First report of toxic *Cylindrospermopsis raciborskii* and *Raphidiopsis mediterranea* (Cyanoprokaryota) in Egyptian fresh waters

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Abstract

Cylindrospermopsis raciborskii, a potentially toxic and highly adaptable freshwater cyanobacterium, was believed to have been misidentified in the Nile at the end of the 19th century. This study reports the presence of *Cylindrospermopsis raciborskii* and *Raphidiopsis mediterranea* for the first time in Egyptian fresh waters since that time. *Cylindrospermopsis raciborskii* appeared in the El-Dowyrat fish pond during May 2002, when bottom waters reached, as a result of climatic change, sufficiently high temperatures to allow the germination of its akinetes in the sediments. Both *C. raciborskii* and *R. mediterranea* showed seasonal variations, with highest densities recorded in August of each year. The count of the two species correlated positively with pH, temperature and conductance, and negatively with nutrients, during the study period. The densities of *C. raciborskii* and *R. mediterranea* varied significantly along the depth profile of this pond, with peaks obtained at 1 and 0.5m, respectively. Isolates of *C. raciborskii* and *R. mediterranea* from this pond exhibited toxicity to *Artemia salina*, *Daphnia magna* and mice. *Cylindrospermopsis raciborskii* extracts had hepatotoxic effects on mice, but *R. mediterranea* extracts showed neurotoxic effects on mice. The identification of toxic *C. raciborskii* and *R. mediterranea* in this pond should be considered during the monitoring of cyanobacteria in drinking and recreational water sources in Egypt.

Introduction

The *Cyanobacteria* are a very successful phytoplankton group associated with high eutrophication levels in lakes, rivers and reservoirs. The temporary or permanent dominance of members of this group is sometimes considered as a clear sign of severe eutrophication (Dokulil & Mayer, 1996). As certain species of the *Cyanobacteria* are well known to produce potent hepatotoxins and neurotoxins, cyanobacterial blooms can pose a significant threat to the health of animals and humans (Sivonen & Jones, 1999).

Cylindrospermopsis raciborskii is one of these toxic cyanobacteria, and was originally described as a species of only tropical interest (Woloszynska, 1912). Since the initial description, the species has been found in many tropical and subtropical regions and appears to be spreading around the world (Padisak, 1997; Briand *et al.*, 2002; Saker *et al.*, 2003). However, *C. raciborskii* has been found in several temperate areas, such as Hungary (Borics *et al.*, 2000), Austria (Dokulil & Mayer, 1996), France (Briand *et al.*, 2002), Germany (Fastner *et al.*, 2003), Greece (Hindak & Moustaka, 1988), Portugal (Saker *et al.*, 2003), Slovakia (Horecka & Komarek, 1979), and Spain (Romo & Miracle, 1994). More recently, *C. raciborskii* was recorded for the first time in Canadian fresh waters (Hamilton *et al.*, 2005).

The presence of *C. raciborskii* in water supplies used for human and stock consumption is of particular concern because of its potential for production of a potent hepatotoxin, cylindrospermopsin (Saker *et al.*, 2003). This toxin has been implicated in outbreaks of human poisoning (Haymann, 1992) and cattle mortality (Saker *et al.*, 1999), and it can accumulate in the tissues of other aquatic organisms (Saker & Eaglesham, 1999). It has also been reported that some strains of *C. raciborskii* from Brazil can produce paralytic shellfish poisons, including saxitoxins, neosaxitoxins and gonyautoxin 2/3 isomers, similar to those produced by another freshwater cyanobacterium, *Anabaena circinalis* (Lagos *et al.*, 1999).

Raphidiopsis is a nonheterocystous planktonic cyanobacterium, and was reported for the first time by Fritsch & Rich (1929). *Raphidiopsis* species are less frequent in the natural environment than are other planktonic cyanobacteria (Kersner 1997). So far, only two species of this genus have been reported to produce toxins, including cylindrospermopsins and anatoxins. Cylindrospermopsin and deoxycylindrospermopsin were detected from *R. curvata* Fritsch and Rich strain HB1 isolated from a fish pond in Wuhan, China (Li *et al*., 2001a), and anatoxin-a and homoanatoxin-a were isolated and identified from *R. mediterranea* Skuja strain LBRI 48, isolated from Lake Biwa, Japan (Namikoshi *et al*., 2003; Watanabe *et al*., 2003).

The naturally expanding distribution of *C. raciborskii* is due to its invasive behavior (Padisak, 1997), and the incidence of such a tropical species in temperate waters might be an indicator of global warming (Padisak, 1998). However, the existence of strains or populations of *C. raciborskii* adapted to low temperatures strongly suggests that *C. raciborskii* is not only an ongoing invasive species but also a species with different physiologic strains or ecotypes tolerant to temperature (Chonudomkul *et al*., 2004). The authors also reported that cylindrospermopsin is synthesized without any relation to phylogenetic or genetic clusters and to geography, and suggested isolation of different strains of *C. raciborskii* from various areas of the world to support these speculations.

Toxic cyanobacteria have been investigated in Egyptian fresh waters, including the Nile River and irrigation canals (Mohamed & Carmichael, 2000). Most of these studies have dealt with toxic *Microcystis* blooms, and to my knowledge, there is no published literature reporting the presence of toxic *Cylindrospermopsis* and *Raphidiopsis* in Egyptian fresh waters. However, it is believed that *C. raciborskii* was misidentified as *Cylindrospermum kaufmannii* in the Nile at the end of the 19th century (Huber-Pestalozzi, 1938). Therefore, Padisak (1997) reported that the world's earliest floristic record of the occurrence of *C. raciborskii* was probably that from the Nile.

The El-Dowyrat fish farm is a natural pond covered every year during the warm season with a heavy bloom of *Microcystis aeruginosa* (Mohamed *et al*., 2003). Until April 2002, during regular investigation and monitoring of cyanobacteria in Egyptian fresh waters, no trichome of *Cylindrospermopsis* or *Raphidiopsis* was recorded either in this pond (Z.A. Mohamed, unpublished data) or in the neighboring areas of the Nile River and irrigation canals running to this pond (Ali, 2004). The present study documents and confirms the presence of toxic *Cylindrospermopsis raciborskii* and *Raphidiopsis mediterranea* in this fish pond in Egypt. The study also describes some environmental conditions related to the success of these species in the natural environment.

Materials and methods

Study site

The El-Dowyrat fish farm is a shallow freshwater pond, 15 km southeast of Sohag city (26°30′N, 31°50′E). It is about 87 500 m^2 in area, and has a maximum depth of about 4 m. The pond has no natural superficial inflow or outflow, and is dependent on groundwater exchange. At the end of 2001, this fish pond was exposed to human activities, including removal of surrounding trees and elimination of macrophytes, probably for restoration purposes. The pond water is used mainly for irrigation, fisheries and recreation purposes. Near this pond is a restaurant, where large numbers of fish are taken from the pond for food.

Sampling and environmental parameters

Phytoplankton samples were collected monthly between 10:00 and 11:30 a.m. from May 2002 to August 2003, using a plankton net (25-μm mesh size) lowered to a depth of 1 m. The physical, chemical and biological properties of the pond water were also studied along the vertical axis of this pond. This was performed by taking water samples using a Van Dorn bottle-like container from the surface and at seven different depths (0.5, 1, 1.5, 2, 2.5, 3 and 3.5 m) in August 2003 only, when the highest densities of *C. raciborskii* and *R. mediterranea* were recorded. Each phytoplankton or water sample was a composite of three samples collected from different stations in the pond. An aliquot of each phytoplankton sample was filtered through a Whatman GF/C fiberglass filter. The pigments were extracted from these filters in methanol (90%), and chlorophyll *a* concentrations were measured spectrophotometrically according to the method of Talling & Driver (1963). Five hundred milliliters of each phytoplankton sample was preserved in Lugol's iodine solution and stored in the dark for 24 h. After sedimentation, the supernatant was siphoned away, and the remaining solution (50 mL) was well mixed and used for identification and enumeration of phytoplankton. Algal counts were made with a Sedgwick–Rafter counting chamber (APHA, 1995) and an Olympus binocular microscope. The density was calculated for natural taxonomic units (cells, colonies, or filaments) per liter of original pond water. Algae were identified to the species level, when possible, according to Desikachary (1959), with the aid of some floristic papers, e.g. Hill (1970), Anagostidis & Komarek (1988), Komarek & Kling (1991), Komarek & Anagostidis (1989, 1998), Komarek & Hindak (1988) and Hindak (1992).

Water temperature, pH and conductivity were measured in real time during each sampling using a Cyberscan Waterproof pH/conductivity/TDS/ °C/°F meter PC series (Eutech Instruments, PTE LTD, BIK 55, Ayer Rajah Cresent # 4–16/24, Singapore), and dissolved oxygen was measured using an

O_2-meter type CG 867 (Schott, Gerate, Gmbh, D6238 Hofheim a.t.s, Germany). Water samples for ammonium, nitrate and phosphate concentrations were taken using 2.5-L glass bottles, filtered through Whatman GF/C fiberglass filters, and analyzed in the algal laboratory, Department of Botany, Faculty of Science, Sohag University, by standard methods according to APHA (1995).

Morphologic characteristics

The morphologic characteristics of *C. raciborskii* and *R. mediterranea* were assessed microscopically in phytoplankton samples collected from the pond during August 2003 and preserved in Lugol's solution. The average lengths and widths of trichomes, vegetative cells, akinetes and heterocysts were obtained from 50 to 100 measurements for each structure.

Isolation and culture of cyanobacteria

Cylindrospermopsis raciborskii and *R. mediterranea* were isolated from water samples taken in August 2003. Single filaments of *C. raciborskii* and *R. mediterranea* were isolated separately using a Pasteur pipette, washed several times with culture medium. *Cylindrospermopsis* filaments were then transferred into sterile screwcap test tubes containing 5 mL of nitrogen-free BG11 medium (Stanier *et al*., 1971), and *Raphidiopsis* filaments were placed in test tubes with nitrogen-containing BG11 medium. Cultures were maintained at $25 \pm 2\,^{\circ}C$ under a 14 : 10 light/dark cycle with a photon flux density of $24\,\mu mol\,m^{-2}\,s^{-1}$ provided by fluorescent lamps. For mass cultivation of these cyanobacteria, the cells of each species in the late exponential phase were used to inoculate 4-L culture flasks containing 2 L of BG11 medium with nitrogen for *Raphidiopsis* and without nitrogen for *Cylindrospermopsis*. These flasks were incubated under the same conditions described above and aerated with filtered air (filter pore size 0.2 μm). Cells were harvested in the late exponential growth phase (after 3 weeks) by centrifugation (10 000 ***g***, 15 min), freeze-dried and stored at $-20\,^{\circ}C$ until workup.

Toxicity test

Mouse bioassay

The freeze-dried cell mass (2.5 g) of *Cylindrospermopsis* or *Raphidiopsis* was homogenized in 50 mL of saline solution until cells were completely lysed. Different doses (1, 5, 10, 50 and 100 mg of lyophilized cells per mL) of these extracts were administered intraperitoneally to male Albino mice (weight 20–25 g). Control animals were injected with physiologic saline solution. Six mice were used for each dose as replicates. Signs of toxicity were looked for every hour during the first 12 h, and at 24, 48 and 72 h. Moribund mice were subjected to postmortem autopsy to examine abnormalities in organs. LD_{50}, expressed as the dry weight of cyanobacterial mass per kg of mouse, was calculated according to the method of Meier & Theakston (1986).

Artemia salina assay

Freeze-dried cells (2.5 g) of *Cylindrospermopsis* and *Raphidiopsis* were homogenized in 50 mL of sterile distilled water. The broken cell suspensions were then centrifuged (10 000 ***g***, 10 min), and cell-free extracts were diluted with seawater to give five concentrations of 20, 10, 5, 2.5, 1.25 $mg\,mL^{-1}$ in terms of dry weight of original sample. The toxicity of these extracts to *Artemia salina* larvae was determined according to the method of Metcalf *et al*. (2002). Briefly, 10–20 of the 1-day hatching larvae of brine shrimp were pipetted in glass culture tubes that had been dosed with different concentrations of each extract. Each treatment was performed in triplicate, and seawater was used as a control. The culture tubes were incubated under fluorescent lamps at room temperature ($25 \pm 2\,^{\circ}C$). The numbers of dead or atypically moving larvae were counted after 24, 48 and 72 h to calculate mortality (Meier & Theakston, 1986). The toxicity was expressed as the percentage of dead larvae minus the mortality in control samples. The LC_{50} ($mg\,mL^{-1}$) was determined by probit analysis (Finney, 1963).

Daphnia magna test

Daphnia magna specimens collected from the El-Dowyrat fish pond were used in toxicity tests. The animals were grown in 1-L glass jars containing filtered pond water at room temperature ($25 \pm 2\,^{\circ}C$) under a 14 : 10 light/dark cycle. Daphnids were fed with an algal suspension of *Ankistrodesmus falcatus* at a concentration of 10^5 cells mL^{-1}. Acute toxicity to *Daphnia magna* was determined by exposure of 10 daphnids to different doses of *C. raciborskii* and *R. mediterranea* extracts at the same concentrations used in the *Artemia salina* assay. Each treatment was performed in triplicate, and filtered pond water was used as a control. Survival rates were recorded after 24, 48 and 72 h. The toxicity was expressed as the percentage of dead daphnids minus the mortality in control samples. The LC_{50} ($mg\,mL^{-1}$) was determined by probit analysis (Finney, 1963).

Statistical analysis

Relationships between environmental parameters and biological variables, including counts of cyanobacterial species, were statistically analyzed by one-way ANOVA ($P < 0.05$) using SPSS 9.0 software for Windows. Spearman rank correlation coefficients were also used to measure the degree of association between the physical and chemical properties, and the biotic variables.

Results

Environmental parameters and seasonal variation of *C. raciborskii* and *R. mediterranea*

The results of physicochemical analysis of El-Dowyrat fish pond water showed that surface temperature, dissolved oxygen and nonconservative nutrients (nitrate and phosphate) varied ($P < 0.001$) seasonally during the study period (Fig. 1), whereas conductivity, pH and ammonium did not change significantly ($P > 0.05$) during the time of this study.

Algal biomass, measured as chlorophyll *a* content, of the pond water changed dramatically with time ($P < 0.001$) and correlated positively with pH, temperature and conductivity ($r = 0.5$–0.8), whereas it correlated negatively with dissolved oxygen and nutrient concentrations ($r = -0.3$ to -0.7). Chlorophyll *a* showed two peaks, both in August, in two summers during the study period (Fig. 1c). These two peaks of chlorophyll *a* were associated with the highest counts of *C. raciborskii* ($r = 0.87$), *R. mediterranea* ($r = 0.81$) and *M. aeruginosa* ($r = 0.54$). Counts of *C. raciborskii* correlated positively with pH, temperature and conductance ($r = 0.5$–0.87), but correlated negatively with dissolved oxygen and nutrients ($r = -0.3$ to -0.77). Although many cyanobacterial species were found with *C. raciborskii* in the fish pond during this study, *C. raciborskii* showed a strong correlation with *R. mediterranea* ($r = 0.8$). Furthermore, *R. mediterranea* responded to all environmental parameters of the pond water in the same way as *C. raciborskii*.

The first trichome of *C. raciborskii* was recorded in May 2002. The density of this cyanobacterium increased gradually until reached its maximum (18×10^6 trichomes L^{-1}) in August 2002, and decreased dramatically during September and October 2002. The species disappeared totally from the pond during the period from November 2002 to February 2003 (Table 1). In 2003, the species appeared earlier in March, but with low density (3.5×10^6 trichomes L^{-1}). After that, the density of *C. raciborskii* increased gradually, and reached its maximum (29×10^6 trichomes L^{-1}) again in August, as in the previous year, whereas the first trichome of *R. mediterrenea* was observed in June 2002 and reappeared in March 2003. The density of *R. mediterrenea* increased and decreased during the study period in the same way as that of *C. raciborskii* (Table 1). *Microcystis aeruginosa* bloom was observed year-round on the water surface of this pond (Table 1). *Pseudanabaena limnetica* was also dominant year-round and was associated with *Microcystis* bloom on the water surface. Other algal species, such as chlorophyceans, euglenophyceans, bacillariophyceans and dinoflagellates, were intermittently present in the pond during the study period. Some chlorophycean and bacillariphycean species replaced *C. raciborskii* and *R. mediterranea* as dominant species from November 2002 to February 2003 (Table 1).

Depth profiles of environmental parameters and *C. raciborskiii* and *R. mediterranea*

The results presented in Fig. 2 show the depth profile of environmental parameters of the El-Dowyrat fish pond during the strongest stagnation period (i.e. no water renewal) in August 2003. The measured temperatures did not exhibit marked variation with depth ($P > 0.05$). The fish pond was not stratified, with a difference $< 2\,^{\circ}C$, between the surface and the bottom. Likewise, pH values did not differ significantly among seven depths ($P > 0.05$). In contrast, dissolved oxygen changed dramatically with depth ($P < 0.001$). The dissolved oxygen concentration at the bottom was about 3.5 times less than that at the surface, indicating the eutrophic nature of this pond. The conductivity of the pond water showed significant variation ($P < 0.001$) along the vertical profile, with high values recorded at the surface. Dissolved nutrient concentrations showed high variation along the depth profile of this pond ($P < 0.01$). Ammonium represented a larger amount of nitrogen in the pond than nitrate, and its concentration was higher at the surface than at the bottom (Fig. 2b). Nitrate concentrations were generally very low in the pond, and nitrate was not detectable at depths of 0.5 and 1 m. Dissolved phosphate concentrations were also low in the pond; they decreased from the surface to a depth of 2 m, and then increased again to a depth of 3.5 m (Fig. 2b). The level of chlorophyll *a*, as a trophic indicator, was generally high in this pond, and showed great vertical difference between the surface ($405.9\,\mu g\,L^{-1}$) and the bottom ($15.3\,\mu g\,L^{-1}$) (Fig. 2b). Chlorophyll *a* content correlated positively with all environmental parameters along the depth profile of the pond ($r = 0.25$–0.95). On the other hand, chlorophyll *a* content correlated negatively with the counts of *C. raciborskii* and *R. mediterranea* ($r = -0.8$ and -0.4, respectively), and correlated positively with the count of *M. aeruginosa* ($r = 0.98$) along the vertical profile of the pond.

The species composition of phytoplankton did not significantly change along the vertical axis in the pond. Nevertheless, percentages of some species showed marked variations ($P < 0.01$) with depth (Fig. 3). It is of particular importance that *C. raciborskii* and *R. mediterranea* were represented by lower percentages at the surface (7.4% and 13%, respectively). The highest percentages were obtained at a depth of 1 m for *C. raciborskii* (28.2%) and at a depth of 0.5 m for *R. mediterranea* (40.3%). In contrast, the highest percentage of *M. aeruginosa* (36.1%) was obtained at the

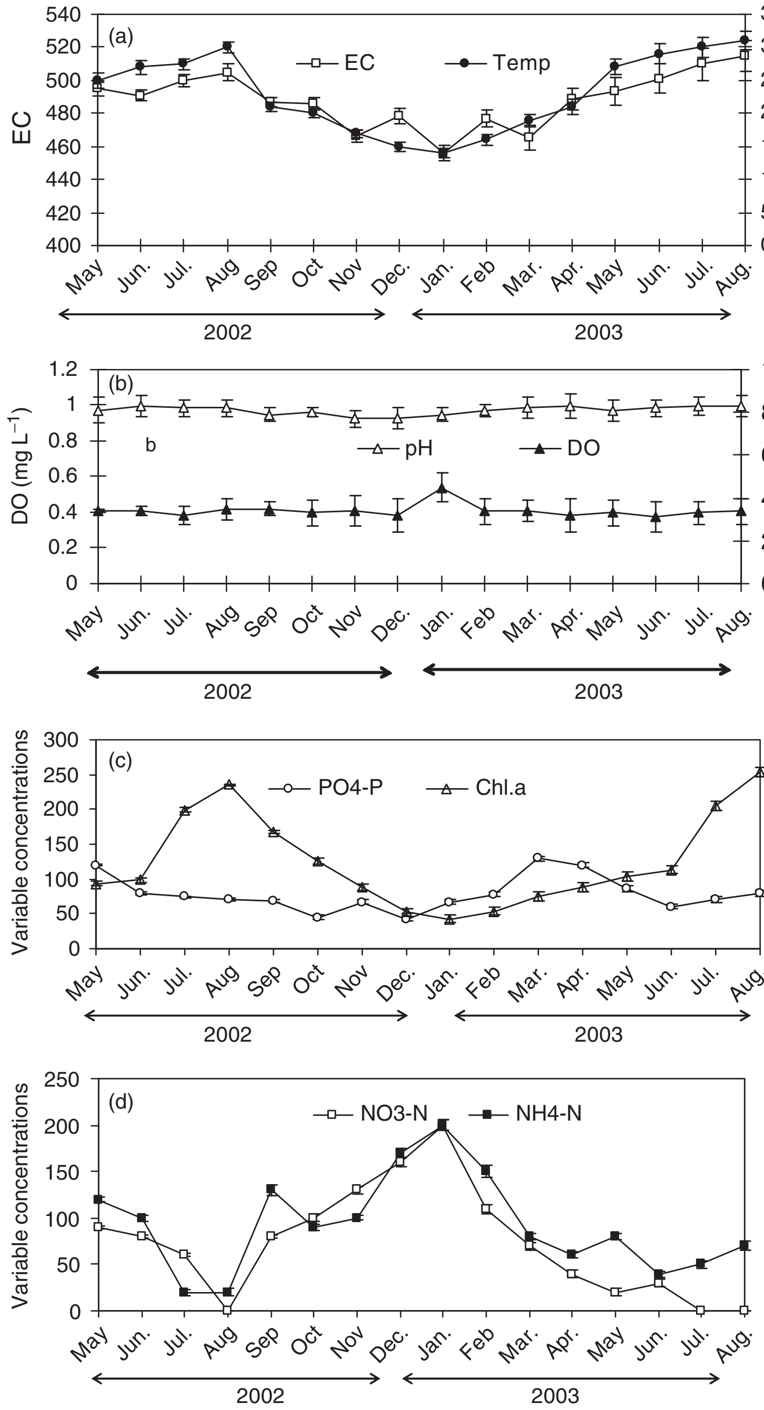

Fig. 1. Seasonal variation of environmental parameters in the El-Dowyrat fish pond during the study period (May 2002 to August 2003). (a) Temperature (°C) and conductivity (μmoh $cm^{-1}\,s^{-1}$). (b) Dissolved oxygen (DO, $mg\,L^{-1}$) and pH. (c) PO_4^{3-}, and chlorophyll *a* ($\mu g\,L^{-1}$). (d) NO_3^-, NH_4^+ ($\mu g\,L^{-1}$).

surface and the lowest was obtained at the bottom (11.8%). *Pseudanabaena limnetica* showed a good association with *M. aeruginosa* ($r=0.7$) rather than with *C. raciborskii* and *R. mediterranea* at all depths in the pond. The results also revealed that both *C. raciborskii* and *R. mediterranea* had a marked association with each other ($r=0.5$), and correlated negatively ($r=-0.8$ and -0.5, respectively) with *M. aeruginosa* at the depths studied.

Table 1. Counts of phytoplankton species (organism L^{-1}) recorded in the El-Dowyrat fish pond during the study period (May 2002 to August 2003)

Species	May	June	July	August	September	October	November	December	January	February	March	April	May	June	July	August
Cyanobacteria																
Cylindrospermopsis raciborskii	6	9.5	14	18	13	10.2	–	–	–	–	3.5	9	14	14.6	27	29
Raphidiopsis mediterranea	–	8	11	28	21	16	5	–	–	–	1.3	3.2	11.6	15.8	26	32
Cylindrospermum sp.	–	–	–	12	–	–	–	–	–	–	–	–	–	–	8.6	4.5
Pseudoanabena limnetica	22	23.5	34.5	44	27	22	10.2	2.3	1	3.6	9.6	13.4	23.4	38	48	54
Merismopedia glauca	5	7.6	11.2	14	9	3.4	1.2	–	2	1.1	4.3	6.8	7.6	14.5	16	18
Microcystis aeruginosa	23	58	72	78	64	43	21	11	2.1	1	16.3	34	43	65	77	84
Nodularia spumigena	–	–	–	4	–	–	–	–	–	–	–	–	–	2.1	5.3	6.2
Chlorophyceae																
Ankistrodesmus sp.	1.2	1	–	2	–	–	–	2.5	4.3	4	3.1	1.8	1	–	1	–
Cosmarium sp.	–	–	1	1.5	1.6	1	1	3.4	4.1	3.2	3.1	2	1.3	–	–	–
Gomphonema sp.	–	1.4	–	1	–	–	–	1.3	2.5	4.1	2.8	1	–	–	–	–
Pediastrum sp.	–	–	–	1	–	–	–	2.3	3.5	5.3	2.5	1	1.2	–	–	–
Scenedsmus sp.	1	1	1.3	2.3	1.2	2	1.1	3.2	3.8	6.8	4	2.4	1.5	1	–	–
Staurastrum sp.	–	–	–	1.2	–	1.3	–	4.4	4.8	5.3	2.1	1.5	1.1	–	–	–
Tetraedron sp.	–	–	–	1.1	–	–	–	1.8	2.3	3.2	1.2	1.1	1	–	–	–
Bacillariophyceae																
Nitzschia sp.	7.2	6.7	6.1	6	1.3	1	–	–	5.4	6.6	8.3	8.7	6.2	4.5	2.1	1.3
Synedra sp.		–	2.3	5.5	1.2	1	1	–	4.2	4.8	6.5	5.6	4.3	3.1	1.4	1
Euglenophyceae																
Euglena sp.	–	–	1.5	2.4	–	–	1.5	2.4	2.7	1.4	–	–	–	–	1.2	1
Phacus sp.	–			–	1.2	1.8	1.4	–	–	–	–	2.1	–	–	–	–
Dinophyceae																
Ceratium sp.	–	–	2.5	1.1	–	–	–	–	–	–	–	–	–	–	–	–
Peridinium sp.	–	–	–	0.6	–	–	–	–	–	–	–	–	–	–	1.1	0.8

Count of all algae, the number $\times 10^6 L^{-1}$ for cyanobacterial species, and $\times 10^3 L^{-1}$ for other algal species.

Morphology of *C. raciborskii* and *R. mediterranea*

Only the morphologic coiled form of *C. raciborskii* was observed in the El-Dowyrat fish pond during the study period (Fig. 4a–d). According to the morphologic descriptions of this species presented in Table 2, this morphotype is consistent with that observed by other authors elsewhere (Komarek & Kling, 1991; McGregor & Fabbro, 2000) and was thus identified as *C. raciborskii* (Woloszynska) Seenaya and Subba Raju. In contrast, only the straight form of *Raphidiopsis* was observed in this pond during the study period (Fig. 4e–h). On the basis of the morphologic characteristics shown in Table 2 and the species characteristics described by Hill (1970), Hindak (1992) and Watanabe *et al.* (2003), the strain was identified as *R. mediterranea* Skuja.

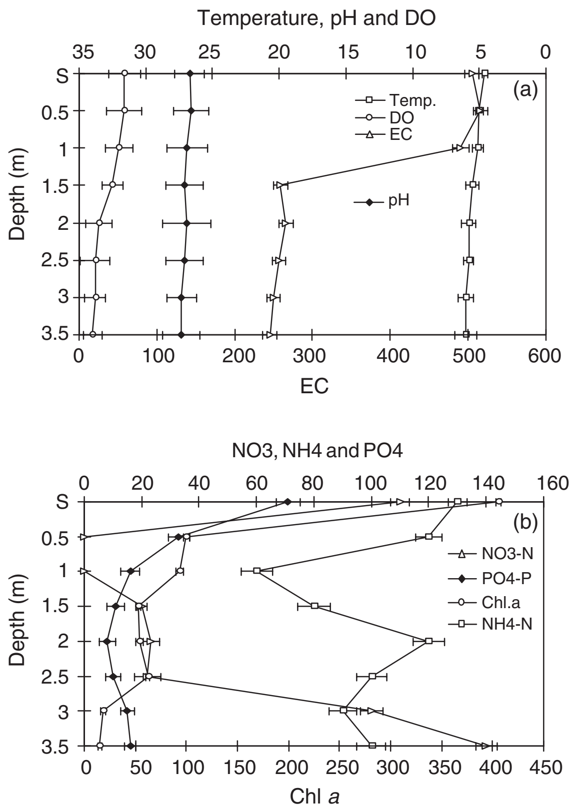

Fig. 2. Depth profile of environmental parameters in the El-Dowyrat fish pond during August 2003. (a) Temperature, dissolved oxygen (DO, mg L^{-1}), pH and conductivity (μmoh cm^{-1} s^{-1}). (b) NO_3^-, NH_4^+, PO_4^{3-} and chlorophyll *a* concentrations (μg L^{-1}).

Toxicity of *C. raciborskii* and *R. mediterranea*

The results of the mouse bioassay for *C. raciborskii* extract did not show any signs of neurotoxic effects, but revealed symptoms of weakness, depressed appetite and loss of weight. Postmortem examination showed signs of hemorrhage within the liver, kidneys and small intestine, and congestion of lungs (data not shown). Histopathologic tests were not performed for any organ. Three LD_{50}s were reported for this extract at different times (24, 48 and 74 h). The LD_{50} value is time-dependent; the highest value was obtained at 24 h, and the lowest at 72 h (Table 3). In addition, *C. raciborskii* extract showed toxicity to *Artemia salina* with different values of LC_{50} at different exposure times. The extract of this cyanobacterium was also found to be toxic to *D. magna*, with LC_{50} values higher than those for *Artemia salina* (Table 3).

Mice injected intraperitoneally with *R. mediterranea* extract exhibited symptoms of neurotoxic effects, including muscle fasciculation, staggering, twitching, exaggerated abdominal breathing, convulsions, and death by respiratory arrest within 8 min. The LD_{50} of this extract was estimated at

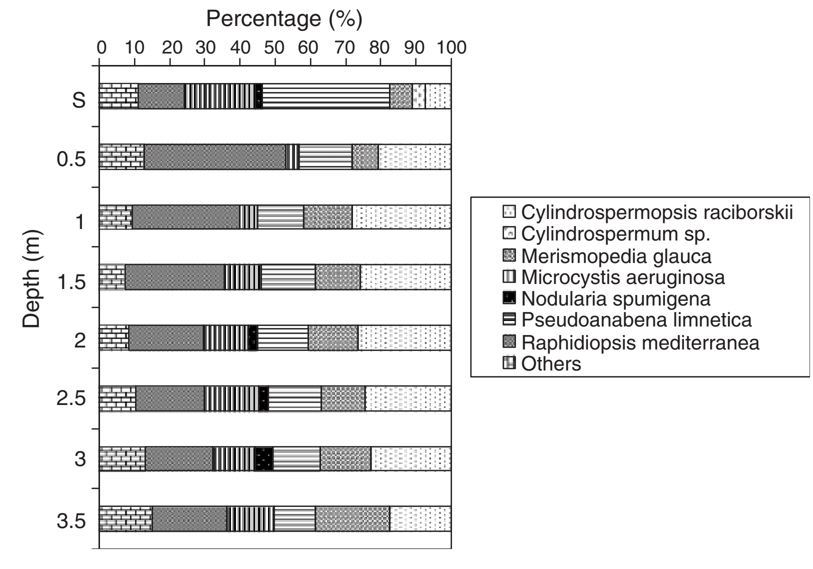

Fig. 3. Percentages and species composition of phytoplankton present along the depth profile of the water column of the El-Dowyrat fish pond during August 2003.

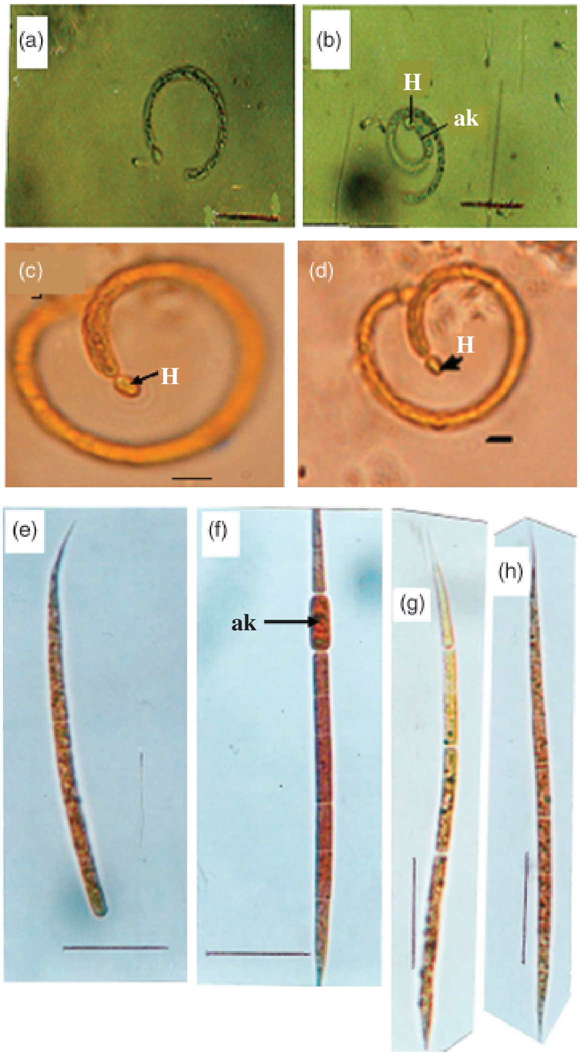

Fig. 4. Micrographs of *Cylindrospermopsis raciborskii* (a–d) and *Raphidiopsis mediterranea* (e–h). Bar = 20 µm, except for (c) and (d), where bar = 10 µm.

Table 2. Morphologic characteristics of *Cylindrospermopsis raciborskii* and *Raphidiopsis mediterranea* isolated from the El-Dowyrat fish pond during the study period

Character	*Cylindrospermopsis raciborskii*	*Raphidiopsis mediterranea*
Morphologic form	Coiled, terminal cells conical, akinetes cylindrical, separated or adjacent to heterocysts, heterocysts drop-like, bluntly pointed, found at one or both ends	Straight, terminal cells gradually attenuated with hair-like ends, akinetes with tiny granules, found near the ends, heterocysts absent
Trichome length (µm)	83–170 (180)	78–179 (230)
Cell length (µm)	4.5–6.5 (7.5)	5.2–16.5 (18)
Cell width (µm)	2.8–3.6 (4.5)	2.3–3.5 (4)
Akinete length (µm)	9–11 (12)	7.5–18 (20)
Akinete width (µm)	3–3.8 (4.2)	3.5–5 (5.5)
Heterocyst length (µm)	4.5–5.5 (7.5)	Absent
Heterocyst width (µm)	2.3–2.9 (3.2)	Absent

Numbers in parentheses are mean outliers.

360 mg kg^{-1} (Table 3). The extract of this cyanobacterium did not show any signs of hepatotoxicity (data not shown). *Raphidiopsis mediterranea* extract was also toxic to *Artemia salina* and *D. magna*, with different LC_{50} values that decreased with increase of exposure time (Table 3). The results also showed that *R. mediterranea* extract was more toxic to *Artemia salina* than to *D. magna* at all exposure times.

Discussion

Changes in climatic factors (e.g. wind, light intensity, temperature) can accelerate algal blooms and modify the phytoplankton structure in freshwater ecosystems. Cyanobacteria are strongly driven by physical factors such as local weather conditions (Briand *et al.*, 2004). Previous studies on phytoplankton structure did not reveal any trichome of either *C. raciborskii* or *R. mediterranea* in the El-Dowyrat fish pond until April 2002 (Mohamed *et al.*, 2003; Z.A. Mohamed, unpublished data). During the present study, a change in phytoplankton composition was observed, with the appearance of *C. raciborskii* and *R. mediterranea* for the first time in this pond. At the end of 2001, human activities, including the removal of trees around the pond and elimination of macrophytes, might have led to the direct exposure of the pond to sun irradiation and high air temperature, increasing the temperature of the surface water (data not shown). In addition, as the pond is shallow, the temperature of the bottom waters may have been sufficiently high to allow the germination of *Cylindrospermopsis* akinetes in the sediments. Thus, these changes could provide suitable conditions for the proliferation and growth of *C. raciborskii* in the El-Dowyrat fish pond. Previously, Ryan *et al.* (2003) reported that loss of macrophytes in Lakes Waahi, Nagroto and Whangape, New Zealand, provided ideal conditions for the proliferation of *C. raciborskii*. Other shallow lakes in New Zealand that have changed to an 'alternative stable state' (Scheffer, 1998), signaled by nutrient enrichment, high turbidity, and loss of macrophytes, could also be expected to be susceptible to blooms of *C. raciborskii*, depending on water temperature. Therefore, the appearance of *C. raciborskii* in the El-Dowyrat fish pond could be related to the

Table 3. Toxicity of isolates of *Cylindrospermopsis raciborskii* and *Raphidiopsis mediterranea* taken from the El-Dowyrat fish pond during a bloom in August 2003

Species	Toxicity at time		
	24 h	48 h	72 h
Cylindrospermopsis raciborskii			
Mouse bioassay (LD_{50}, mg kg^{-1})	450 ± 8	285 ± 6.5	205 ± 3.5
Artemia assay (LC_{50}, mg mL^{-1})	60 ± 3.4	20 ± 1.2	6 ± 0.8
Daphnia assay (LC_{50}, mg mL^{-1})	138 ± 4.5	86 ± 2.3	22 ± 1.8
Raphidiopsis mediterranea			
Mouse bioassay (LD_{50}, mg kg^{-1})	360 ± 7.5*	–	–
Artemia assay (LC_{50}, mg mL^{-1})	8 ± 0.6	4 ± 0.4	1.5 ± 0.3
Daphnia assay (LC_{50}, mg mL^{-1})	13 ± 1.3	7 ± 0.8	3 ± 0.4

*Death occurred within 8 min of injection.

increase in water temperature, and supports the hypothesis that the increase in water temperature in temperate lakes is the key factor in the expanding growth area of *C. raciborskii* (Briand *et al.*, 2004). The present study also confirms the observation of Padisak (1997) showing that a water temperature ranging from 22 to 23.5 °C is most suitable for the germination of *Cylindrospermopsis* akinetes. Such temperatures were reached and exceeded in the El-Dowyrat fish pond in May 2002 (25 °C), and were associated with the appearance of *C. raciborskii* in this pond. In addition to warming of the pond water, water stagnation (lack of water renewal) in this fish pond could have participated in creating suitable conditions for the appearance of *C. raciborskii* and *R. mediterranea* in this pond. This hypothesis was supported by comparing the results of the present study with those of Ali (2004), who revealed the absence of these species in the running waters of irrigation canals surrounding this fish pond.

Based on a monthly survey, both *C. raciborskii* and *R. mediterranea* showed seasonal variations in the El-Dowyrat fish pond during the present study. The high densities of these two species correlated with high temperatures (25–30 °C) during the period from May to August in 2002 and 2003. *Cylindrospermopsis raciborskii* and *R. mediterranea* disappeared from the pond water during the period from November 2002 to February 2003, when the water temperature decreased to below 17 °C. These results agree with previous studies showing that *C. raciborskii* seems to be limited to warm months in temperate regions (Briand *et al.*, 2002; Saker *et al.*, 2003), and that their akinetes can survive the cold winters and only germinate when water temperatures reach 22–23 °C (Padisak, 1997). Likewise, numbers of *C. raciborskii* and *R. mediterranea* correlated with pH and conductivity of the pond water. This is consistent with most previous studies, which showed that these species occur in lakes with a pH of 8–8.7 (Padisak, 1997) and a wide range of salinity (Bouvy *et al.*, 2003; Hamilton *et al.*, 2005). During the present study, the abundance of *C. raciborskii* and *R. mediterranea* correlated negatively with nutrient levels in the fish pond, supporting the results obtained by Briand *et al.* (2002). *Cylindrospermopsis raciborskii* was present in high densities at low ammonium concentrations in the fish pond, corroborating the suggestion that this cyanobacterium can assimilate ammonium at low ambient concentrations (Présing *et al.*, 1996). At the same time, *Cylindrospermopsis* organisms occurred at high densities, and their trichomes had low percentages of heterocysts, in spite of low concentrations or lack of nitrate in the pond water during the current study (data not shown). This finding agrees totally with the hypothesis that although *C. raciborskii* is a nitrogen fixer, it does not seem to be highly dependent on nitrogen fixation and prefers ammonium to nitrate as a nitrogen source (Briand *et al.*, 2002). Although the phosphate values detected in the El-Dowyrat fish pond during this study were higher than 10 μg L^{-1}, indicating that this pond was not phosphorus-deficient, according to Sas (1989), *Cylindrospermopsis* organisms were found in high numbers. Thus, these results are not consistent with those obtained by Padisak & Istvanovics (1997), who reported that *C. raciborskii* dominates in phosphorus-limited reservoirs. Those authors attributed this finding to the ability of this cyanobacterium to store phosphorus through luxury uptake (uptake of more than is needed) in order to gain an advantage over other cyanobacteria. However, the results of the present study are in agreement with the supposition of Borics *et al.* (2000) that the competitive advantage for *Cylindrospermopsis* could not be any specialized phosphorus-uptake strategy, as supposed earlier by Padisak & Istvanovics (1997), because of the high phosphorus concentrations of the water, but rather high ammonium uptake (Présing *et al.*, 1996).

Unlike most toxic cyanobacteria, *Cylindrospermopsis* does not form scums on the surface of the water, but is present in highest concentrations below the surface (St Amand, 2002). The distribution of cyanobacteria throughout the water column of the El-Dowyrat fish pond was investigated on a single sampling date during a severe bloom in August 2003. Although the pond does not seem to have thermal stratification, *C. raciborskii* showed a great difference in its distribution along the depth profile, with a peak of density at a depth of 1 m. This finding can be explained by the hypothesis of Paerl (1988) that in absence of thermal stratification in lakes, other factors can potentially influence the development and dominance of cyanobacteria, such as limitation by light, weather conditions and turbulence. The abundance of *Cylindrospermopsis* in the El-Dowyrat fish pond below the surface is expected, and agrees with all previous studies, which attributed this finding to the shade tolerance of this species and its ability to proliferate in water with low transparency, giving it an indirect competitive advantage over other heterocystous cyanobacteria (Briand *et al.*, 2002). Although the water transparency was not measured during the present study, the presence of a heavy bloom of *M. aeruginosa* on the water surface of such a shallow pond most likely makes the pond water less transparent. Other parameters, such as dissolved oxygen, electric conductivity (EC), NO_3^-, NH_4^+, and PO_4^{-3}, varied negatively with depth during the present study. *Cylindrospermopsis raciborskii* correlated negatively with most of these parameters, particularly dissolved nutrients. The negative relationship between nutrient levels and *C. raciborskii* abundance supports the results obtained during the seasonal period of the present study and corroborates the results of previous studies demonstrating that this species proliferates when nutrient concentrations are low (Présing *et al.*, 1996; Padisak & Istvanovics, 1997; Komarkova *et al.*, 1999; Briand *et al.*, 2002).

During this study, *R. mediterranea* was associated with *C. raciborskii* throughout the water column and showed similar behavior under the environmental conditions of the pond. The association of *Raphidiopsis* with *Cylindrospermopsis* was previously reported in tropical and subtropical reservoirs (McGregor & Fabbro, 2000; Bouvy *et al.*, 2003; Chellappa & Costa, 2003). In this respect, Komarkova *et al.* (1999) considered *Raphidiopsis* populations mixed with *C. raciborskii* as most likely to be filaments of *C. raciborskii* lacking heterocysts, whereas McGregor & Fabbro (2000) considered *Raphidiopsis* trichomes as environmental morphotypes of *C. raciborskii* and that these trichomes could be induced to form heterocysts under conditions of abundant phosphorus. *Raphidiopsis mediterranea* is considered as a morphotype corresponding to the straight form of *C. raciborskii*, whereas *R. curvata* is a morphotype corresponding to the coiled form of *C. raciborskii* (McGregor & Fabbro, 2000). The results of the present study did not show such harmony in the association between *Cylindrospemopsis* and *Raphidiopsis* morphotypes, as the straight form of *R. mediterranea* was associated with the coiled form of *C. raciborskii* in the El-Dowyrat fish pond. The *C. raciborskii* morphotype recorded in this pond during the present study was restricted only to the coiled form, with complete absence of the straight form. This finding may be explained by the suggestion that the coiled form of *C. raciborskii* is able to grow faster than the straight form under conditions of lower light availability in more turbid reservoirs (Saker *et al.*, 1999). A previous study reported the dominance of the coiled form in the artificially destratified Solomon dam and the almost complete absence of the coiled form in the strongly stratified and highly stable lake Julius (Saker, 1996).

Cylindrospemopsis raciborskii has been reported to produce several toxins, including cylindrospermopsin, saxitoxins and anatoxin-a (Chorus & Bartram, 1999). Cylindrospermopsin is the primary toxin produced by *C. raciborskii* (St Amand, 2002). It is a hepatotoxin causing liver and kidney damage in mouse bioassays (Hawkins *et al.*, 1997). Although no HPLC analysis was performed for cylindrospermopsin detection in *C. raciborskii* isolated from the El-Dowyrat fish pond during the present study, because of lack of standards, the crude extract of this strain showed signs of poisoning in the mouse bioassay, as has been reported in the literature for other cylindrospermopsin-producing *Cylindrospermopsis* (Hawkins *et al.*, 1997; Li *et al.*, 2001b). The estimated LD_{50}s (mouse, intraperitoneal) of the cells of the Egyptian strain of *Cylindrospermopsis* (CY-Egyp.) during the present study were 450 mg kg^{-1} at 24 h and 205 mg kg^{-1} at 72 h. Compared to other strains, the CY-Egyp. strain is less toxic by an order of magnitude of about nine than the Australian strain (AWT205) (LD_{50}, 52 mg kg^{-1} at 24 h) (Hawkins *et al.*, 1997) and less toxic by an order of magnitude of two than the Thailand strain (LD_{50}, 250 mg kg^{-1} at 24 h) (Li *et al.*, 2001b). The lower toxicity of the CY-Egyp. strain could be attributed to the coiled morphotype of this strain, and confirms the findings of previous authors reporting that the coiled morphotype of *C. raciborskii* is less toxic than the straight morphotype (St Amand, 2002; McGregor & Fabbro, 2000). However, Saker *et al.* (1999) reported that both the straight and coiled morphotypes of *C. raciborskii* were almost equally toxic. The CY-Egyp. strain was more toxic to *Artemia salina* than to *D. magna*, indicating that *Daphnia* is more resistant to this toxic extract. The *Artemia salina* bioassay was suggested to be a useful screen for the toxicity-based detection of cylindrospermopsin (Metcalf *et al.*, 2002). The CY-Egyp. strain was less toxic (LC_{50}, 60 mg mL^{-1} at 24 h) than the *C. raciborskii* strains tested by Metcalf *et al.* (2002) (LC_{50}, 3.24–20 mg mL^{-1} at 24 h). As *C. raciborskii* and *D. magna* are found together in the El-Dowyrat fish farm (data not shown), *Cylindrospermopsis* would be expected to have a toxic effect on those animals that can feed on it. Previously, *C. raciborskii* has been reported to affect the feeding, growth and reproduction of many zooplanktonic organisms (Nogueira *et al.*, 2004). Although cylindrospermopsin from *C. raciborskii* was found to be not lethal to daphnids, *C. raciborskii* itself can promote a decrease in daphnid body sizes (Padisak, 1997). Therefore, the toxic effect of the CY-Egyp. strain on *Daphnia* during the present study could be more related to toxic compounds present in the algal crude extract than to cylindrospermopsin.

Raphidiopsis mediterranea isolated from the El-Dowyrat fish pond during the present study showed neurotoxic effects on mice. Although the neurotoxic agent in this algal extract was not identified, the symptoms exhibited by mice were similar to those of cyanobacterial extracts containing neurotoxins such as anatoxin-a (Carmichael, 1992; Namikoshi *et al.*, 2003). Recently, *R. mediterranea* was identified in Lake Biwa, Japan Watanabe *et al.*, 2003), and found for the first time to produce homoanatoxin-a and anatoxin-a (Namikoshi *et al*., 2003). Therefore, the present study is the second to report the neurotoxicity of this cyanobacterium. On the other hand, Li *et al*. (2001a) found that *Raphidiopsis curvata* isolated from a fish pond in Wuhan, China produced the alkaloid hepatotoxins cylindrospermopsin and deoxycylindrospermopsin, but its extract did not show lethal toxicity to mice at doses up to 1500 $mg\,kg^{-1}$. The present study also showed that *R. mediterranea* extract was highly toxic to *Artemia* and *Daphnia*. The toxicity of this species to *Daphnia* is reported here, and needs to be studied for other zooplankton organisms in aquatic ecosystems.

In conclusion, the present study reports and confirms the presence of toxic *C. raciborskii* and *R. mediterranea* in Egyptian fresh waters. The recent appearance of these species in this pond may be attributed to the warming of the pond water as a result of human activities, including removal of trees and loss of macrophytes at the end of 2001. In addition to warming, water stagnation (lack of water renewal) could participate in creating suitable conditions for the appearance of *C. raciborskii* and *R. mediterranea* in this pond. Together with the temperature, other environment factors (e.g. pH, conductivity, nutrients) also affected the abundance of *C. raciborskii* and *R. mediterranea* in this pond. These two species showed also a significant variation in distribution along the depth profile of the El-Dowyrat fish pond. Only the coiled form of *C. raciborskii* was found in this pond during the present study. Isolates of *C. raciborskii* and *R. mediterranea* from this pond exhibited toxicity to *Artemia salina* and *D. magna*. Also, the extracts of *C. raciborskii* and *R. mediterranea* showed hepatotoxic and neurotoxic effects, respectively, on mice. The identification of toxic *C. raciborskii* and *R. mediterranea* in this Egyptian fish pond should be a matter of concern for phycologists, and water and public health authorities in Egypt, as these species are likely to spread to other water sources in Egypt. Therefore, populations of *Cylindropsermopsis* and *Raphidiopsis* should be considered upon monitoring of toxic cyanobacteria in recreation and drinking water sources in Egypt.

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