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Original Research

Population Dynamics of *Raphidiopsis raciborskii* and Cylindrospermopsin Concentration in Ea Nhai Reservoir in Dak Lak Province, Vietnam

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Abstract

Raphidiopsis raciborskii is a globally distributed bloom-forming cyanobacterium and produces toxin cylindrospermopsin (CYN), which results in public and environmental health consequences. piece of evidence To understand their blooming dynamics and to identify the environmental factors that determine their success in the tropical reservoir, a monthly monitoring program was conducted from May 2019 to April 2020. Variations in phytoplankton communities, environmental parameters (temperature, pH, dissolved oxygen, turbidity, dissolved inorganic nutrients) and CYN were simultaneously determined from a total of 36 samples in the Ea Nhai reservoir in Vietnam. The phytoplankton is composed of a dominant cyanobacteria community formed mainly by filamentous *R. raciborskii* with the biovolume of up to 66.8 mm^3 L^{-1}. PCA and Pearson correlation results showed the biovolume and variation of *R. raciborskii* in the Ea Nhai reservoir were mainly related to temperature and nutrients ($N\text{-}NH_4$, $P\text{-}PO_4$, TP, TN). Analysis of the toxins by ELISA demonstrated the presence of CYN in all water samples with an average value of 1.24 µg L^{-1} and it was significantly correlated with the biovolume of the invasive species *R. raciborskii.* The results of the HPLC analysis confirmed the production of CYN in nine isolated *Raphidiopsis* strains with concentrations ranging from 0.054 to 0.584 µg g^{-1}DW. The relatively

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high biovolume of *R. raciborskii* that is associated with the level of CYN in the water and the capacity to produce CYN of the isolated *R. raciborskii* strains represent a potential threat to the public health and the life of domestic and wild animals in the reservoir catchment.

Keywords: *Raphidiopsis raciborskii*, cylindrospermopsin, tropical reservoir, Dak Lak province

Introduction

Harmful algal blooms (HABs) occur around the world, causing significant damage in aquatic ecosystems and contributing to the loss of biodiversity globally [1-3]. Cyanobacterial harmful algal blooms (CyanoHABs) are particularly problematic due to their ability to reduce water quality, disrupt nutrient cycling and produce toxic secondary metabolites (cyanotoxins) that are released into the aquatic environment [4, 5]. *Raphidiopsis raciborskii* (formerly known as *Cylindrospermopsis raciborskii*) is a harmful, planktonic cyanobacterium. *R. raciborskii* was originally described in Java, Indonesia and is considered a typical tropical species. Currently, this species is increasingly reported in the tropics, subtropics and temperate lakes and reservoirs worldwide [2, 6-8]. The invasion of *R. raciborskii* has been hypothesized from the tropical zone to the northern latitudes based on morphology, toxicity, and phylogeography studies [9]. The successful invasion of the temperate zone may be due to its phenotypic flexibility, nitrogen fixation capacity and persistence of different ecotypes [3]. Global warming and eutrophication have also been shown to influence the spread of *R. raciborskii* in temperate regions [5]. *R. raciborskii* can also produce several different toxins, such as cylindrospermopsin (CYN), saxitoxin (STX) and anatoxin-a. These toxins have chronic health effects and are associated with the fatal poisonings of many animals and humans [4].

Cylindrospermopsin (CYN) is one toxin produced by *R. raciborskii*. It is an alkaloid ($C_{15}H_{21}N_5O_7S$; 415.43 Da) with tricyclic guanidine radical, one sulfate group and one uracil ring [10]. At present, five analogues of CYN are known [1]. The impact of this compound's biological activity is wide. CYN can negatively affect organs, such as the liver, eyes, kidneys, lung, heart, thymus, spleen, adrenal glands, and intestine. Moreover, it is toxic to cells, genes, the immune system, the nervous system, and the endocrine system. The major toxicity mechanism is implemented by inhibiting protein synthesis, interacting with cytochrome P450 (CYP450), causing oxidative stress and DNA strand breakage, linking estrogen receptors, and influencing acetylcholinesterase activity (AChE) [11, 12]. Unlike microcystins (MC) which are susceptible to photodegradation, most CYNs have high chemical stability, a high release capacity into the water, and a slow rate of decomposition due to their influence on abiotic factors in nature [13]. Therefore, it creates potential risks in using and managing water resources. In addition, the lack of visual monitoring signs i.e., no surface scums, no change of water color, rapid germination with large numbers of cells, metamorphosis, relative toxicity and the year-round existence makes the species more challenging to manage, with managers reliant on chemical and microscopic analyses which require significant time and money.

Toxic cyanobacterial blooms occur consistently and at a higher frequency in Vietnam's inland and coastal eutrophic waters. Previous studies have shown that bloom-forming mainly by genus *Microcystis* and variants of MCs in bloom and cultural samples have been reported in lakes and reservoirs [14, 15]. *R. raciborskii* blooms have also been found in some water-bodies in Vietnam [16, 17]. However, studies of this species and its toxins in Vietnam are limited. The Ea Nhai reservoir in the Dak Lak province of Vietnam is located in the central highland region. It was built in 1986-1988 to provide drinking water supplies and agricultural irrigation for aquaculture and public usage for Dak Lak province and the surrounding areas. The phenomena of yellow discoloration, odor and streaks suspended under surface water have frequently been observed in the Ea Nhai reservoir during the dry season. However, no data on cyanotoxins exists for this system. The present study therefore aimed to determine the seasonal fluctuations in *Raphidiopsis raciborskii* biovolume from May 2019 to April 2020 at three sampling sites in the Ea Nhai reservoir. In addition, the correlation between the abundance of cyanobacteria, toxin concentrations (CYN) and environmental factors in the reservoir was examined.

Methodology

Study Site

The Ea Nhai Reservoir (12°44'41"; 108°11'53") located in Dak Lak province, Vietnam has a surface area of 250 ha and an effective water storage capacity of 15 million m^3 (Fig. 1). The average depth of the reservoir is 6 m, with the deepest site at full supply capacity being 17 m. The catchment area of the reservoir is 165 km^2. The average annual air temperature in the area is 26ºC with minimum and maximum air temperature values that are between 21ºC and 33ºC. The climate of the basin has the characteristics of both a humid tropical climate as well as the hot, dry southwest monsoon, which divides this area into two distinct seasons: the dry season from November to April; the rainy season from

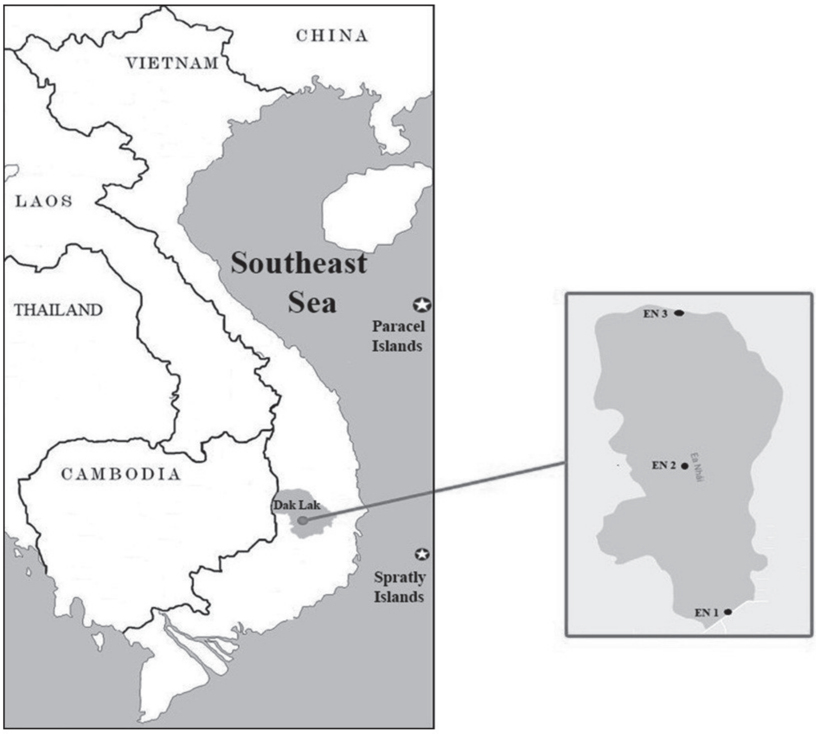

Fig. 1. Sampling location in the Ea Nhai reservoir, Dak Lak province.

May to October with a cool and humid climate (90% of annual rainfall). The main uses of the reservoir are as a public water supply and as agricultural irrigation for Dak Lak province and the surrounding areas. Agriculture (mainly coffee and rice crops) is what the land is predominantly used for in the basin.

Sample Collection and Analysis

Field sampling in the Ea Nhai reservoir was conducted monthly at three sampling sites (EN1, EN2 and EN3) from May 2019 to April 2020. Using a calibrated multi-parameter probe, environmental variables (water temperature, pH, dissolved oxygen (DO) and turbidity) were measured *in situ*. Water samples for nutrient analyses (P-PO_4, N-NH_4, N-NO_3, TP and TN) were collected by immersion of the laboratory cleaned polypropylene sample bottles below the surface water. Samples were kept cool in the dark at 4ºC before they were immediately transferred to the laboratory for further analyses. Chemical analyses were conducted according to International Standards (ISO). Water samples were analyzed within 24 h at the Institute of Biotechnology, Tay Nguyen University. Samples for qualitative phytoplankton examination were collected using plankton net (20 µm mesh size) and immediately fixed with formaldehyde solution at a 4% (v/v) final concentration. For phytoplankton enumeration, samples were collected with a plastic tube, 2 m long and 10 cm in diameter. Then, the water samples (0-2 m in depth) were mixed in a bucket and 100 mL sub-samples were transferred to glass bottles, fixed with the Lugol's iodine solution, and then sedimented for 48 h before counting.

Phytoplankton cell density was counted using a Sedgewick-Rafter counting chamber under a light microscope (Olympus BX51) [18]. The cell biovolume was estimated by measuaring geometric shape and/or size to each taxon from representative cell counts in field samples [19]. Cyanobacteria species were identified according to their morphology using light microscopic observation. Cyanobacteria species were identified according to their morphology-based on standard references [20]. At each sampling site, a live sample (sample without fixing with formaldehyde solution) was also collected for cyanobacteria isolation. Samples were kept cool and delivered to the laboratory within five h after sampling. For isolation, samples were then concentrated by centrifugation and/or filtering through nylon mesh. Isolation of *Raphidiopsis* species from living phytoplankton samples was carried out by selectively picking out filamentous *Raphidiopsis* forms using a modified and sterilized Pasteur pipette under an inverted microscope (Olympus CK40). Single filamentous cyanobacteria were rinsed in several drops of sterilized Z8 media [21] to remove the contaminating cells or suspended particles. The isolated strains were cultured at 24±4ºC, dark/light cycle 12 pm:12 am at an intensity of 2.000-3.000 lux. The biomass of cultured strains was harvested at the end of the exponential growth phase by centrifugation for 10 min at 6000 rpm at room temperature. The concentrated material was lyophilized by freeze-drying under vacuum at -55ºC for 24 h, then stored at -20ºC before toxins analysis. 1.5 mL field and culture medium water samples were taken for toxin analysis. Samples were stored in Eppendorf tubes and frozen at -18ºC until analysis.

Cylindrospermopsin Analysis

Enzyme-Linked Immunoabsorbent Assay (ELISA) Analysis

Cylindrospermopsin (CYN) concentrations in water samples from the Ea Nhai reservoir were examined by the ELISA method using an Abraxis Cylindrospermopsin ELISA kit (Microtiter Plate) (Abraxis, USA). The optical density of the sample was measured at 450 nm on an automatic ELISA reader system (CODA, Bio-Rad, USA) and the concentration of cylindrospermopsin ($\mu g\ L^{-1}$) in the samples was determined against the standard curve of cylindrospermopsin-HRP. If the cylindrospermopsin concentration in the samples was higher than a standard substance (2 $\mu g\ L^{-1}$), the samples were diluted until within the range of the standard curve.

HPLC Analysis

The freeze-dried samples from culturing were extracted in 2.5 mL methanol (MeOH - 99.9%) containing 0.1% trifluoroacetic acid (TFA) in an ultrasonic bath for 15 min. The sample then underwent a probe ultrasonication in ice for one min. Extracts were filtered through C_{18} column; and the column was cleaned with methanol. After filtration, the filtrate was evaporated at a low temperature (30ºC) for five min. After evaporation, the residue was re-suspended in 250 µL of water and purified by centrifugation prior to HPLC analysis [17]. The HPLC system Thermo consisted of an UltiMate 3000 autosampler, and an UltiMate 3000 Variable Wavelength Detector (VWD); column BDS Hypersil C_{18} (250x4.6 mm, 5.0 µ*m*), mobile phase: MeOH (A) 30 - water containing 10 mM ammonium acetate (B) 70% (v/v) (30:70), flow rate: 0.8 mL min^{-1}; sample volume: 10 µL; column chamber temperature: 30ºC; analysis time was seven minutes. The samples were injected into the mobile phase just before the column. In the column, the components were separated and the detector measured the absorbance peak at 262 nm. Cylindrospermopsin was identified by absorption spectra and retention time and quantified at 262 nm using CRM-CYN as an external standard.

Data Analysis

Pearson correlation matrix analyses were performed to quantitatively examine the correlations between the relative biovolume of *Raphidiopsis* species, cylindrospermopsin concentrations and environmental factors (pH, DO, temperature, turbidity, $N\text{-}NO_3$, $N\text{-}NH_4$, $P\text{-}PO_4$, TN and TP). One-way ANOVA was applied to check the significant difference at $p<0.05$. The IBM-SPSS Statistics Version 22.0 software was used to analyze the results at a 5% significance level.

Results and Discussion

Physical and Chemical Characteristics

The physico-chemical characteristics of the Ea Nhai reservoir from May 2019 to April 2020 are presented in Table 1. Water temperatures ranged from 25.5ºC to 32.0ºC with an average value of 29.0ºC during the investigation period. The pH values varied between a minimum of 7.1 to a maximum of 8.3 and did not differ significantly over the year. The DO ranged from 4.1 to 8.0 mg L^{-1} and higher values were measured in the dry season than the rainy season. Higher values of turbidity were recorded during the rainy period (May to October). Mean concentrations of ammonium and nitrate across the study were 0.23±0.05 and 0.21±0.08 mg L^{-1}, respectively.

Lower ammonium concentrations were found during the rainy period compared to the dry season. While there were no clear seasonal variations in nitrate concentrations. Dissolved orthophosphate-P concentration varied from 0.06 to 0.1 mg L^{-1}.

The Ea Nhai reservoir was characterized by relatively high concentrations of TN and TP, with mean TP concentrations ranging from 0.16 to 0.4 mg L^{-1} and mean TN concentrations ranging from 1.4 to 3.67 mg L^{-1}. Monthly TN:TP ratios ranged from 13.9 to 35, with a mean value of the study of 22.3. Agricultural activities and intensive fish cage aquaculture seem to be the main sources of nutrient enrichment in the Ea Nhai reservoir. According to the trophic classification proposed by the Organization for Economic Cooperation and Development criteria (OECD, 1982), the water quality of the Ea Nhai reservoir was classified to be eutrophic (based on TP values).

Phytoplankton Community and *R. raciborskii* Species in the Ea Nhai Reservoir

This study has provided fundamental information on the spatial and temporal distribution of cyanobacteria community in the poorly researched Ea Nhai reservoir. The microscopic examination of a total of 36 phytoplankton samples collected from the Ea Nhai reservoir from three sampling sites during the period of May 2019 to April 2020 showed the dominance of cyanobacteria during the study period. During the study, 39 taxa of phytoplankton were identified distributed in six groups: Cyanobacteria, Chlorophyceae, Bacillariophyceae, Euglenophyceae, Dinophyceae and Chrysophyceae. Throughout the study period, cyanobacteria were the most dominant contributor to the phytoplankton community accounting for more than 96% of the total phytoplankton and cyanobacteria cell density varied from 380 x 10^6 to 5009 x10^6 cells L^{-1}

Table 1. Environmental variables in the Ea Nhai reservoir during the year 2019-2020 (average values and min –max values).

Month	Temp (ºC)	DO ($mg\ L^{-1}$)	pH	Turbidity (NTU)	$N\text{-}NH_4$ ($mg\ L^{-1}$)	$N\text{-}NO_3$ ($mg\ L^{-1}$)	$P\text{-}PO_4$ ($mg\ L^{-1}$)	TN ($mg\ L^{-1}$)	TP ($mg\ L^{-1}$)
May-19	**25.33** *(25-25.5)*	**5.92** *(5.84-6.02)*	**8.34** *(8.11-8.71)*	**48.73** *(45.8-50.9)*	**0.15** *(0.14-0.16)*	**0.29** *(0.27-0.32)*	**0.07** *(0.066-0.075)*	**1.86** *(1.85-1.87)*	**0.17** *(0.15-0.2)*
Jun-19	**25.97** *(25.9-26.1)*	**5.81** *(5.74-5.88)*	**8** *(7.92-8.07)*	**52.63** *(49.5-56)*	**0.11** *(0.11-0.11)*	**0.2** *(0.2-0.21)*	**0.06** *(0.063-0.065)*	**1.76** *(1.68-1.85)*	**0.18** *(0.176-0.1885)*
July-19	**27.5** *(27-28)*	**5.37** *(5.16-5.78)*	**8.09** *(8.01-8.15)*	**62.73** *(53.2-77.1)*	**0.14** *(0.104-0.182)*	**0.22** *(0.196-0.252)*	**0.06** *(0.059-0.064)*	**1.38** *(1.28-1.54)*	**0.16** *(0.154-0.171)*
Aug-19	**28.17** *(28-28.5)*	**5.82** *(5.72-5.9)*	**8.25** *(8.21-8.3)*	**66.73** *(61.2-70.5)*	**0.21** *(0.196-0.235)*	**0.24** *(0.219-0.28)*	**0.1** *(0.094-0.099)*	**2.55** *(2.34-2.76)*	**0.4** *(0.395-0.408)*
Sep-19	**28.33** *(28-29)*	**5.51** *(5.38-5.6)*	**7.72** *(7.62-7.81)*	**74.17** *(56.2-100)*	**0.16** *(0.154-0.168)*	**0.2** *(0.196-0.203)*	**0.1** *(0.092-0.102)*	**1.65** *(1.61-1.68)*	**0.22** *(0.218-0.226)*
Oct-19	**30.17** *(30-30.5)*	**4.14** *(4.08-4.18)*	**7.94** *(7.9-7.98)*	**44.2** *(41.9-47.5)*	**0.18** *(0.18-0.184)*	**0.36** *(0.355-0.359)*	**0.1** *(0.099-0.104)*	**2.1** *(1.96-2.24)*	**0.22** *(0.206-0.233)*
Nov-19	**29.73** *(29.2-30.1)*	**7.21** *(7.14-7.3)*	**7.97** *(7.92-8)*	**52.83** *(50.6-55)*	**0.2** *(0.195-0.2)*	**0.17** *(0.163-0.17)*	**0.1** *(0.092-0.103)*	**2.16** *(2.04-2.34)*	**0.25** *(0.233-0.261)*
Dec-19	**31.4** *(31.3-31.6)*	**5.63** *(5.46-5.84)*	**8.05** *(8-8.1)*	**39.08** *(38.52-40.15)*	**0.15** *(0.148-0.162)*	**0.22** *(0.21-0.231)*	**0.09** *(0.089-0.097)*	**2.96** *(2.82-3.07)*	**0.26** *(0.255-0.268)*
Jan-20	**29.3** *(29-29.7)*	**5.96** *(5.88-6.02)*	**7.14** *(7.09-7.23)*	**37.49** *(36.17-39.08)*	**0.17** *(0.159-0.182)*	**0.26** *(0.245-0.275)*	**0.09** *(0.088-0.092)*	**3.43** *(3.31-3.53)*	**0.21** *(0.203-0.221)*
Feb-20	**32** *(30-34)*	**6.46** *(6.36-6.6)*	**8.25** *(8.2-8.31)*	**39.51** *(38.79-40.63)*	**0.29** *(0.29-0.294)*	**0.27** *(0.261-0.282)*	**0.1** *(0.091-0.116)*	**2.92** *(2.86-2.98)*	**0.29** *(0.282-0.304)*
Mar-20	**28.83** *(28.7-29)*	**7.77** *(7.74-7.84)*	**8.18** *(8.01-8.3)*	**40.14** *(40.06-40.24)*	**0.38** *(0.361-0.392)*	**0.19** *(0.19-0.198)*	**0.11** *(0.103-0.114)*	**3.67** *(3.5-3.75)*	**0.35** *(0.321-0.372)*
Apr-20	**31.73** *(31.7-31.8)*	**8.03** *(7.64-8.42)*	**8.29** *(8.27-8.3)*	**40.09** *(40.06-40.11)*	**0.36** *(0.36-0.368)*	**0.18** *(0.179-0.182)*	**0.1** *(0.098-0.102)*	**3.51** *(3.46-3.56)*	**0.28** *(0.279-0.281)*

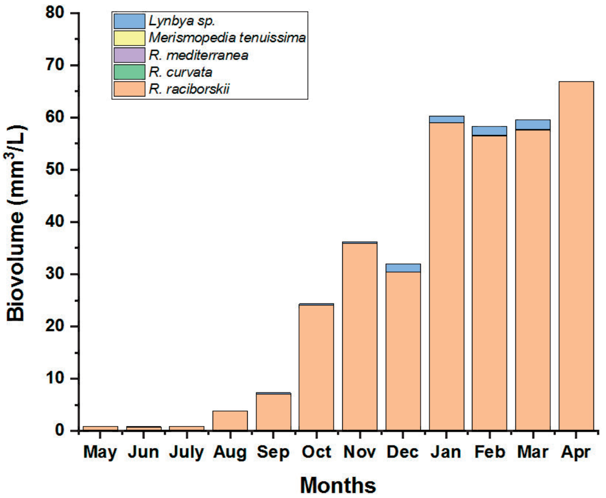

Fig. 2. Changes in occurrence of five most common cyanobacterial biovolume in the Ea Nhai reservoir from May 2019 to April 2020.

with the lowest density occurring in the rainy season period (May to July 2019) and higher cyanobacteria cells observed in the dry season from January to April 2020. Other groups such as Chlorophyceae, Bacillariophyceae, Euglenophyceae, Dinophyceae and Chrysophyceae were represented but in much lower cell densities. A total of 19 cyanobacteria taxa belonging to the orders of Nostocales, Oscillatoriales, and Chroococcales were recorded, including the known potential cyanotoxin producers, *Microcystis aeruginosa* (Kutzing) Kutzing*;* *M. wesenbergii* (Komarek) Komarek in Kondrateva*;* *M. botrys* Teiling*;* *M. flos-aquae* (Wittrock) Kirchner*;* *M. panniformis* Komárek, Komárková-Legnerová, Sant'Anna, M.T.P.Azevedo & P.A.C.Senna*;* *Oscillatoria limosa* C.Agardh ex Gomont*;* *Pseudanabaena mucicola* (Naumann et Huber-Pestalozzi) Schwabe*;* *R. Raciborskii* (Woloszynska) Aguilera, Berrendero Gomez, Kastovsky, Echenique & Salerno; *R. mediterranea* Skuja and *R. curvata* Fritsch et. Rich. Among the cyanobacteria*,* *R. raciborskii* consistently dominated the cyanobacterial community throughout the sampling period, accounting for 97.82% of the total cyanobacterial biovolume. Whereas *R. curvata*, *R. mediterranea, Merismopedia tenuissima* Lemmermann, and *Lyngbya* sp. accounted for a significantly lower proportion with 0.08%, 0.05%, 0.02% and 2.08%, respectively (Fig. 2).

Trichomes of the *R. raciborskii* from the Ea Nhai reservoir are free-floating, slightly curved and tightened at the cell wall, tapering toward the ends of trichomes with rounded conical cells (Fig. 3). Vegetative cells

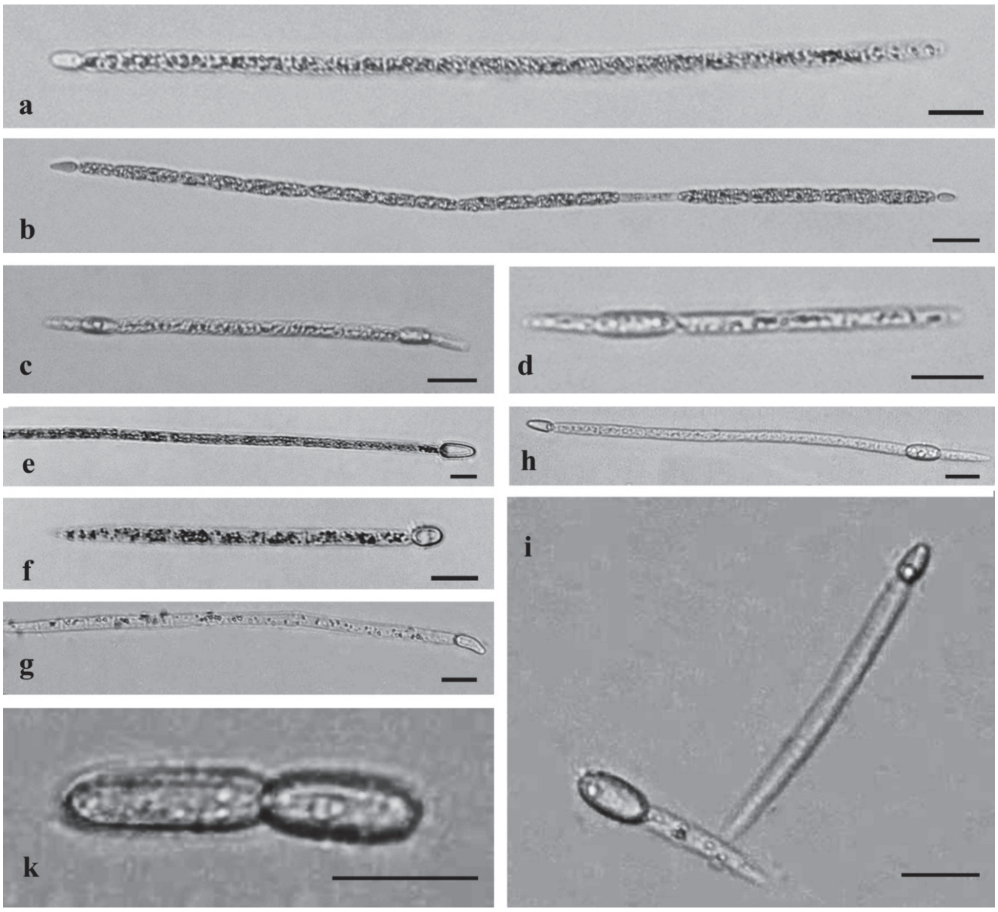

Fig. 3. The morphology of *Raphidiopsis raciborskii* in the Ea Nhai reservoir: a-d. Filaments with heterocytes and akinetes in nature; e-i. Filament with heterocytes and akinetes in cultures. Scale bars = 10 µm.

are cylindrical, 4.5-10.1 µm long, 2.5-4.1 µm wide and single heterocytes, conical or arrow-shaped, 2.5-4.5 µm wide, 3.2-9.2 µm long in the field samples. Akinetes are a long oval-shape, 8-12 µm long, 3.5-4 µm wide and located just behind the heterocyte or 3-4 vegetative cells from the heterocyte. In culture, vegetative cells are 6-12 µm long, 3-4.5 µm wide. Heterocytes morphology changes a lot and appears endlessly at one or both ends of the trichome, 7.5-12 µm long, 4-5 µm wide. Akinetes are 10-20 µm long and 4.5-5.5 µm wide. *R. raciborskii* have been known in their morphology to exist in many forms (straight, curved, coiled) in the natural environment. All isolated strains from the Ea Nhai reservoir were straight form in culture conditions with the average length of trichomes longer than that in the wild and will sometimes form into tufts. *R. raciborskii* has become increasingly prevalent and appeared in many tropical, subtropical and even in temperate water bodies worldwide [3, 22] and is known to cause blooms in Vietnamese freshwaters such as in Xuan Huong lake, Dau Tieng, Tri An reservoirs and some water bodies in Hue [16, 23]. *R. raciborskii* biovolume exhibited distinct temporal variation with the lowest biovolume of 0.77 $mm^3 L^{-1}$ and the highest value of 66.80 $mm^3 L^{-1}$ in June 2019 and January 2020, respectively. Although *R. raciborskii* did not form a scum on the surface of the water in the Ea Nhai reservoir, they were found in high biovolume and had a year-round presence in this tropical climate. *R. raciborskii* accounted for more than 97% of the total cyanobacterial biovolume. Other studies on *R. raciborskii* in tropical and subtropical regions found similar results. In a study in New Zealand, *R. raciborskii* blooms occurred in lakes Waahi, Waikare and Whangape with varying intensities and frequencies. In Lake Waahi blooms occurred in April 2007 (4.5 $mm^3 L^{-1}$). In lake Waikare, *R. raciborskii* has formed dense summer blooms, with the most severe occurring in February 2011 (12.5 $mm^3 L^{-1}$) and March 2013 (7.4 $mm^3 L^{-1}$), while *R. raciborskii* blooms were most frequent and dense in Lake Whangap with biovolume peaked at 144 $mm^3 L^{-1}$ [24]. Based on a seasonal survey in lakes in Yunnan Province, China, Jia et al. (2021) [25] investigated *R. raciborskii* populations, where *R. raciborskii* blooms were found in Lake Xihu at low water temperatures between 10-15ºC. The biovolume of *R. raciborskii* in Lake Xihu ranged from 0.01-42.44 $mm^3 L^{-1}$ [25]. The persistent dominance and occurrence of *R. raciborskii* throughout the year in this study have also been reported in many other previous studies [3, 5, 22]. However, in several temperate regions where *R. raciborskii* only occurs in warmer months [26, 27].

Concentrations of Cylindrospermopsin

Cylindrospermopsin (CYN) concentrations in water surface samples collected from the Ea Nhai reservoir were analyzed using ELISA. The ELISA test revealed CYN presence in all of the water samples throughout the year from May 2019 to April 2020 with a mean value of 1.24±0.08 µg L^{-1} (Fig. 4). Higher CYN concentrations were found during the dry season (November to April) than during the rainy season. Mean concentrations of 1.24 µg L^{-1} measured in this study were comparable to 0.11 to 1.12 µg L^{-1} reported from six lakes in Washington, USA [19] and 0-1.58 µg $L^{-1,}$ which was referenced from Huong river, Vietnam [16]. However, this value was much lower than those reported an urban reservoir used for drinking water supply, south China (8.25 µg L^{-1}); a temperate lake, Germany (11.8 µg L^{-1}) [28, 29] and a farm water supply in central Queensland (1050 µg L^{-1}) [30]. The CYN detected in this study was higher than the lifetime drinking water guideline value for CYN of 0,7 µg L^{-1} that was proposed by the World Health Organization [31]. CYN is an alkaloid cyanotoxin by which may be produced by some filamentous freshwater cyanobacteria species, including *R. raciborskii; R. curvata* Fritsch et. Rich;

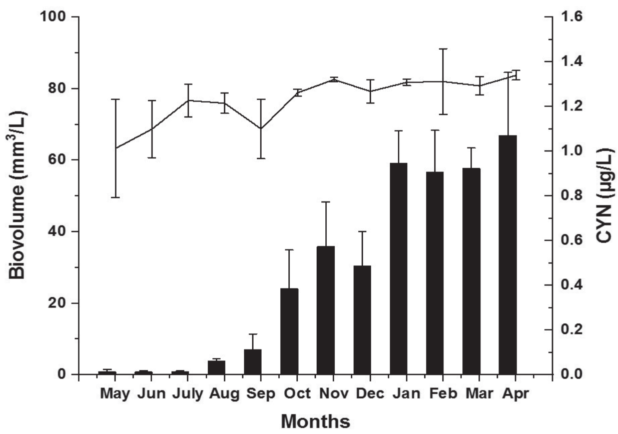

Fig. 4. Seasonal variation of the *Raphidiopsis raciborskii*, *R. curvata* and *R. mediterrancea* biovolumes and CYN concentrations in the Ea Nhai reservoir during the study period from May 2019-April 2020. Bar. Biovolume; line. CYN concentrations.

Table 2. Cylindrospermopsin (CYN) concentrations in nine *Raphidiopsis* isolated strains from the Ea Nhai reservoir.

Species	Strain codes	CYN concentrations (µg g^{-1} DW)
R. raciborskii	CENG	0.235
	CEN0	0.504
	CEN7	0.054
	CEN10	0.444
R. curvata	RCEN0	0.267
	RCEN1	0.314
	RCEN2	0.172
R. mediterranea	RMEN2	0.584
	RMEN3	0.398

R. mediterranea Skuja*; Aphanizomenon ovalisporum* Forti*; A. flos-aquae* Ralfs ex Bornet & Flahault*; A. gracile* Lemmermann*; A. klebahnii* Elenkin ex Pechar*; Umezakia natans* Watanabe*; Anabaena bergii* Ostenfeld*; A. planctonica* Brunnthaler*; A. lapponica* Borge*; Lyngbya wollei* (Farlow ex Gomont) Speziale and Dyck; *Dolichospermum* sp. and *Oscillatoria* sp. Vaucher ex Gomont [32, 33]. Of the potential CYN producers, *R. raciborskii, R. curvata* and *R. mediterranea* which were identified in the Ea Nhai reservoir, a significant correlation was found between CYN and *R. raciborski*, *R. curvata and R. mediterranea* species biovolumes ($p<0.01$, Table 3). However, the biovolume of *R. cuvarta* and *R. mediterranea* were very low, 0.08 $mm^3 L^{-1}$ and 0.02 $mm^3 L^{-1}$, respectevely. Therefore, the relatively high biovolume of the invasive species *R. raciborskii*, up to 66.8 $mm^3 L^{-1}$, in the phytoplankton community of the Ea Nhai reservoir could be the source of the CYN detected in the water during the study period.

The WHO provided Guideline values for CYN in lifetime drinking and recreational water are 0.7 µg L^{-1} and 6 µg L^{-1}, respectively [31]. To determine the level of the risk associated with the CYN contamination from three toxin-producing species (*R. raciborskii, R. cuvarta* and *R. mediterranea*) in the Ea Nhai reservoir, we calculated the average toxic concentration in a cell from nine strains of the three species. From there, we can estimate the harmful density of the species in accordance with the limits set by WHO. The average toxin concentration in a cell reached $1.87x10^{-8}$ µg $cell^{-1}$. With the limit for drinking water of 0.7 µg L^{-1}, the harmful density of the three species reached $37.4x10^6$ cells L^{-1}. For recreational water, the toxic density was $320x10^6$ cells L^{-1}. Compared with the above threshold, the *R. raciborskii* densities in the Ea Nhai reservoir ($377x10^6$ cells L^{-1} to $4.928x10^6$ cells L^{-1}) were higher for both drinking water and recreational water as suggested by WHO. Therefore, the presence of *R. raciborskii* and CYN in the reservoir makes the water source polluted and represents a potential threat to public health, community, and wildlife species around the reservoir catchment.

To confirm the identity of the potential producer of CYN in the Ea Nhai reservoir, nine *Raphidiopsis* strains belonging to three potential CYN producer species were successfully isolated from water samples collected from the Ea Nhai reservoir, including *R. raciborskii* (4 strains), *R. curvata* (3 strains) and *R. mediterranea* (2 strains). All *Raphidiopsis* strains investigated in the present study produced CYN and toxin concentrations that varied from 0.054 to 0.584 µg g^{-1} DW (Table 2). The CYN concentrations of the nine strains with positive results were estimated at the highest value of 0.584 µg g^{-1} DW, which was much lower than 1.

2 mg g^{-1} DW and 1.7-2 mg g^{-1} DW reported for Thai (*R. raciborskii* CY-Thai) and China strains (*R. curvata* CHAB1150), respectively [34, 35] and 917 µg g^{-1} DW noted for a Queensland strain (*R. mediterranea* FSS1-150/1) [36].

The abiotic variables (CYN, Temp., DO, $N\text{-}NH_4$, $P\text{-}PO_4$, TN and TP) were significantly correlated with the *R. raciborskii* biovolume (R = 0.60, $p<0.01$; R = 0.66, $p<0.01$; R = 0.60, $p<0.01$; R = 0.73, $p<0.01$; R = 0.65, $p<0.05$, R = 0,84, $p<0.01$; R = 0.34, $p<0.01$, respectively). Beside, *R. curvata* and *R. mediterranea* biovolume also showed a significant correlation with abiotic variables (CYN, Temp., DO, $N\text{-}NH_4$, $P\text{-}PO_4$, TN, TP) (Table 3). Cyanobacterial proliferation is typically caused by multiple drivers occurring simultaneously instead of a single environmental factor. Field and laboratory investigations have found that *R. raciborskii* abundance may be affected by environmental factors such as light, temperature and nutrients [3, 37]. In our study, the temperature was positively correlated with *R. raciborskii* biovolume. The result suggested that the high water temperature may play a key role in regulating the presence of *R. raciborskii* in Ea Nhai reservoir. Similar results were found in previous studies [38-40]. So far, *R. raciborskii* has successfully invaded many regions from tropical and subtropical towards temperate zones. Laboratory researches have shown that the optimum temperature for the proliferation of *R. raciborskii* is relatively high, between 25ºC and 35ºC. Typically, they formed blooms at temperatures greater than 25ºC [1, 25]. The temperature in the studied reservoir ranged from 25.5ºC to 32ºC and *R. raciborskii* blooms occur during the in-between seasons and the dry season of the year. The biological volume reached the highest value at the end of the dry season. The species has also formed dense summer blooms in Lake Waikare [24] and a shallow pond in France [38]. However, in the Xihu Lake, *R. raciborskii* blooms were also found at low water temperatures between 10ºC and 15ºC [25]. Those were below the temperature threshold (15ºC-17ºC) for the growth of *R. raciborskii* in the natural environment [39]. Several studies have shown that *R. raciborskii* was capable of growing at low temperatures of 11ºC in subtropical lakes [41], 13ºC-20ºC in tropical lakes [22]. The contrasting results related to the effect of temperature on *R. raciborskii* seem to be associated with the occurrence of genetically and ecophysiologically different ecotypes of *R. raciborskii* and its greater phenotypic plasticity in response to environmental factors [1, 42]. Moreover, climate warming is considered an important driver that enhances the expansion of the so-called phenotypically plastic species to new areas. Nitrogen (N) and phosphorus (P) have been shown to affect the dominance of *R. raciborskii* in freshwater systems [43]. Some studies suggested that *R. raciborskii* can dominate in both low and high phosphorus and nitrogen conditions. The abiotic variable that played an important role and had a significant influence on the biovolume of *R. raciborskii* in the present study were dissolved and total phosphorus and nitrogen. This cyanobacteria species have been found to dominate when the concentration of total phosphorus and total nitrogen are high [16, 42]. A recent study demonstrated that high pH

Table 3. Person correlation between relative abundance of species *R. raciborskii* and environmental factors in Ea Nhai reservoir from May 2019 to April 2020.

	Temp.	DO	pH	$N-NH_4$	$N-NO_3$	$P-PO_4$	TN	TP	Turbidity	*R. raciborskii*	*R. curvata*	*R. mediterranea*	CYN
Temp.	1												
DO	0.260	1											
pH	-0.012	0.262	1										
$N-NH_4$	***0.518*****	***0.754*****	***0.349****	1									
$N-NO_3$	-0.008	***-0.642*****	-0.057	-0.195	1								
$P-PO_4$	***0.672*****	***0.331****	-0.013	***0.666*****	0.043	1							
TN	***0.582*****	***0.603*****	-0.070	***0.749*****	-0.090	***0.634*****	1						
TP	***0.376****	***0.411****	0.299	***0.637*****	-0.134	***0.677*****	***0.585*****	1					
Turbidity	***-0.444*****	-0.280	0.039	***-0.373****	-0.181	-0.233	***-0.620*****	-0.047	1				
R. raciborskii	***0.662*****	***0.602*****	-0.134	***0.729*****	-0.110	***0.648*****	***0.844*****	***0.343****	***-0.615*****	1			
R. curvata	***0.494*****	***0.734*****	0.170	***0.909*****	-0.223	***0.576*****	***0.796*****	***0.452*****	***-0.504*****	***0.842*****	1		
R. mediterranea	***0.612*****	***0.728*****	0.069	***0.796*****	-0.274	***0.563*****	***0.857*****	***0.427*****	***-0.614*****	***0.905*****	***0.928*****	1	
CYN	***0.698*****	0.309	-0.099	***0.474*****	-0.199	***0.515*****	***0.492*****	***0.356****	-0.298	***0.596*****	***0.438*****	***0.506*****	1

* Correlation is significant at the 0.05 level (2 tailed), ** Correlation is significant at the 0.01 level (2-tailed).

facilitated the release of phosphorus from sediment that provided the phosphorus source for the abundance of *R. raciborskii* in Dongqian, China [2]. As mentioned by Posselt et al. (2009) [44] addition of dissolved inorganic phosphate (DIP) in field experiments conducted in a subtropical reservoir in Queensland, Australia, has also promoted *R. raciborskii* dominance. However, the present study results are contrary to other studies that reported *R. raciborskii* dominated in phosphorus-limited reservoirs [45, 46]. This is due to their high affinity for P and high phosphate uptake capacity, which allows them to outcompete other cyanobacteria and eukaryotic phytoplankton species [3, 46]. Ammonium concentrations were positively correlated with *R. raciborskii* biovolume in the present study. Ammonium concentrations were considered the preferred nitrogen source for the growth of R. raciborskii. The highest growth rate of this species was found in the presence of ammonium. Similarly, as mentioned by, high ammonium concentration (up to 700 mg L^{-1}) was the main factor that boosted the bloom of *R. raciborskii* in two Brazilian reservoirs [5]. However, the dominance of a heterocyclic species such as *R. raciborskii* can be found under low nitrate concentration [38]. Although *R. raciborskii* is able to fix atmospheric nitrogen, this species can maintain high growth rates under diazotrophic and non-diazotrophic conditions [47]. In addition, other studies have reported that the biomass of *R. raciborskii* remains high in abundance under low phosphorus (P) and nitrogen (N) concentrations [2, 5, 48]. The reason for such contradiction in nutritional requirements for the same species may be due to variation between strains (ecotypes) in populations. Recent studies have demonstrated that *R. raciborskii* strains isolated in the same lake can dramatically alter their morphological, physiological and genetic properties to enhance the potential of populations, to adapt quickly to changing environmental conditions [1, 3, 48, 49]. Although the relationship between *R. raciborskii* and different nutrients is complex, these studies showed that *R. raciborskii* can proliferate over other phytoplankton species in a wide range of nutrient concentration due to their flexible physiological behavious including: high phosphorus and ammonium uptake affinity; and a high storage capacity for phosphorus [49]. More studies are necessary to better understand the factors that promote the dominance of this species in the Ea Nhai reservoir.

Conclusions

This study indicated that the eutrophic conditions of the Ea Nhai reservoir in Vietnam were dominated by the cyanobacterium species *R. raciborskii* with the biovolumes up to 66.8 mm^3 L^{-1}. CYN was also measured throughout the study period, representing a risk for aquatic and human health. Nine *Raphidiopsis* strains belonging to three potential CYN producer species (*R. raciborskii, R. curvata* and *R. mediterranea*) were successfully isolated and confirmed to produce CYN. Moreover, such abiotic factors as temperature and nutrients (N-NH_4, P-PO_4, TP, TN) played an essential role in the occurrence and variation of *R. raciborskii* abundance in the Ea Nhai reservoir.

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Conflict of Interest

The authors declare no conflict of interest.

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