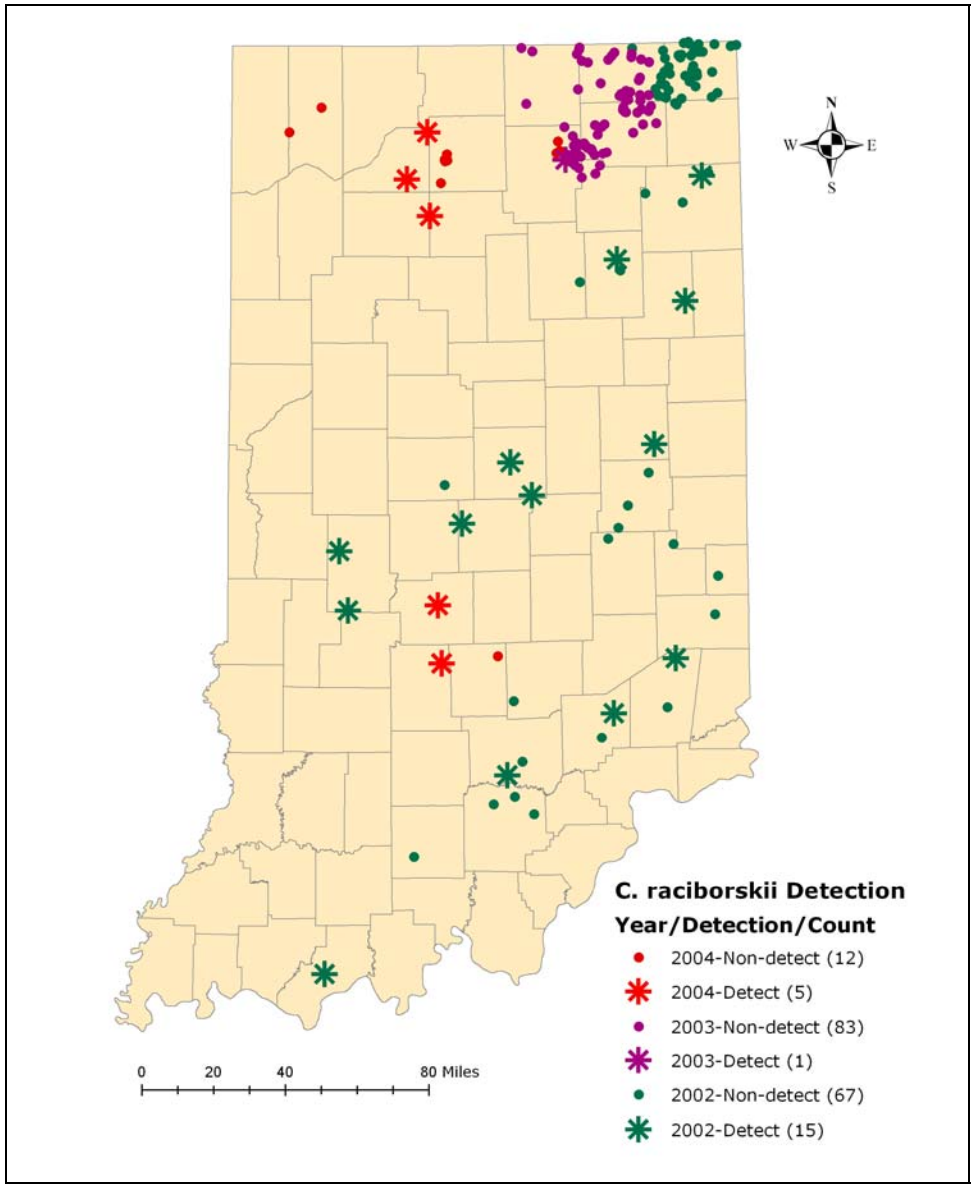


Figure 5.3. Detection of *C. raciborskii* by year.

Our own data show a strong correlation between warm, bottom temperatures and positive detection of *C. raciborskii* (Figure 4-9). In addition to warm late-summer temperatures, the temperature of bottom waters (hypolimnion) in lakes is influenced by springtime weather. Interestingly enough, a cool, prolonged spring season can allow for an extended period of mixing (spring turnover) in lakes. This prolonged period of mixing can significantly warm up the bottom waters. In contrast, a warm spring can cause a rapid onset of thermal stratification in lakes, before the bottom waters have much chance to warm up during a extended period of circulation.

A second reason for the differences in detected populations may be a function of our sampling routine. In 2002 and 2003, 'non-targeted' lakes were sampled as a part of the routine Clean Lakes Program (CLP) July and August sampling protocol. The Indiana CLP is currently on a rotation that samples each lake in the state once every five years. In 2004, we targeted lakes for sampling, during our routine lake assessment in August only and using volunteer monitors in August and September. The 'targeted' sampling was tailored to sample only lakes we believed exhibited conditions characteristic to a *C. raciborskii* bloom (decreased Secchi depth and dark, green water below the surface, with no conspicuous surface bloom). Sampling only during late summer and using a targeted protocol would be expected to increase the percentage of detected lakes.

5.2 Characteristics of lakes with 'detected' C. raciborskii populations

The lakes and reservoirs with detected *C. raciborskii* samples in this study shared three main characteristics, lake type, lake depth, and eutrophic characteristics. First, *C. raciborskii* has been detected in more reservoirs than in lakes within Indiana. Reservoirs tend to have more extensive shallows than lakes and these shallow areas can be warmer at the sediments, a major factor in successful germination of *C. raciborskii* akinetes. Reservoirs also tend to be long and narrow. This facilitates long wind fetches that can increase water circulation, prevent thermal stratification, and promote warmer temperatures at the sediments.

Second, lakes with detected populations were shallower than 'non-detect lakes. This is partially a function of lake type (see preceding paragraph) but there are also shallow natural lakes. Shallow lakes have warmer temperatures at the sediments than do deep lakes at the same latitude. Sediments of shallow lakes can reach temperatures of 22-23.5 °C, which is the optimal temperature necessary for akinete germination (Padisák 1997). The mean temperature in the deepest bottom waters of lakes in which we detected *C. raciborskii* was 18.2 ^{o}C but it was only 11.8 ^{o}C in non-detect lakes. One would expect warmer bottom temperatures in shallow areas of these lakes. There was little difference between the mean surface water temperature in detect (26.5 ^{o}C) and non-detect (26.9 ^{o}C) lakes. Other researchers have reported that *C. raciborskii* survives in temperate regions when water temperatures are above 25°C (Saker et al. 2003; Briand et al. 2002; Padisák 1997). However, in Austria, maximum *C. raciborskii* populations occurred at water temperatures of only15-18 °C (Padisák 1997 [Dokulil and Mayer 1996]).

Without germination, populations of *C. raciborskii* should not be anticipated. Also, *C. raciborskii* is sensitive to internal variations in a lake, due to either polymictic changes or wind disturbances which suggests that artificial destratification may work (Padisák 1997).

A third characteristic common to all the detect lakes was that they all exhibited eutrophic characteristics. Most of the lakes with detected populations were eutrophic according to Carlson's TSI . Each of the detect lakes had significantly lower Secchi depths, higher epilimnetic total phosphorus (TP), and higher chlorophyll-*a*. Our observations of higher TP with detected populations is contrary to what Dobberfuhl (2003) found in Florida. He saw that *C. raciborskii* declined with increasing TP values and that above 0.02 mg P/L *C. raciborskii* populations declined. The correlation of *C. raciborskii* and high chlorophyll-*a* is intriguing. *C. raciborskii* has a relatively low content of chlorophyll-*a* (Saker and Griffiths 2001) yet we saw a strong relationship here.

5.3 Current Risk

The question that this paper begs is what is the current human health risk that *C. raciborskii* is placing on Indiana lakes and reservoirs. According to World Health Organization (WHO) guidelines, lakes with over 20,000 cyanobacterial cells/mL present a relatively mild risk with a low probability of adverse health effects. From *C. raciborskii* numbers alone three lakes have this risk. The WHO further suggests that over 100,000 cyanobacterial cells/L present a moderate probability of adverse health effects. Only Lake Lemon and Glen Flint fall into this category.

However, our study did not have the funds to perform toxin analysis on the samples with detected *C. raciborskii* populations. Without these data, we can only speculate about the potential human health risk. From Ball Lake, we know that *C. raciborskii* in Indiana is capable of producing toxins. However, that is the only toxin test that has been conducted in Indiana. *C. raciborskii* populations do not necessarily produce toxins, even when densities are high. In Wisconsin, concentrations of up to 244,000 cells/mL have been measured, but analysis did not detect the presence of toxins (Richard Lathrop, pers. comm.).

In our lakes, the populations of large zooplankton (*Cladocera and Copepoda*) were similar for both detect and non-detect lakes (Figure 5-4). If our populations of *C. raciborskii* were producing toxins, we would expect to find fewer large zooplankton since these grazers are sensitive to the toxins (Nogueira et al. 2004). The lack of large zooplankton in the water was a primary clue to the presence of algal toxins during the Ball Lake incident (St. Amand, pers. comm.). Of course, the only certain way to know whether a toxic cyanobacteria is producing toxins is to analyze for them.

5.4 Future Studies

Clearly, additional studies are needed of the distribution, abundance, seasonal dynamics, and toxin production in *C. raciborskii* and other toxin-producing cyanobacteria in Indiana. Such information is essential before lake managers and policy makers can develop effective policies to protect the public and the environment from these nuisances. An important component of future studies should be toxin analysis. Currently, the costs for toxin analysis can be prohibitively

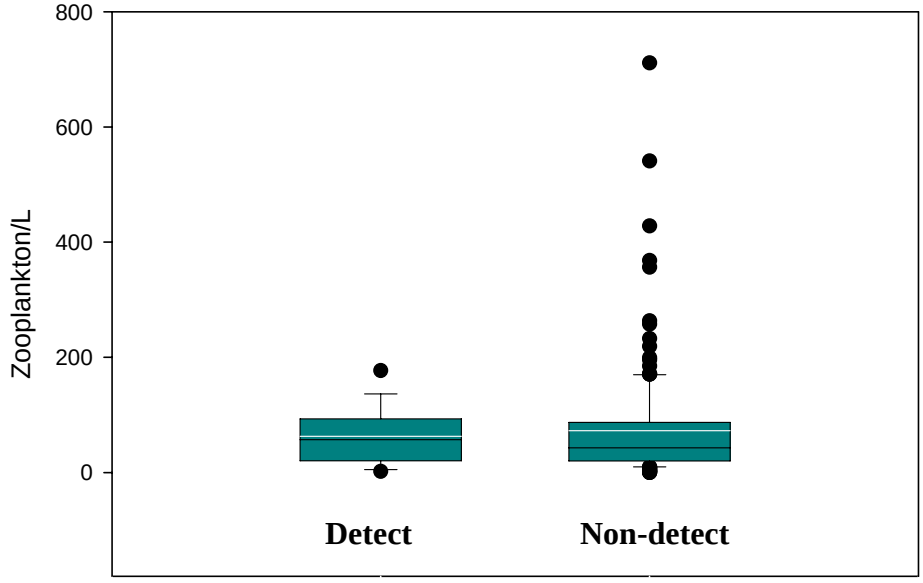


Figure 5-4. Distribution of large zooplankton in detect and nondetect lakes.

expensive and are available for only a handful of laboratories in the U.S. However, costs are coming down as demand for services increases.

The sampling design must also be carefully considered. While random sampling has the benefit of generating statistically significant results, we believe that targeted sampling would yield greater results and public protection. This study has shown that targeted sampling yielded a higher percentage of detect lakes.

Future studies should also incorporate a temporal design that includes multiple samples during August and September from the same lake. This will help us better understand the seasonal dynamics of *C. raciborskii.* Water quality parameters such as surface and bottom temperature, Secchi disk transparency, total phosphorus, nitrate, ammonia, and chlorophyll-a should be collected concurrently.

Preliminary results from Wisconsin suggest that there may be a great deal of spatial variation of *C. raciborskii* populations within a given lake. In this study, we only sampled at the maximum depth of each lake. At least one of our volunteer monitors informed us that while they did not see conditions indicating a *C. raciborskii* bloom at their lake's maximum depth, they saw symptoms near the shores or in bays.

In those lakes identified in this study as having detectable populations of *C. raciborskii,* we would further recommend follow-up monitoring, especially in those lakes with significant recreational activity and those serving as public drinking water supplies.

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APPENDIX A:

LAKES SAMPLED DURING THIS STUDY

LAKE	COUNTY	SAMPLE DATE
Appleman	Lagrange	06-Aug-02
Ball	Steuben	05-Aug-02
Barton	Steuben	08-Jul-02
Beaver Dam	Steuben	29-Jul-02
Big Blue #13 (Westwood)	Henry	20-Aug-02
Big Long	Lagrange	12-Aug-02
Big Otter	Steuben	01-Jul-02
Big Turkey	Lagrange	06-Aug-02
Bischoff	Ripley	22-Jul-02
Boones Pond	Boone	14-Aug-02
Booth	Steuben	30-Jul-02
Bower	Steuben	08-Jul-02
Brookville Reservoir	Franklin	23-Jul-02
Brush Creek	Jennings	16-Jul-02
Cagles Mill (Cataract)	Putnam	07-Aug-02
Cedar Lake	Lagrange	01-Jul-02
Cedarville Reservoir	Allen	13-Aug-02
Chrisney	Spencer	15-Jul-02
Clare	Huntington	19-Aug-02
Clear	Steuben	02-Jul-02
Crooked	Steuben	30-Jul-02
Crosley	Jennings	16-Jul-02
Eagle Creek Res	Marion	14-Aug-02
Elk Creek	Washington	15-Jul-02
Everett	Allen	13-Aug-02
Fish	Steuben	02-Jul-02
Fox	Steuben	12-Aug-02
Geist Res.	Marion	14-Aug-02
George	Steuben	08-Jul-02
Glen Flint	Putnam	07-Aug-02
Golden Lake	Steuben	06-Aug-02
Green Lake	Steuben	29-Jul-02
Grouse Ridge	Bartholomew	16-Jul-02
Hamilton	Steuben	05-Aug-02
Henry	Steuben	12-Aug-02
Hog	Steuben	08-Jul-02
Hogback	Steuben	06-Aug-02
Huntington Reservoir	Huntington	19-Aug-02
Hurshtown Res.	Allen	13-Aug-02
Jimmerson	Steuben	02-Jul-02
John Hay	Washington	15-Jul-02
Knightstown (Big Blue #7)	Henry	23-Jul-02
Knob Lake	Jackson	15-Jul-02
Kunkle	Wells	20-Aug-02
Lake Gage	Steuben	02-Jul-02
Lake James	Steuben	02-Jul-02

Lake of the Woods	Lagrange	05-Aug-02
Lake Pleasant	Steuben	29-Jul-02
Lemon (Reed Pt)	Monroe	07-Jul-02
Lemon (Riddle Pt)	Monroe	07-Jul-02
Lime (Gage)	Steuben	02-Jul-02
Little Otter	Steuben	01-Jul-02
Little Turkey	Steuben	05-Aug-02
Little Turkey (LaGrange)	Lagrange	06-Aug-02
Long (Clear)	Steuben	02-Jul-02
Long (Pleasant)	Steuben	08-Jul-02
Loon	Steuben	30-Jul-02
Manlove	Fayette	23-Jul-02
Marsh	Steuben	08-Jul-02
McClish	Steuben	05-Aug-02
Mollenkramer Res.	Ripley	23-Jul-02
Morse Res	Hamilton	14-Aug-02
Otter	Steuben	30-Jul-02
Pigeon	Steuben	08-Jul-02
Prairie Creek Reservoir	Delaware	20-Aug-02
Pretty	Lagrange	12-Aug-02
Salamonie Reservoir	Huntington	19-Aug-02
Silver	Steuben	30-Jul-02
Snow	Steuben	02-Jul-02
Spring Valley (Tucker)	Orange	15-Jul-02
Spurgeon Hollow	Washington	16-Jul-02
St. Joseph	Allen	19-Aug-02
Starve Hollow	Jackson	16-Jul-02
Stayner/Gannon	Steuben	29-Jul-02
Story (Lower)	Dekalb	12-Aug-02
Story (Upper)	Dekalb	12-Aug-02
Summit	Henry	20-Aug-02
Syl-van	Steuben	08-Jul-02
Versailles	Ripley	22-Jul-02
Wall	Lagrange	29-Jul-02
Whitewater	Union	23-Jul-02
Wood's Lake (Big Blue #3)	Rush	23-Jul-02
Adams	Lagrange	21-Jul-03
Allen	Kosciusko	04-Aug-03
Atwood	Lagrange	08-Jul-03
Backwater	Kosciusko	05-Aug-03
Banning	Kosciusko	18-Aug-03
Barrel and a half	Kosciusko	04-Aug-03
Bear	Noble	11-Aug-03
Big Barbee	Kosciusko	18-Aug-03
Bixler	Noble	21-Jul-03
Blackman	Lagrange	30-Jun-03
Brokesha Lake	Lagrange	14-Jul-03
Buck	Lagrange	14-Jul-03

Cass Lake	Lagrange	14-Jul-03
Cree	Noble	21-Jul-03
Dallas	Lagrange	08-Jul-03
Diamond	Noble	28-Jul-03
Duely	Noble	29-Jul-03
Eagle	Noble	28-Jul-03
Emma	Lagrange	07-Jul-03
Engle	Noble	28-Jul-03
Fish	Elkhart	15-Jul-03
Fish Lake (Plato)	Lagrange	01-Jul-03
Gilbert	Noble	11-Aug-03
Gordy	Noble	29-Jul-03
Hackenberg	Lagrange	08-Jul-03
Hammond	Kosciusko	04-Aug-03
Harper	Noble	12-Aug-03
Heaton	Elkhart	15-Jul-03
High	Noble	11-Aug-03
Hindman	Noble	11-Aug-03
Hunter	Elkhart	14-Jul-03
Indian Village	Noble	29-Jul-03
Irish	Kosciusko	18-Aug-03
James	Kosciusko	19-Aug-03
Jones	Noble	22-Jul-03
Kiser	Kosciusko	05-Aug-03
Knapp	Noble	12-Aug-03
Kuhn	Kosciusko	18-Aug-03
Latta	Noble	21-Jul-03
Little Barbee	Kosciusko	18-Aug-03
Little Bause	Noble	12-Aug-03
Little Knapp	Noble	12-Aug-03
Martin	Lagrange	07-Jul-03
Mateer Lake	Lagrange	30-Jun-03
Messick	Lagrange	08-Jul-03
Mongo Mill Pond	Lagrange	30-Jun-03
Moss	Noble	12-Aug-03
Nasby Mill Pond	Lagrange	30-Jun-03
Nauvoo	Lagrange	21-Jul-03
Old	Whitley	11-Aug-03
Olin	Lagrange	07-Jul-03
Oliver	Lagrange	07-Jul-03
Ontario Mill	Lagrange	30-Jun-03
Oswego	Kosciusko	19-Aug-03
Pigeon Lake	Lagrange	01-Jul-03
Rider	Noble	29-Jul-03
Ridinger	Kosciusko	19-Aug-03
Robinson	Kosciusko	19-Aug-03
Rothenberger	Kosciusko	04-Aug-03
Royer Lake	Lagrange	01-Jul-03

Sacarider	Noble	28-Jul-03
Sawmill	Kosciusko	18-Aug-03
Sechrist	Kosciusko	18-Aug-03
Shipswewana	Lagrange	14-Jul-03
Shock	Kosciusko	05-Aug-03
Simonton	Elkhart	15-Jul-03
Skinner	Noble	28-Jul-03
Smalley	Noble	29-Jul-03
South Twin	Lagrange	01-Jul-03
Sparta	Noble	11-Aug-03
Steinbarger	Noble	22-Jul-03
Stone Lake	Lagrange	14-Jul-03
Sylvan	Noble	21-Jul-03
Syracuse	Kosciusko	04-Aug-03
Tamarack (Noble)	Noble	22-Jul-03
Tippecanoe	Kosciusko	19-Aug-03
Troy Cedar	Whitley	19-Aug-03
Waldron	Noble	22-Jul-03
Wawasee	Kosciusko	05-Aug-03
Webster	Kosciusko	05-Aug-03
Westler	Lagrange	08-Jul-03
Wible	Noble	21-Jul-03
Witmer	Lagrange	08-Jul-03
Yellow Creek	Elkhart	15-Jul-03
Bass	Starke	16-Aug-04
Bruce	Fulton	16-Aug-04
Cook	Marshall	11-Aug-04
Cordry Lake	Brown	7-Sep-04
Dewart	Kosciusko	11-Aug-04
Flint	Porter	18-Aug-04
James	Kosciusko	10-Sep-04
Koontz	Starke	17-Aug-04
Kreighbaum	Marshall	11-Aug-04
Lemon	Monroe	18-Aug-04
Maxinkuckee	Marshall	16-Aug-04
Mill Pond	Marshall	11-Aug-04
Ole Swimming Hole	Morgan	16-Aug-04
Pretty	Marshall	10-Aug-04
Sweetwater	Brown	2-Sep-04
Tippecanoe	Kosciusko	10-Sep-04
Lake on the Green	Lake	03-Aug-04
Oswego	Kosciusko	10-Sep-04

Lakes positive for *Cylindrospermopsis raciborskii* are in red.