A snapshot of alien cyanobacteria found in northeastern European freshwaters - Lithuania case

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ABSTRACT

In recent years, cyanobacterial invasions have increased in European temperate lakes. Climate warming is highlighted as a key driver of the distribution and establishment of alien cyanobacteria, and water bodies in poor ecological status, characterized by meso-eutrophic conditions, are underlined as a suitable habitat for invasion. The establishment of newly arrived species is directly related to fluctuating physicochemical factors such as temperature, and nutrient concentrations, especially phosphorus and nitrogen, or biotic factors such as competition. Cyanobacterial invasions can have profound ecological impacts, such as displacing native species, causing the biodiversity loss of local communities, and modifying the ecosystem's cyanotoxins profile. This study presents the occurrence of four alien bloom-forming potentially toxic cyanobacteria - *Chrysosporum bergii, Cuspidothrix isstaschenkoi, Raphidiopsis raciborskii* and *Sphaerospermopsis aphanizomenoides* - in the northern parts of their current range in temperate Europe and provides insights into their ecology. Special attention was paid to the lakes of Lithuania (Simnas, Jieznas and Gineitiškės), which is the northernmost location of distribution zone for some alien cyanobacteria and provides as a dispersion route to Norther Europe via the continental area. We i) described dynamics of indigenous cyanobacteria community invaded by alien cyanobacteria; ii) assessed concentrations of cyanotoxins in field and culture samples, and suggested toxin producers; and iii) detailed the correlation between the biomass of alien cyanobacteria and environmental conditions. These species were found in three human-affected shallow hyper-eutrophic lakes during warm period of the year; however, their biomass was low. We assume that present temperatures do not limit the occurrence of these alien species but are insufficient for their successful proliferation. In addition, we provided the first evidence of anatoxin-a production by isolated strains of *C. issatschenkoi* in Lithuania. Alien cyanobacteria were detected at an early stage of their development, however, a rise in global temperatures and the spread of strains with toxigenic potential could lead to increased proliferation and further northward expansion of these alien species.

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Key words: aquatic invasions; freshwater; *Chrysosporum*; *Cuspidothrix*; *Raphidiopsis*; *Sphaerospermopsis*.

Citation: Šuikaitė I, Karosienė J, Koreivienė J. A snapshot of alien cyanobacteria found in northeastern European freshwaters - Lithuania case. *J Limnol 2024;83:2183*.

Edited by: Diego Fontaneto, *National Research Council, Water Research Institute (CNR-IRSA), Verbania Pallanza, Italy.*

Received: 5 March 2024.
Accepted: 26 April 2024.

J. Limnol., 2024; 83:2183
DOI: 10.4081/jlimnol.2024.2183

INTRODUCTION

Environmental changes caused by human activity disrupt the natural distribution of species by increasing their establishment into non-native regions and, therefore, affect native biodiversity (Sukenik *et al.,* 2012, 2015; Milardi *et al.,* 2019). Those species that have been introduced to habitats beyond their natural geographic range are defined as alien species (Ricciardi & Cohen, 2007; Sukenik *et al.,* 2012). In the context of freshwater alien cyanobacteria, this phenomenon can be attributed to the global expansion of trade, human mobility, fishing, and aquaculture (Nunes *et al.,* 2015). Invasive alien species are species that cause harmful effects on the environment. They can have profound ecological impacts, such as displacing native species, affecting trophic interactions, and modifying habitat structure (Sukenik *et al.,* 2015; Weithoff *et al.,* 2017; Buchberger and Stockenreiter, 2018).

During the last decade, cyanobacteria have increased their distribution into non-native habitats (Sukenik *et al.,* 2012). Of particular interest are cyanobacteria within the Nostocales order that are recognized as species with strong competitive advantage for invasion (Sukenik *et al.,* 2012). In this study we focus on four bloom-forming Nostocalean cyanobacteria, *Chrysosporum bergii* (Ostenfeld) Zapomelová *et al.* (2012), *Cuspidothrix issatschenkoi* (Usačev) Rajaniemi *et al.* (2005), *Raphidiopsis raciborskii* (Wołoszyńska) Aguilera *et al.* (2018), and *Sphaerospermopsis aphanizomenoides* (Forti) Zapomelová *et al.* (2009), all considered as alien species due to their non-European origin (Stüken *et al.,* 2006, Kaštovský *et al.,* 2010; Zapomělová *et al.,* 2012; Napiórkowska-Krzebietke *et al.,* 2023).

Cyanobacteria can produce cyanotoxins, which production varies geographically. *Raphidiopsis raciborskii* produces cylindrospermopsin (CYN) and saxitoxins (STXs) in South America and Australia (Vico *et al.,* 2020). While cyanotoxin production by *R. raciborskii* has not been confirmed in Europe, some strains have been shown to produce yet undefined toxic metabolites (Antal *et al*., 2011; Poniedziałek *et al.,* 2015). Microcystins (MCs) production by *Sphaerospermopsis aphanizomenoides* has been reported just once without molecular confirmation (Sabour *et al.,* 2005), and there are no reports of cyanotoxin production by *Chrysosporum bergii*. Only *Cuspidothrix issatschenkoi* is considered a cyanotoxin producer in Europe, with its ability to produce anatoxin-a (ATX-a) confirmed by molecular analysis (Stoyneva-Gärtner *et al.,* 2022).

Raphidiopsis raciborskii is the most often reported species in Europe among other alien cyanobacteria (Šuikaitė *et al.,* 2023) and is predicted to spread further in Central and Northern Europe (Meriggi *et al.,* 2022). In contrast, *Chrysosporum bergii, Cuspidothrix issatschenkoi*, and *Sphaerospermopsis aphanizomenoides* are less prevalent species due to their specific environmental preferences (Hodoki *et al.,* 2013; Šuikaitė *et al.,* 2023). However, all the species have expanded towards northern temperate regions in Europe. The northern range limits for *C. bergii* and *S. aphanizomenoides* are in Lithuania (Savadova *et al.,* 2018; Karosienė *et al.,* 2020). In contrast, *C. issatschenkoi* and *R. raciborskii* have successfully extended their range further northward into Russia for *R. raciborskii* (Babanazarova *et al.,* 2011) and Finland for *C. issatschenkoi* (Rajaniemi *et al.,* 2005). Lithuania's terrestrial landscape, lacking natural barriers like seas or mountains, serves as a potential corridor for species dispersal into Northern Europe.

Climate warming is highlighted as a key driver of the distribution and establishment of alien cyanobacteria, and water bodies in poor ecological status, characterised by meso-eutrophic conditions, are underlined as a suitable habitats for invasion (Kokociński and Soininen, 2012; Babanazarova *et al.,* 2015; Budzyńska and Gołdyn, 2017; Kokociński *et al.,* 2017; Budzyńska *et al.,* 2019; Napiórkowska-Krzebietke *et al.,* 2023). However, the literature on factors facilitating the establishment of alien cyanobacteria in northern parts of Europe is sparse, with only two studies addressing the establishment of these species above the 54°N latitude (Babanazarova *et al.,* 2015; Kokociński *et al.,* 2017). The aim of this study is to present new occurrences of alien cyanobacteria in the northern range of their distribution in temperate Europe, describe stages of establishment, and provide insights into their ecology. Special attention was paid to the lakes of Lithuania, which is the northernmost location of distribution zone for some alien cyanobacteria and provides as a dispersion route to Norther Europe *via* the continental area. Our research has: i) examined the diversity of cyanobacterial communities invaded by alien species during their growth season; ii) assessed concentrations of cyanotoxins in field and culture samples and suggested toxin producers; and iii) detailed the correlation between the biomass of alien cyanobacteria and environmental conditions.

METHODS

Study sites and sampling

The study was conducted for three shallow lakes in Lithuania: Lakes Simnas, Jieznas, and Gineitiškės (Tab. 1). All three lakes are used for recreational purposes and are areas surrounded by settlements and agricultural fields. Water surface samples were collected at a depth of 0.5 m, while the integrated column samples collected from the whole water column at the deepest point of the lake using a stoppered hose known as the "Anaconda" (Mantzouki *et al.,* 2018). Sampling occurred every two weeks from June to September in 2021 for Lakes Simnas and Jieznas, and in 2022 for Lake Gineitiškės. A total of 22 samples were collected for environmental conditions, cyanobacterial composition, and cyanotoxin analysis.

Environmental variables and chlorophyll-*a*

Ecologically important environmental parameters from water surface such as water temperature, ph, conductivity, dissolved oxygen, Secchi depth, total nitrogen (TN), total phosphorus (TP), TN:TP ratio, chlorophyll-*a* (chl-*a*) were measured in three studied lakes (Tab. 2). Water temperature, pH, conductivity, dissolved oxygen values were recorded in-situ by a WTW F/Set-3 portable multiline meter with selective electrodes. Transparency of the water was measured using a Secchi disk. TN and total TP were analysed according to standard methods (LST ENISO 10304; LST EN ISO 14911) in the certificated laboratory. For the determination of chl-*a,* samples from both the water surface and integrated column were transported to the laboratory and measured using a fluorometer algae lab analyser (bbe Moldaenke GmbH, Schwentinental, Germany).

Phytoplankton collection and analysis

Samples for phytoplankton analysis (0.5 L) were preserved by adding 10% (v/v) formaldehyde solution for fixation. The preserved samples were decanted prior counting after they had been allowed to settle for at least 7 days. Cyanobacterial species identification and counting were performed using a Nageotte chamber under a light microscope. At least 400 cells per sample were estimated. Biovolume was calculated based on the cell

Tab. 1. Morphometric characteristics of the studied lakes.

Lake	Maximum depth, m	Surface area, ha	Coordinates
Simnas	4.1	244	54°39'95.36", 23°63'83.37"
Jieznas	4.4	74	54°59'27.01", 24°18'03.96"
Gineitiškės	3	14.19	54°73'79.55", 25°18'53.21"

numbers and mean cell volumes of species using formulas for geometric shapes (Hillebrand *et al.,* 1999). Taxonomic identification of cyanobacterial species was based on morphology according to the descriptions of Komárek (2013). Species that biomass exceeded 10 % of total cyanobacterial biomass were considered as dominant. Potentially toxin-producing species were determined according to Bernard *et al.* (2017) and Sabour *et al.* (2005).

Isolation of strains

Cuspidothrix issatschenkoi, known as a producer of ATX-a in Europe (Stoyneva-Gärtner *et al*., 2022), was isolated from Lake Jieznas in 2021. Two *C. issatschenkoi* strains (NRC/JIE/2021/D5 and NRC/JIE/2021/F5) were isolated using a glass microcapillary pipette. The strains were inoculated in modified MWC medium (Lebret *et al.,* 2012) and cultured at 20°C, illuminated with 100 µmoL photons m^{-2} s^{-1} and with a photoperiod regime of 12 h:12 h light:dark cycle. The cyanobacterial strains were deposited in the culture collection of algae and cyanobacteria at the Nature Research Centre (Lithuania).

Cyanotoxins analysis

Microcystins, saxitoxins, and anatoxin-a analysis were analysed by the enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instruction. The Beacon Analytical Systems (Saco, ME, USA) ELISA test was used for MCs analysis (a maximum detection limit of 5 µm L^{-1}), and the Eurofins Abraxis (Warminster, PA, USA) ELISA test was used for ATX-a and STXs (a maximum detection limit of 5 µm L-1 and 0.4, respectively). ELISA analysis was performed on water field samples collected from the water surface of Lakes Simnas, Jieznas, and Gineitiškės, as well as on cultures of isolated *Cuspidothrix issatschenkoi* strains collected from Lake Jieznas. Analysis was conducted to determine total concentration of cyanotoxins that includes both intracellular and extracellular content. For the isolated cyanobacterial cultures, 40 mL of each culture was centrifuged at 8000× g for 10 min. Subsequently, the resulting wet biomass was promptly frozen at -20°C until analysis. A positive control and negative control supplied in the ELISA kit were used for quality control measures. The absorbance was recorded using a labsystems Multiscan RC (Thermo Fisher Scientific, Waltham, MA, USA) plate reader at 450 nm.

Data visualization and statistical analysis

Statistical analysis and data plotting were performed using software R (version 3.5.2), with a significance level set at $p<0.05$. Shannon-Weaver diversity index (H) of cyanobacteria was calculated in the *vegan* package. Differences of environmental variables (physical and chemical parameters, and chl-*a*), Shannon-Weaver diversity index and total concentrations of cyanotoxins between three lakes were determined using a one-way ANOVA after the positive normality test (Shapiro-Wilk test) and homogeneity of variance test (Levene test), while non-parametrical data were analysed using the Kruskal-Wallis test in the *dplyr* and *car* packages. Data visualization of dominant cyanobacteria was performed using *ggplot2, ggpubr* and *patchwork* packages. Non-parametrical Mann-Whitney U test was used to compare the biomass of dominant and alien cyanobacteria between water surface and column samples together among three lakes using the *dplyr* package. The relation of the environmental factors and biomass of cyanobacteria, and the relation of the cyanotoxins and biomass of potentially toxic cyanobacteria in the studied area were analysed using Spearman's rank correlation in the *Hmisc* package.

RESULTS

Environmental variables

During the sampling season, water temperatures in all three lakes were similar (Tab. 2). It ranged from 17.9 to 24.7°C in Lake Simnas, from 17.7 to 26.6°C in Lake Jieznas, and from 14.2 to 26.9°C in Lake Gineitiškės. Statistical analysis revealed that among the environmental parameters studied, only conduc-

Tab. 2. Summary of environmental variables, chlorophyll-*a* (chl-*a*) concentrations (mean± SD), Shannon-Weaver diversity index (H) and trophic status in the three lakes during the study period, and results of one-way ANOVA (F) and Kruskal-Wallis (χ^2). Bold values indicate statistical significance at the p<0.05 level.

	Simnas	Jieznas	Gineitiškės	Test statistic	p-value
Water temperature, °C	21.64±2.60	22.66±3.34	20.31±4.30	F=0.80	0.465
pH	8.74±0.32	8.64±0.37	8.58±0.90	χ^2=1.58	0.455
Conductivity, µs cm^{-1}	419.57±52.71	440.00±5.77	303.13±26.07	χ^2=14.72	**<0.001**
Dissolved oxygen, mg L^{-1}	12.52±1.26	11.00±2.30	9.77±2.29	F=3.28	0.060
Secchi depth, m	0.64±0.19	0.60±0.06	0.54±0.15	F=1.03	0.378
TN, mg N L^{-1}	1.51±0.05	2.34±0.78	1.53±0.35	χ^2=2.49	0.288
TP, mg P L^{-1}	0.04±0.03	0.03±0.01	0.10±0.15	χ^2=6.58	**0.037**
TN:TP atomic ratio	91.84±41.96	160.29±70.85	71.74±22.36	χ^2=5.73	0.057
Chl-*a* surface water, µg L^{-1}	32.87±7.12	22.29±3.79	59.53±19.44	F=9.88	**0.001**
Chl-*a* column water, µg L^{-1}	33.18±6.90	23.55±3.00	59.70±19.19	F=10.21	**0.001**
Shannon-Weaver diversity index	2.11±0.28	0.60±0.25	1.10±0.30	F=53.98	**<0.001**
Trophic status*	Hypertrophic	Eutrophic	Hypertrophic		

TN, total nitrogen; TP, total phosphorus; *trophic status was assessed according to Vollenweider and Kerekes, 1982.

tivity, TP, and chl-*a* showed significant differences ($p<0.05$) among three lakes (Tab. 2). Lake Gineitiškės exhibited significantly lower electrical conductivity but higher TP and chl-*a* concentrations. According to mean and maximum values of Secchi disk depth, TP and chl-*a* concentrations, Lakes Simnas and Gineitiškės were classified as hypertrophic and Lake Jieznas as eutrophic (Tab. 2).

Diversity and dynamics of cyanobacterial community

In this study, four alien cyanobacterial species were identified. *Sphaerospermopsis aphanizomenoides* was found only in Lake Simnas, while both *Chrysosporum bergii* and *Raphidiopsis raciborskii* were present in Lakes Jieznas and Gineitiškės (Fig. 1) (Tab. 3). *Cuspidothrix issatschenkoi* was detected in all three lakes studied. In all studied lakes, the biomass of four alien cyanobacterial species was low, with concentrations not exceeding 1.5 mg L^{-1} (Tab. 3). A total of 54 species of native cyanobacteria were identified in three investigated water bodies, including taxa from genera *Aphanizomenon, Aphanocapsa, Aphanoteche, Anabaenopsis, Chroococcus, Cyanodyction, Dolichospermum, Geitlerinema, Jaaginema, Limnothrix, Merismopedia, Microcystis, Phormidium, Planktothrix, Planktolyngbya, Pseudanabaena, Rhabdoderma, Romeria, Snowella, Spirulina*, and *Woronichinia*. Out of them, 12 species were found common in all three lakes. No statistically significant difference was found between the biomass of dominant and alien cyanobacteria in water surface and

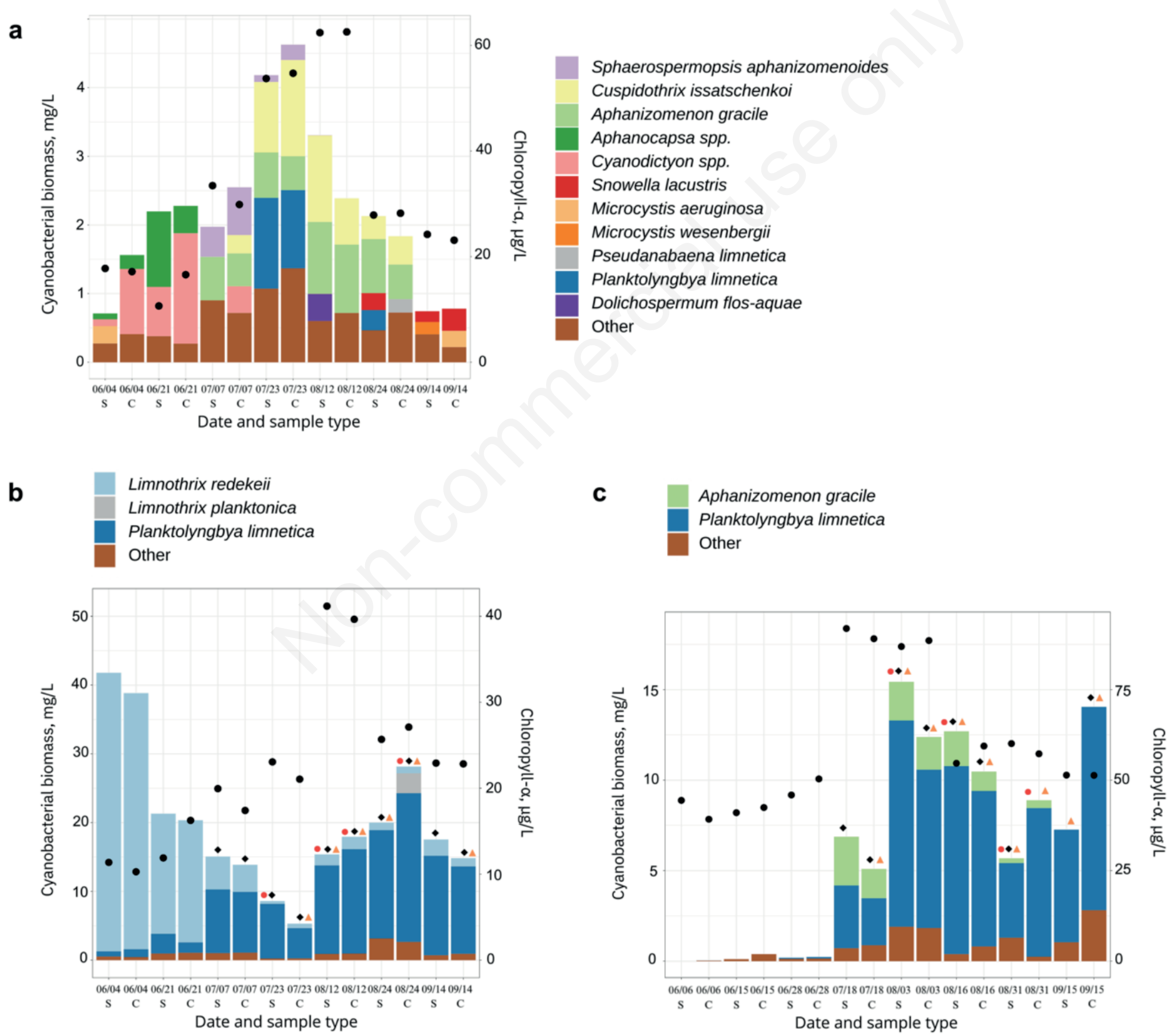

Fig. 1. Cyanobacterial composition from surface (S) and column (C) water in Lake Simnas (a), Lake Jieznas (b) and Lake Gineitiškės (c) during the studied period. Only cyanobacteria that exceeds 10% of total cyanobacterial biomass are shown in graphs. Alien species that reached less than 1.5 % of total cyanobacterial biomass are presented under the symbols: red circle, *C. bergii*; black square, *R. raciborskii*; orange triangle, *C. issatschenkoi*. Black dots represent chlorophyll-*a* values from water surface samples.

column samples (p>0.05) (Tab. 4). Shannon-Weaver diversity index (H) of cyanobacterial population significantly differed between the lakes (F=53.98, p<0.001) (Tab. 2). The highest diversity index was reported in Lake Simnas (H=2.11) with polidominant cyanobacterial community that included at least 10 dominant species (Fig. 1A). In Lake Simnas, alien *Sphaerospermopsis aphanizomenoides* reached a considerable amount of the total cyanobacterial biomass in July (up to 27 % of total cyanobacterial biomass). However, its dominance was short-lived and was replaced by alien *Cuspidothrix issatschenkoi* that accounted for 38% of the total cyanobacterial biomass. Despite considerable contributions to the total cyanobacterial biomass, the biomass of *S. aphanizomenoides* and *C. issatschenkoi* were low, reaching up to 0.70 and 1.26 mg/l, respectively. Moreover, the total biomass of the cyanobacterial community in Lake Simnas was lower compared to Lakes Jieznas and Gineitiškės - it reached the maximum biomass of 4.63 mg L^{-1} (Tab. 3) in the second part of July. Lakes Jieznas and Gineitiškės showed lower diversity index (H=0.60 and H=1.10, respectively), although exhibited higher maximum biomass of the cyanobacterial community, reaching up to 41.77 mg L^{-1} in early June and 14.67 mg L^{-1} in early August, respectively (Tabs. 2 and 3). In June, Lake Jieznas was dominated by native *Limnothrix redekeii,* reaching a maximum biomass of 40.50 mg L^{-1}. In July, its domination was replaced by native *Planktolyngbya limnetica* (maximum biomass of 21.62 mg L^{-1}), followed by the presence of three alien cyanobacteria, *Chrysosporum bergii, Cuspidothrix issatschenkoi* and *Rapidiopsis raciborskii*, with maximum biomasses of 0.07, 0.02 and 0.07 mg L^{-1}, respectively (Fig. 1) (Tab. 3). In Lake Gineitiškės, a cyanobacterial bloom occurred in the latter part of summer, with a considerable amount of native *Planktolyngbya limnetica* (maximum biomass of 11.39 mg L^{-1}), which co-dominated with native *Aphanizomenon gracile* (maximum biomass of 2.69 mg L^{-1}). The bloom was followed by the presence of low amount of alien cyanobacteria, with maximum biomasses of 0.13, 0.22 and 0.08 mg L^{-1}, for *Chrysosporum bergii, Cuspidothrix issatschenkoi* and *Raphidiopsis raciborskii,* respectively. The peak values of chl-*a* were 62.56 µg L^{-1} for Lake Simnas, 41.18 µg L^{-1} for Lake Jieznas, and 91.89 µg L^{-1} for Lake Gineitiškės, indicating the highest biomass of phytoplankton in Lake Gineitiškės, although the highest biomass of cyanobacterial community was identified in Lake Jieznas (Tab. 3).

Tab. 3. Maximum values chlorophyll-*a* (chl-*a*) concentrations, biomass of total cyanobacteria and alien species, and their contribution to total cyanobacterial biomass (%) from the surface and column water samples in the studied lakes during the study period.

	Simnas		Jieznas		Gineitiškės	
	Surface	Column	Surface	Column	Surface	Column
Chl-*a*, µg L^{-1}	62.56	62.56	41.18	39.65	91.89	89.07
Total cyanobacteria, mg L^{-1}	4.18	4.63	41.77	38.82	14.67	12.43
C. bergii, mg L^{-1} (%)	—	—	0.07 (<1%)	0.05 (<1%)	0.03 (<1%)	0.12 (<1%)
C. issatschenkoi, mg L^{-1} (%)	1.26 (38%)	1.40 (30%)	0.02 (<1%)	0.02 (<1%)	0.22 (<1.5%)	0.06 (<1%)
R. raciborskii, mg L^{-1} (%)	—	—	0.07 (<1%)	0.04 (<1%)	0.08 (<1%)	0.02 (<1%)
S. apahanizomenoides, mg L^{-1} (%)	0.44 (17%)	0.70 (27%)	—	—	—	—

Tab. 4. Mann-Whitney U test results for biomass of dominant and alien cyanobacteria in water surface and column samples in the studied lakes. Text in bold represents alien species.

Species	Z	p-value
Aphanizomenon gracile	-0.01	0.990
Aphanocapsa spp.	0.21	0.831
Cyanodictyon spp.	-0.67	0.505
Chrysosporum bergii	0.77	0.443
Cuspidothrix issatschenkoi	-0.16	0.872
Dolichospermum flos-aquae	-0.02	0.982
Limnothrix planktonica	-0.35	0.724
Limnothrix redekei	0.19	0.848
Microcystis aeruginosa	0.94	0.346
Microcystis wesenbergii	0.14	0.890
Pseudoanabena limnetica	-0.89	0.372
Planktolyngbya limnetica	0.14	0.890
Raphidiopsis raciborskii	0.68	0.494
Snowella lacustris	0.79	0.428
Sphaerospermopsis aphanizomenoides	0.16	0.873
Total cyanobacteria	0.14	0.890

Cyanotoxins and potential toxin producers

ELISA analysis revealed the presence of MCs, STXs and ATX-a in the water surface samples of Lakes Simnas, Jieznas and Gineitiškės during the study period (Tab. 5). The highest average concentration of MCs was found in Lakes Simnas at 0.352 μg L^{-1}, with a peak value of 0.941 μg L^{-1} at the end of July. Lakes Jieznas and Gineitiškės had lower mean concentrations of MCs at 0.187 and 0.258 μg L^{-1}, respectively, although Lake Gineitškės showed significant variation in MCs, ranging from 0.045 to 0.484 μg L^{-1} (with the maximum value in September). ATX-a concentrations were relatively consistent between Lakes Simnas and Jieznas, with average values around 0.013 μg L^{-1}, while Lake Gineitiškės had a notably higher average concentration at 0.044 μg L^{-1}, reaching peak at 0.183 μg L^{-1} in September. STXs concentrations were similar in Lakes Simnas and Jieznas with average values of 0.020 and 0.021 μg L^{-1}, respectively (Tab. 5). Lake Gineitiškės showed a higher average STXs concentration of 0.058 μg L^{-1}, with the highest level recorded at 0.441 μg L^{-1} in August. Statistical analysis revealed that none of total concentrations of cyanotoxins significantly differed among the lakes (Tab. 5). Spearman's rank correlation analysis indicated relationships between cyanotoxins and the presence of both potentially toxic, alien and native cyanobacterial species (Tab. 6). MCs correlated positively with native *Microcystis aeruginosa* and ATX-a with alien *Cuspidothrix issatschenkoi* and native *Pseudanabaena limnetica.* STXs showed correlations with native *Aphanizomenon gracile* and *Dolichospermum circinalis*, as well as with alien *Raphidiopsis raciborskii*. ELISA results confirmed the presence of ATX-a in the strains of *Cuspidothrix issatschenkoi* strains (NRC/JIE/2021/D5 and NRC/JIE/2021/F5) isolated from Lake Jieznas.

Relationship between environmental and biotic variables

Spearman's rank correlation analysis was used to investigate the relationship between cyanobacterial biomass, chl-*a* and environmental variables (Tab. 7). The analysis revealed positive

Tab. 5. Total concentration of cyanotoxins (mean±SD, μg L^{-1}) during the study period and results of Kruskal-Wallis (χ^2).

	Simnas	Jieznas	Gineitiškės	χ^2	p-value
MCs	0.352±0.272	0.187±0.042	0.258±0.148	3.28	0.194
ATX-a	0.013±0.008	0.013±0.024	0.044±0.077	2.86	0.239
STXs	0.020±0.024	0.021±0.009	0.113±0.155	2.76	0.252

MCs, microcystins; ATX-a, anatoxin-a; STXs, saxitoxins.

Tab. 6. Results of Spearman's rank correlation between and potentially toxic cyanobacteria. Bold values indicate statistical significance at the p<0.05 level.

	MCs	ATX-a	Stxs
Cuspidothrix issatschenkoi	—	**0.453**	—
Raphidiopsis raciborskii	—	—	**0.574**
Sphaerospermopsis aphanizomenoides	0.211	—	—
Aphanizomenon gracile	—	—	**0.732**
Dolichospermum circinale	0.114	0.014	**0.631**
Dolichospermum flos-aquae	0.277	0.172	—
Microcystis aeruginosa	**0.483**	0.171	—
Microcystis flos-aquae	0.329	—	—
Planktothrix agardhii	-0.056	-0.508	—
Pseudanabaena limnetica	—	**0.353**	—

MCs, microcystins; ATX-a, anatoxin-a; STXs, saxitoxins.

Tab. 7. Spearman's rank correlation showing relationship between cyanobacterial biomass, chlorophyll-*a* (chl-*a*), and environmental variables. Bold values indicate statistical significance at the p<0.05 level.

	TN	TP	T	Conductivity
Chrysosporum bergii	0.41	-0.06	0.03	-0.06
Cuspidothrix issatschenkoi	0.07	0.24	0.05	-0.04
Raphidiopsis raciborskii	**0.64**	0.03	0.16	0.05
Sphaerospermopsis aphanizomenoides	-0.04	0.03	0.24	0.23
Total cyanobacterial biomass	0.25	-0.12	0.14	0.28
Chl-*a*	0.14	**0.61**	-0.19	**-0.68**

TN, total nitrogen; TP, total phosphorus; T, temperature.

correlations between the presence of alien *Raphidiopsis raciborskii* and TN, as well as a positive correlation between chl-*a* and TP. Furthermore, chl-*a* demonstrated a negative correlation with conductivity.

DISCUSSION

In this study we presented the occurence four alien nostocalean species - *Chrysosporum bergii, Cuspidothrix isstaschenkoi, Sphaerospermopsis aphanizomenoides,* and *Raphidiopsis raciborskii* - in northern regions of their distribution in Europe. These alien cyanobacteria were identified in shallow, human-affected lakes: urbanized, recreationally used, and surrounded by agricultural fields, thereby resulting in a hypereutrophic condition (Tab. 2). Eutrophication of inland waters is an example of anthropogenically altered ecosystems, which are common habitats for invasion of planktonic algae (Wilk-Woźniak and Najberek, 2013).

Historical records indicate that *Chrysosporum bergii, Cuspidothrix issatschenkoi*, and *Sphaerospermopsis aphanizomenoides* were first recorded in Lithuania less than 20 years ago. The first report of *C. bergii* in Lithuania was in Lake Gineitiškės in 2008 (Koreivienė and Kasperovičienė, 2011) and later was found in Lake Rėkyva in 2015 (Savadova *et al.,* 2018). The earliest occurrences of *C. issatschenkoi* and *S. aphanizomenoides* in Lithuania were documented in Lakes Gauštvinis, Širvys, and Jieznas in 2014 and 2015 (Karosienė *et al.,* 2020). However, *R. raciborskii* in Lake Jieznas was already reported 45 years ago (Kavaliauskienė, 1996) and have not been found in other localities in the country (Kokociński *et al.,* 2017). In this study we report the presence of *C. bergii* in Lake Jieznas, *C. issatschenkoi* in Lakes Simnas and Gineitiškės, and *R. raciborskii* in Lake Gineitiškės for the first time. These new findings indicate a recent expansion of their distribution range in Lithuania. Interestingly, while we did not observe the occurrence of *S. aphanizomenoides* in Lake Jieznas as previously reported (Karosienė *et al.,* 2020), we showed its presence in Lake Simnas for the first time. None of the studies recorded the blooms formation by any of alien cyanobacterial species in Lithuania.

In the three lakes investigated in this study, the biomass of the four alien cyanobacterial species was low, with values not exceeding 1.5 mg L^{-1} (Tab. 3). This finding aligns with other comparable studies in temperate Europe around the 54°N latitude and above. For instance, *Chrysosporum bergii* has been reported in 20 water bodies across Germany and Poland, yet it has never become a dominant species (Stüken *et al.,* 2006; Budzyńska *et al.,* 2019). Likewise, *Sphaerospermopsis aphanizomenoides* has been documented in at least 16 water bodies in Poland (Stefaniak and Kokocinski, 2005; Kokociński and Soininen, 2012; Budzyńska *et al.,* 2019) and 10 water bodies in Germany (Stüken *et al.,* 2006). However, it exceeded 10% of total phytoplankton biomass in only three lakes in Poland, reaching a maximum biomass of 22.3 mg L^{-1} (Budzyńska and Gołdyn, 2017; Budzyńska *et al.,* 2019; Napiórkowska-Krzebietke *et al.,* 2023). *Cuspidothrix issatschenkoi* has been reported in at least one water body in Germany (Ballot *et al.,* 2010), two in Poland (Napiórkowska-Krzebietke *et al.,* 2023) and one in Finland (Rajaniemi *et al.,* 2005). While this species has expanded its distribution beyond Lithuania, its presence in northern regions remains infrequent, and it has not been reported to form blooms.

Contrary to the other species, there has been an increasing number of reported blooms of *Raphidiopsis raciborskii* in around the 54°N latitude, including Germany (Nixdorf *et al.,* 2003) and Poland (Kokociński *et al.,* 2017; Kokociński and Soininen, 2012; Budzyńska and Gołdyn, 2017), showing its potential for proliferation in north-east distribution zone. This species has expanded its range further north than Lithuania, with its northernmost location in the European part of Russia, where it has also formed blooms (Babanazarova *et al.,* 2015). Similar to our study, some reports describe the low abundance of *R. raciborskii* in temperate lakes (Stefaniak and Kokocinski, 2005; Kokociński and Soininen, 2012).

To better understand what restricts the establishment and proliferation of non-native cyanobacterial species, a number of limiting factors have to be considered. Successful invasion is influenced by the biological traits of the invader. Elevated nutrient levels have been linked to increased growth rates in species such as *Cuspidothrix issatschenkoi* (Gagnon and Pick, 2012), *Raphidiopsis raciborskii* (Kokociński and Soininen, 2012), and *Sphaerospermopsis aphanizomenoides* (Budzyńska *et al.,* 2019; Savadova-Ratkus *et al.,* 2021). In contrast, studies suggest that the growth rates of *Chrysosporum bergii* do not necessarily correlate with nutrient levels (Savadova-Ratkus *et al.,* 2021), although it is frequently found in meso- or eutrophic shallow lakes (Stüken *et al.,* 2006; Kaštovský *et al.*, 2010; Koreivienė and Kasperovičienė, 2011). In our study, none of the analysed parameters significantly affected the biomass of alien cyanobacteria, except for a positive relationship found between *R. raciborskii* and TN (Tab. 7). Despite high nutrient concentrations in the investigated lakes, none of these invaders produced significant biomass (Fig. 1) (Tab. 3).

It is important to emphasize that not only the separate effects of TN and TP influence proliferation of cyanobacteria, but also the ratio of these nutrients. *Sphaerospermopsis aphanizomenoides* is considered as highly P-limited species (Budzyńska *et al.,* 2019), which may explain its limited presence in N-rich Lake Simnas, with a mean N:P atomic ratio of 91.84 (Tab. 2).

There is a common belief that temperature positively affects the proliferation of alien cyanobacteria (Mehnert *et al.,* 2010; Sukenik *et al.,* 2012; Savadova *et al.,* 2018). However, our study did not identify temperature as a significant factor influencing the growth of alien species (Tab. 7). The mean water temperatures recorded in the studied lakes ranged from around 20 to 23 °C, which, according to laboratory studies, is below the range typically associated with high growth rates for *Chrysosporum bergii*, *Raphidiopsis raciborskii* and *Sphaerospermopsis aphanizomenoides* (Mehnert *et al.,* 2010; Savadova *et al.,* 2018). This may explain the low biomass of the alien species found in the three lakes, as these species are known to be more competitive at higher temperatures compared to native species. Notably, previous studies that recorded the bloom formation of *Sphaerospermopsis aphanizomenoides* near the border of its current temperate geographical range have demonstrated a strong correlation between its proliferation and water temperatures, reaching up to 30°C (Budzyńska and Gołdyn, 2017), therefore low temperature (average 21.64°C in Lake Simnas) might be among the factors limiting the proliferation of *S. aphanizomenoides* in our study (Tab. 2). In accordance with our study, it occurred in low amounts under water temperatures between 20-25°C in the northern areas of its distribution (Budzyńska *et al.,* 2019).

Moreover, the biomass of *Raphidiopsis raciborskii* is often positively related to the temperature (Stüken *et al.,* 2006). In addition, in its northernmost recorded location in Russia, *R. Raciborskii* formed blooms during an unusually warm summer (average temperature 27.2°C) (Babanazarova *et al.,* 2015). However, some studies have found no direct relationship between the blooms of this species and temperature in temperate lakes (Kokociński and Soininen, 2012; Budzyńska and Gołdyn, 2017). Furthermore, *R. raciborskii* has been reported to form blooms at temperatures as low as 17°C in southern Poland (Budzyńska and Gołdyn, 2017), contradicting our findings where *R. raciborskii* was present in low amounts at temperatures around 20-23°C (Tab. 2). That suggest that strains of *R. raciborskii* found in this study may represent distinct ecotypes with preferences for warmer environments.

Cyanotoxins, including MCs, ATX-a, and STXs were quantified in the studied lakes, however the quantities did not exceed the alert levels set by the World Health Organization for recreational water (Tab. 5) (World Health Organization, 2021). Potentially toxic native cyanobacteria identified in this study include species from the genera *Microcystis, Dolichospermum, Planktothrix, Pseudanabaena* and *Aphanizomenon* (Bernard *et al.,* 2017 and references therein). Statistical analysis results were consistent with the literature, identifying *Microcystis aeruginosa* as a producer of MCs (Vezie *et al.,* 1998), *Pseudanabaena limnetica* as a producer of ATX-a (Osswald *et al.,* 2009), and *Aphanizomenon gracile* and *Dolichospermum circinale* as a producer of STXs (Pomati *et al.,* 2006; Ledreux *et al.,* 2010) (Tab. 6).

Statistical analysis revealed a positive relationship between alien *Raphidiopsis raciborskii* and concentrations of STXs (Tab. 6). This species is known to produce STXs in South America (Vico *et al.,* 2020). However, since its production of STXs has never been reported in Europe, the likelihood of its production in Lakes Jieznas, and Gineitiškės is considered low. *Chrysosporum bergii* and *Sphaerospermopsis aphanizomenoides* have never been confirmed as producers of any cyanotoxins worldwide, which was cosistent with our observations (Tab. 6). The absence of cyanotoxins production may contribute to the low competitive advantage of these alien species, resulting in their low abundance.

On the other hand, the alien *Cuspidothrix issatschenkoi* is capable of producing ATX-a in Europe (Stoyneva-Gärtner *et al.,* 2022). Our study confirmed the presence of ATX-a of two isolated strains of this species, suggesting its contribution to the presence of ATX-a in Lakes Simnas, Jieznas and Gineitiškės. Moreover, only one study has ever confirmed the presence of ATX-a in Lithuania (Lakes Širvys and Jieznas) (Karosienė *et al.,* 2020). However, none of the isolated *C. issatschenkoi* strains tested were shown to produce anatoxin-a (Karosienė *et al.,* 2020). Therefore, this is the first report confirming ATX-a production by the strains of *C. issatschenoi* in Lithuania.

In this study, we focused solely on cyanobacteria and did not analyse the entire phytoplankton community. The peaks values of chl-*a* did not coincide with peaks values of cyanobacterial biomass, thus, indicating community dynamics of other phytoplankton members unanalysed in this study (Fig. 1). Therefore, we lack information on the diversity of other phytoplankton species and the other trophic levels, such as zooplankton diversity, which could influence the successful establishment of newly arrived cyanobacteria through competition and grazing interactions. Future investigations, considering the full diversity of phytoplankton and zooplankton structure, will contribute to a more comprehensive understanding of the biotic factors that contribute to ecosystem resilience to newly arrived species.

CONCLUSIONS

This study reported the presence of four alien cyanobacterial species - *Chrysosporum bergii, Cuspidothrix issatschenkoi, Raphidiopsis raciborskii* and *Sphaerospermopsis aphanizomenoides* - in the northern locations of their distribution. These species were found in human-affected shallow hyper-eutrophic lakes in temperate region during warm period of the year. However, it is likely that present temperatures do not limit the occurrence of these alien species but are insufficient for their successful proliferation. The absence of cyanotoxin production by *C. bergii, R. raciborskii* and *S. aphanizomenoides* might also limit their competitive abilities. Importantly, the present work provides the first evidence of ATX-a production by isolated strains of *C. issatschenkoi* in Lithuania, although the toxin production did not coincide with the dominance of this species. In general, certain alien cyanobacteria showed a tendency to spread into northern habitats but fail to reach high abundances. A rise in global temperatures and the spread of strains with toxigenic potential could lead to increased proliferation and further northward expansion of these alien species or their ecotypes. It is, therefore, important to detect these species at an early stage of their development in order to monitor their distribution and preserve from the ecological and health risks they pose.

ACKNOWLEDGMENTS

The authors would like to thank Jūratė Kasperovičienė for her help in identifying cyanobacterial species and to Elżbieta Wilk-Woźniak for her contributions and expert advice that enhanced the quality of this manuscript.

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