Cyanotoxins associated with macrophytes in Berlin (Germany) þÿwater bodies Occurrence and risk assessment

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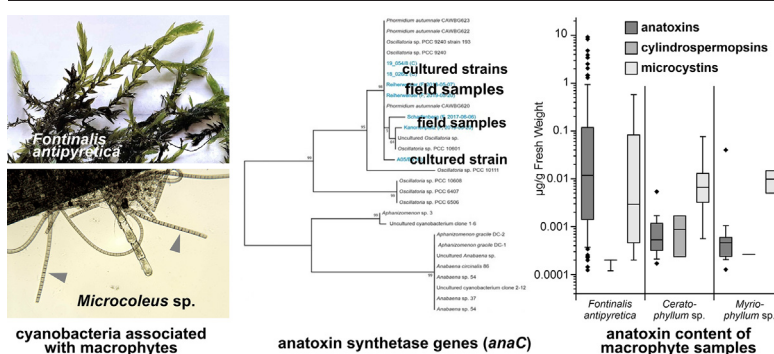[f] Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Str. 24/25, 14476 Potsdam-Golm, Germany

HIGHLIGHTS

- From 2017 to 2021, a total of 398 macrophyte samples were analysed for associated cyanobacteria and cyanobacterial toxins.
- Anatoxin-producing *Microcoleus* sp. (cyanobacteria) is often associated with the water moss *Fontinalis antipyretica*.
- Anatoxin contents of macrophyte samples are highly variable in space and time.
- Hazard assessment suggests that toxigenic cyanobacteria/macrophyte assemblages are a relatively minor risks for humans.

GRAPHICAL ABSTRACT

ARTICLE INFO

ABSTRACT

Fatal dog poisoning after uptake of neurotoxic cyanobacteria associated with aquatic macrophytes in Tegeler See (Berlin, Germany) raised concerns about critical exposure of humans, especially children, to cyanotoxins produced by macrophyte associated cyanobacteria during recreational activity. From 2017 to 2021 a total of 398 samples of macrophytes washed ashore at bathing sites located at 19 Berlin lakes were analysed for anatoxins, microcystins, and cylindrospermopsins, as were 463 water samples taken in direct proximity to macrophyte accumulations. Cyanotoxins were detected in 66 % of macrophyte samples and 50 % of water samples, with anatoxins being the most frequently detected toxin group in macrophyte samples (58 %) and cylindrospermopsins in water samples (41 %). *Microcoleus* sp. associated with the water moss *Fontinalis antipyretica* was identified as anatoxin producing cyanobacterium in isolated strains as well as in field samples from Tegeler See. Anatoxin contents in macrophyte samples rarely exceeded 1 μg/g macrophyte fresh weight and peaked at 9. 2 μg/g f.w. Based on established toxicological points of departure, a critical anatoxin content of macrophyte samples of 3 μg/g f.w. is proposed. Five samples, all taken in Tegeler See and all associated with the water moss *Fontinalis antipyretica*, exceeded this value. Contents and concentrations of microcystins and cylindrospermopsins did not reach critical levels. The potential exposure risks to anatoxins for children and dogs are assessed and recommendations are given.

1. Introduction

Planktonic cyanobacteria often dominate the phytoplankton of eutrophic water bodies. For decades, the causes and effects of their occurrence have been intensively studied, one motivation being the ability of some cyanobacterial taxa to produce potent hepato- and neurotoxins (Buratti et al., 2017). Intoxications of domestic and wild animals have been documented worldwide, and in many countries, legislation exists to prevent harm from toxic planktonic cyanobacteria through drinking water or recreational use (Chorus and Welker, 2021b).

In recent years, a number of publications have pointed to an increasing frequency and intensity of cyanobacterial blooms in inland waters attributed to climate change and eutrophication. However, this is only a crude assessment and, while the situation is deteriorating in many parts of the world, water quality has been improved in the last few decades in others (Chorus et al., 2021; Globevnik et al., 2020). One key factor for improvement is the reduction of nutrient loads to waterbodies, in particular phosphorus (Fastner et al., 2016). In most lakes, however, a delay of 10–15 years between load reduction and a stable phytoplankton biomass equilibrium is observed (Jeppesen et al., 2005). In Berlin, a more or less

continuous improvement of water quality has been observed in many lakes and rivers since the 1990's resulting in, for example, in the increase of water transparency and permanent recurrence of macrophytes.

In particular, the water quality in Tegeler See (Fig. 1) has improved significantly following the installation of a phosphorus removal facility in the 1980's to reduce the P-load from treated sewage that constitutes a main water inflow to the lake (Schauser and Chorus, 2007). In due course, the intensity of cyanobacterial blooms gradually declined, water transparency increased (from summer averages of <1 m to >2.5 m), and macrophytes reestablished down to a depth of 5 m and more (Chorus et al., 2020; Hilt et al., 2010). Overall, restoration of Tegeler See was considered a success and was highly appreciated by the public enjoying recreational activity at five bathing sites under surveillance according to the EU Bathing Water Directive (EU, 2006).

In late spring 2017 several dogs died after a walk along the shores of Tegeler See. At first, it was suspected that the dogs were poisoned intentionally as this had happened a few years ago. Upon forensic veterinary examination, microscopic examination of water and stomach content samples, as well as chemical analysis of various samples, evidence demonstrated that the dogs suffered from acute intoxication with anatoxin-a (Fastner et al.,
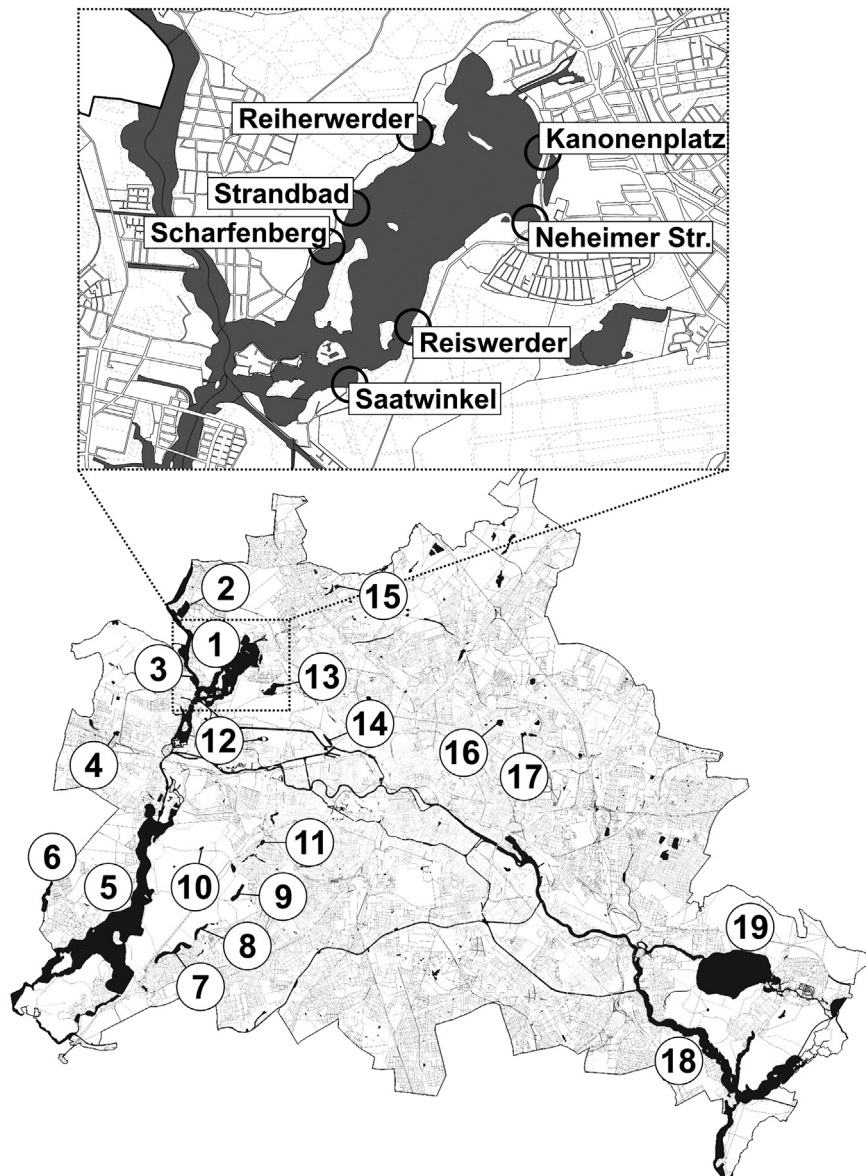

Fig. 1. Location of sampled lakes in Berlin, Germany. The numbers refer to the lakes listed in Table 1. The enlarged section shows the individual sampling sites in Tegeler See.

2018), a cyanobacterial toxin that had not been detected in substantial concentrations (>1 μg/l) in water samples from Berlin lakes previously.

Dog poisonings by anatoxins have been reported repeatedly since the 1990's (Edwards et al., 1992; Faassen et al., 2012; Gugger et al., 2005; Wood et al., 2007). In most of these cases, dogs have ingested the toxigenic cyanobacteria from streams and rivers, where cyanobacteria can build benthic mats on rocky or pebbly river beds (Bouma-Gregson et al., 2019; McAllister et al., 2016). In the sandy sediments of North-Eastern German lakes hard substrates are generally lacking. This may be the reason why benthic cyanobacteria have rarely been reported. Another substrate for benthic cyanobacteria can be macrophytes. This is the case in Tegeler See, where microscopic analyses revealed filamentous cyanobacteria being associated with water moss (*Fontinalis antipyretica*; Fastner et al., 2018). Though cyanobacteria are often present in epiphytic biofilms (Wijewardene et al., 2022), their dominance in macrophytes' biofilms, however, is only rarely reported (Ariosa et al., 2004; Hudon et al., 2009). Beside macrophytes algae can function as substrate for cyanobacteria, e.g., mats of *Cylindrospermum* sp. on *Cladophora* sp. in Californian rivers (Kelly et al., 2019; Power et al., 2015). In Tegeler See, the cyanobacteria appeared rather as single filaments on *Fontinalis* tufts and were tentatively identified as tychoplanktonic *Tychonema* sp. in 2017 (Fastner et al., 2018).

The death of the dogs made it to the headlines of the local press and put high pressure on the authorities to answer the well-justified question whether bathing in Tegeler See is safe for humans. There was a particular interest in the risk to small children, when dogs – with a similar body weight – died shortly after rollicking in the shallow water.

The World Health Organisation has proposed guideline values for maximally tolerable cyanotoxin concentrations and so-called Alert-Level-Frameworks for an efficient mitigation of the cyanotoxin risk primarily from planktonic cyanobacteria (Chorus and Welker, 2021b). While the number of verified cases of human intoxications or epidemiological studies indicating adverse health effects upon chronic exposure is limited (Svirčev et al., 2019; Wood, 2016) the rationale behind the derivation of guideline values is the potent toxicity of these compounds, assessed in toxicological studies with model animals, and their global occurrence in a large number of water bodies (Chorus and Welker, 2021a). Intoxications of wild and domestic animals are considered as sentinel events for potential human intoxications (Hilborn and Beasley, 2015; Svirčev et al., 2019).

The present study was conducted to answer the following questions:

- When and where do toxic cyanobacteria occur associated with (detached) macrophytes, in particular, in close vicinity to frequented bathing sites?
- Is there an association of particular taxa of macrophytes and particular potentially toxigenic cyanobacterial taxa?
- Which cyanotoxins were present and at which contents in samples of macrophytes?
- Do the cyanotoxins present in macrophyte samples pose a health risk for humans, in particular, for young children?
- What recommendations can be derived for authorities and the general public?

From 2017 to 2021 a sampling program was set up with a focus on near-shore macrophyte accumulations at or close to bathing sites in Berlin. On the basis of the results a tentative risk assessment is made and recommendations for comparable conditions are given.

2. Materials and methods

2.1. Study sites

The sampled lakes are located within the city limits of Berlin, Germany and are used for recreational purposes by the public, mainly for bathing and boating. The majority of bathing sites have a shallow morphology, which makes them especially appealing to families with small children. All lakes are under regular surveillance by the Berlin Office for Health and Social

Affairs (Landesamt für Gesundheit und Soziales). Samples are generally taken fortnightly and analysed for faecal indicator organisms (*E. coli*, intestinal enterococci, and coliform bacteria) by standard methods according Berlin state regulations, implementing the EU bathing water directive. Further, water quality is routinely monitored by analysis of chlorophyll *a* concentration, microscopical assessment of phytoplankton composition, determination of biovolumes of cyanobacterial taxa, and cyanotoxin analysis at selected sites (microcystins and cylindrospermopsins, when indicated).

Macrophyte samples and associated water samples were taken primarily from bathing sites that are under surveillance (Fig. 1). Sampling was focused on Tegeler See (see insert Fig. 1), that is represented by five official bathing sites and two further sites where intoxications of dogs presumably have occurred (Kanonenplatz, Neheimer Str.). Further lakes or sites were selected, respectively, where detached macrophytes have been observed during routine surveillance in preceding years, hence the uneven distribution of samples among individual lakes (Table 1).

2.2. Sampling and processing

The lakes listed in Table 1 are routinely monitored according to the EU bathing water directive (EU, 2006) in biweekly to 4-weekly intervals during the bathing season from April to September. When macrophytes were encountered during the routine sampling of recreational sites, samples were taken from May to September in 2017 and 2018, and from April to July (single samples were taken until September) in 2019 to 2021. The largest share of samples was taken in Tegeler See because only there dog intoxications occurred and the lake has the most extensive macrophyte coverage of all Berlin lakes. In other lakes with only few macrophytes, the number of respective samples is accordingly lower. Macrophyte samples of some 50 g were picked manually by random selection from the drift line on lake shores, transferred to wide-mouth polyethylene containers, and stored cool until further processed in the laboratory. Whenever possible under field conditions, the sampled macrophyte taxa were noted. In parallel, water samples were taken in close proximity to macrophyte assemblages (10–30 cm below surface) with wide-mouth polyethylene containers, cooled and transported to the laboratory.

Fresh macrophyte samples were weighted to the nearest 0.01 g remaining in the polyethylene containers after surplus water was decanted into a

Table 1
Sampled lakes and hydrologic characteristics. The number in the first column refers to the numbering in Fig. 1. Trophic state is given for 2021; classification according to guidelines given in LAWA (2014): m: mesotrophic; e: eutrophic; e/p: eutrophic/polytrophic.

Nr.	Water body	Trophic state	Area [ha]	Mean z [m]	Max z [m]	Macrophyte samples	Water samples
1	Tegeler See	m	396	6.6	15.9	190	167
2	Heiligensee	m	32	6.0	9.5	17	22
3	Oberhavel	e	401	3.1	6.5	23	47
4	Spektesee	e	7.1	5.3	11.9	1	1
5	Unterhavel	e/p	1585	4.9	11.1	24	37
6	Groß Glienicker See	m	67	6.8	11.2	52	51
7	Schlachtensee	m	41.6	4.7	8.9	2	5
8	Krumme Lanke	m	14.0	3.9	6.8	7	6
9	Grunewaldsee		17.5			1	1
10	Teufelssee	m	2.3	3.2	5.9	2	4
11	Halensee	e	5.6	3.2	7.8	19	20
12	Jungfernheideteich	m	6.5	1.8	4.0	2	3
13	Flughafensee		30.6	11.8	34.3	5	5
14	Plötzensee	e	7.6	3.4	7.5	18	19
15	Ziegeleisee	e	4.7	6.3	14.1	6	14
16	Weisser See	e	8.3	4.3	10.6	4	4
17	Orankesee		4.1	2.6	6.7	5	5
18	Dahme	e/p	959	3.5	8.5	0	7
19	Müggelsee	e	743	4.9	7.7	20	45
						398	463

cylindrical flask. For the 45 samples from 2017 the exact fresh weights were not been noted, thus 50 g fresh weight has been used as default for these samples (average mass of macrophyte samples: 52.8 g). The water in the flask was filled with lake water to a defined volume of 200 ml and returned to the macrophyte material for resuspension. To release attached epiphytic algae and cyanobacteria the mixture was gently shaken for 20 min and decanted immediately afterwards. Subsamples from these suspensions were preserved with Lugol's solution, subjected to direct microscopic analysis, processed for toxin analysis, and filtered for molecular analyses (see below).

For toxin analysis of single filaments, filaments were isolated from water samples or from strain cultures, respectively, with a pointed Pasteur pipette using an inverted microscope at a magnification of $100\times$ (Axio Observer D1, Zeiss, Germany) and washed in droplets of deionized, sterile water until visually pure. After measurement, filaments were transferred with water into reaction tubes and dried by vacuum centrifugation.

2.3. Microscopic analysis

Phytoplankton analysis was carried out during routine surveillance at selected sites on a fortnightly schedule. Cell densities and biovolumes were estimated according to DIN EN 15204 using an inverted microscope (Olympus IX71).

In suspensions derived from macrophytes cyanobacterial cells of Microcoleaceae were quantified by counting of at least two transects under $400\times$ magnification up to the whole chamber. Cell density of Microcoleaceae was transformed to biovolume by applying an average cell volume of 220,9 μm^3. All cell counts and biovolumes were standardized as per gram macrophyte fresh weight.

2.4. Toxin analysis

For extraction of toxins, aliquots of lake water or macrophyte derived suspension samples (1 ml) were acidified with formic acid to a final concentration of 0.1 % formic acid and subjected to two freeze/thaw cycles. Subsequently, the samples were ultrasonicated for 10 min, shaken for 1 h, centrifuged and the supernatant filtered (0.2 μm, PVDF, Whatman, Maidstone, UK). Extracts were kept frozen ($-20\,°C$) until analysis by liquid chromatography– tandem mass spectrometry (LC-MS/MS).

Toxins were extracted from dried single filaments by adding 200 μl 0.1 % acetic acid, two freeze/thaw cycles and ultrasonication for 10 min. Finally, extracts were filtered using centrifugal filter units (0.45 μm, modified nylon, VWR, Germany).

Anatoxins, cylindrospermopsins and microcystins were analysed by LC–MS/MS as detailed previously (Bauer et al., 2020). Analyses were carried out on an Agilent 2900 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an API 5500 QTrap mass spectrometer (AB Sciex, Framingham, MA, USA) equipped with a turbo-ion spray interface.

For analysis of anatoxins and cylindrospermopsins 10 μl of crude extracts were separated on an Atlantis C18 column (2.1 mm, 150 mm, Waters, Eschborn, Germany) at 30 °C. Compounds were eluted at a flow rate of 0.25 ml/min using a linear gradient of 0.1 % formic acid in water (A) and 0.1 % formic acid in methanol (B) from 1 % to 25 % B within 5 min. Aliquots of 10 μl or 75 μl were injected from the water samples or the filament extracts, respectively. Identification and quantification of anatoxins (anatoxin-a, ATX; dihydro-anatoxin-a, dhATX; homoanatoxin-a, HTX) and cylindrospermopsins (cylindrospermopsin, CYN; deoxy-cylindrospermopsin, dCYN) were performed with the MRM mode using the transitions given in Table S1 (Supplementary information). Calibration standards of CYN and ATX were from the National Research Council (Canada) and dhATX, dCYN and HTX from Novakits (Nantes, France). The limits of detection (LOD) and quantifications (LOQ) for anatoxins and cylindrospermopsins were 0.03 μg/l and 0.1 μg/l, respectively. Values below the LOQ were substituted by LOQ × 0.5. For dihydro-homoanatoxin (dhHTX) no reference material was available for an unequivocal identification and quantification, therefore this structural variant has not been analysed.

Microcystins were separated using a Purospher STAR RP-18 endcapped column (30 mm × 4 mm, 3 mm particle size, Merck, Germany) at 30 °C. The mobile phase consisted of 0.5 % formic acid (A) and acetonitrile with 0.5 % formic acid (B) at a flow rate of 0.5 ml/min with the following gradient program: 0 min 25 % B, 10 min 70 % B, 11 min 70 % B. The injection volume was 10 μl. Identification and quantification of microcystins ([Asp³]-MC-RR, MC-RR, MC-YR, [Asp³]-MC-LR, MC-LR, MC-LW, MC-LF, MC-LA) was performed with the MRM mode using the transitions given in Supplementary information (SI Table 1). Standards were purchased from National Research Council (Canada) and Novakits (Nantes, France). The LOD and LOQ for microcystins differ between congeners ranging from 0.06 to 0.4 μg/l and 0.2 to 1.2 μg/l, respectively. Values below the LOQ were substituted by LOQ × 0.5.

A small number of samples from 2017 was also analysed for saxitoxins but with negative results. Therefore, the analysis of this toxin group was not followed further.

The toxin contents in macrophyte samples were corrected for the background toxin concentrations in corresponding lake water samples.

2.5. Isolation and cultivation of strains

Single filaments were isolated from water samples in 2018 and 2019 with a pointed Pasteur pipette using an inverted microscope at a magnification of $200\times$ (Axio Observer D1, Zeiss, Germany). Individual filaments were serially diluted in droplets of deionized, sterile water until visually pure and transferred into 5 ml polycarbonate tubes containing 1 ml slightly modified Z8 medium (Staub, 1961). Isolates were incubated at 15 °C, 30 μE m^{-2} s^{-1} in an 12 h/12 h light–dark-cycle. Once growth was visible in cultivation tubes, generally after five to nine months, individual filaments were re-isolated to assure monoclonal cultures. Established cultured strains are maintained in Erlenmeyer flasks under the same conditions.

2.6. DNA extraction and amplification

Genomic DNA was extracted from the macrophyte suspension samples filtered through a membrane filter (1.2 μm, mixed cellulose ester, Whatman, Germany). Prior to extraction, filters were cut into small pieces and submerged into appropriate volumes of buffer AL (Qiagen). DNA was extracted by DNeasy Blood and Tissue Kit (Qiagen) and purified with DNA Clean and Concentrator Kit (Zymo Research) according to manufacturers' instructions. DNA from single filaments was released to small amount of MilliQ water with three freeze/thaw cycles followed by ultrasonication (2 × 1 min). Sonicated water/DNA mixture was directly used as a template in PCR reactions.

Detection of anatoxin synthetase (ana) gene cluster was achieved with primers specific for anaC (Rantala-Ylinen et al., 2011). Further, partial regions of 16S rRNA were amplified with cyanobacteria specific primers (Nübel et al., 1997). For both assays Oscillatoria sp. strain PCC 6506 served as positive control (Mejean et al., 2009). PCR mixtures contained 1 μl genomic DNA extract, 5 μl Thermo Scientific™ Maxima™ Hot Start Green 2× PCR Master Mix, 0.5 μM primers in total volume of 10 μl. The PCR program was as following: 98 °C for 6 min; 30 cycles of 98 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min; 72 °C for 5 min. PCR amplification products were qualitatively assessed from 1 % agarose gels stained with ethidium bromide.

2.7. Phylogenetic analysis of anatoxin producers

For sequencing of PCR products, Phusion polymerase with higher accuracy was used. Reactions contained 1 μl of genomic DNA, 5 μl 5 × Phusion Green HF Buffer, 1 μl dNTPs, 0.3 μM of primers, 0.5 μl Phusion polymerase in total volume of 50 μl. The PCR program was as following: 98 °C for 2 min; 35 cycles of 98 °C for 10 s, 58 °C for 20 s, 72 °C for 1 min; 72 °C for 5 min. PCR products were run in 1 % agarose gel with ethidium bromide, were cut from the gel under UV light, and purified using the GeneJET Gel Extraction Kit (Thermo Scientific™) according to manufacturer's instructions. Purified

PCR products were sequenced by ABI 3730 XL analyser at LGC Genomics GmbH, Berlin, Germany using the primer concentration of 5 μM.

Library preparation and sequencing of V3-V4 regions of 16S rRNA genes were conducted at the DNA Sequencing and Genomics laboratory, Institute of Biotechnology, University of Helsinki, Finland. Data analysis was conducted using Mothur v 1.33 software package (Schloss et al., 2009). Low quality reads, reads longer than 471 bases, with ambiguous bases or chimeric structures were removed from the original dataset. Reads were aligned against SILVA reference database release 102 (Pruesse et al., 2007).

Homologous sequences for 16S rRNA and *anaC* genes were acquired from National Center for Biotechnology Information (NCBI) and European Nucleotide Archive. Phylogenetic trees were constructed with MEGA-X (Kumar et al., 2018) after pairwise alignment of sequences using ClustalW (Thompson et al., 2003) with default parameters and manually trimmed.

All sequences were deposited to NCBI under the accession number OP522382-OP522393 (16S rRNA partial) and OP558297-OP558303 (*anaC* partial).

3. Results

3.1. Occurrence and concentrations of toxins

A total of 398 macrophyte and 463 water samples was analysed for cyanobacterial toxins. Cyanobacterial toxins associated to macrophytes could be detected in 265 samples. In the majority of these, anatoxins were detected (230 samples), while cylindrospermopsins and microcystins were less abundant (32 and 69 samples, respectively; Table 2). The toxin content was ≪ 1 μg/g fresh weight of macrophytes (f.w.) in 97.4 % of samples. In 10 out of 398 macrophyte samples the cyanotoxin content exceeded 1 μg/g f.w. (Fig. 2a) and in all cases the toxins were anatoxins.

When comparing samples from Tegeler See to samples from other lakes, the uneven distribution of samples positive for anatoxins is evident (Table 1; Fig. 2a), with higher toxin contents encountered exclusively in macrophyte samples from Tegeler See ($P = 0.002$; two-sided *t*-test). For microcystins, toxin contents were even ($P = 0.36$; two-sided t-test) in Tegeler See compared to other lakes while cylindrospermopsin contents were lower in Tegeler See ($P = 0.005$; two-sided t-test). In summary, toxic cyanobacteria associated with macrophytes, in particular taxa producing anatoxins, were most abundant in Tegeler See. Nevertheless, anatoxins and potential producers from the order Oscillatoriales, were found occasionally in low concentrations in macrophyte samples from other lakes. Toxins presumably originated not in all cases from macrophyte-associated cyanobacteria, but from typically planktonic cyanobacteria like *Aphanizomenon*, *Dolichospermum* or *Microcystis* colonies which have been trapped in the macrophyte assemblages.

An equally heterogeneous distribution was observed when the individual macrophyte taxa were considered. All samples with elevated anatoxin contents were samples of *Fontinalis antipyretica* while samples of other macrophyte taxa consistently had relatively low toxin contents (Fig. 2c). Large

stands of *F. antipyretica* have been reported only from Tegeler See, although this bryophyte is also present in other lakes (Sabine Hilt, IGB Berlin, pers. comm.) but has been rarely found at bathing sites so far.

Total cyanotoxin concentrations in water collected near macrophyte assemblages exceeded 1 μg/l in 46 out of 463 samples. For anatoxins, cylindrospermopsins, and microcystins the numbers were 13, 5, and 26, respectively (Fig. 2b, Table 2). Anatoxin concentration is correlated to the toxin content in corresponding macrophyte samples (i.e., taken the same date at the same site; $P = 0.03$), although this is due to only four water samples with high concentrations of up to 284 μg/l. A high anatoxin content in macrophytes is hence a prerequisite for elevated anatoxin concentrations in water surrounding macrophyte assemblages, but a high anatoxin content of macrophytes not necessarily leads to elevated concentrations in the surrounding water. The highest anatoxin concentration of 1475 μg/l was measured in a sample consisting of pollen (pine, *Pinus sylvestris*) floating on the water surface to which filaments of *Microcoleus* were attached (not included in Fig. 2).

In 2017 and 2018 anatoxins occurred in Tegeler See until late summer with a peak abundance in spring (Fig. 3). Lower contents in summer were not only associated with *Fontinalis*, but also with other macrophytes such as *Myriophyllum* and *Ceratophyllum* (data not shown). Peak abundances occurred every spring when water temperatures were between 15 and 20 °C and around the onset of thermal stratification. Therefore, in the following years sampling was focused on late spring-early summer. Overall, a high variability of anatoxin contents in *Fontinalis* samples between and even within the same sites was observed.

3.2. Species identification and molecular analyses

Cyanobacterial filaments found in macrophyte samples from Tegeler See, were solitary, pale-brownish, around 6–8 μm wide and up to 1.5 mm long. Dense aggregations of granulae at the cell walls gave the trichomes a banded appearance. Three strains isolated from macrophyte samples in 2018 and 2019 show similar dimensions as the field populations (Fig. 4), but are markedly more pigmented (phycoerythrin) and grow as dense mats in culture vessels. Filaments from samples and cultures have thin sheaths and rounded apical cells, occasionally with a calyptra.

Analysis of PCR-amplified partial 16S rRNA sequences resulted in a consistent clustering with sequences characterized as "*Microcoleus*" lineage (Fig. 5a). This cluster constitutes of sequences of strains labelled as *Phormidium*, *Tychonema* or *Microcoleus*, respectively. Strunecký et al. (2013) suggested this cluster to be a monophyletic group and the apparent ambiguity in genus names the result of historic nomenclature that relied primarily on morphological characteristics. Notably, cyanobacterial sequences from Tegeler See share high similarity to sequences of the recently described *Microcoleus anatoxicus*, isolated from Russian River in California (Conklin et al., 2020) and *M.* (*Phormidium*) *autumnalis* isolated from a New Zealand farm pond (Wood et al., 2017; Wood et al., 2012).

The *anaC* gene was detected in all samples and isolated strains in which anatoxins were detected. Sequences of respective amplification products were compared to reference sequences (Fig. 5b). Expectedly, the sequences clustered with *anaC* sequences of Oscillatoriales and were well separated from *anaC* sequences of Nostocales.

We conclude, that the cyanobacteria responsible for the anatoxin production belong to the *Microcoleus* lineage as defined by Strunecký et al. (2013), thereby revising the earlier classification as *Tychonema* sp. (Fastner et al., 2018). A more detailed revision of the taxonomic status of this lineage is beyond the scope of the present study.

3.3. Anatoxin variants and contents in field samples and isolated strains

Of the different anatoxins analysed, ATX and dhATX were predominant in Tegeler See with varying proportions in macrophyte samples. While in 2017 and 2018 the proportion of ATX was 50 % or more in a number of samples, the proportion of dhATX reached nearly 100 % in the majority of samples in 2019. In 2020 and 2021 the proportion of ATX increased
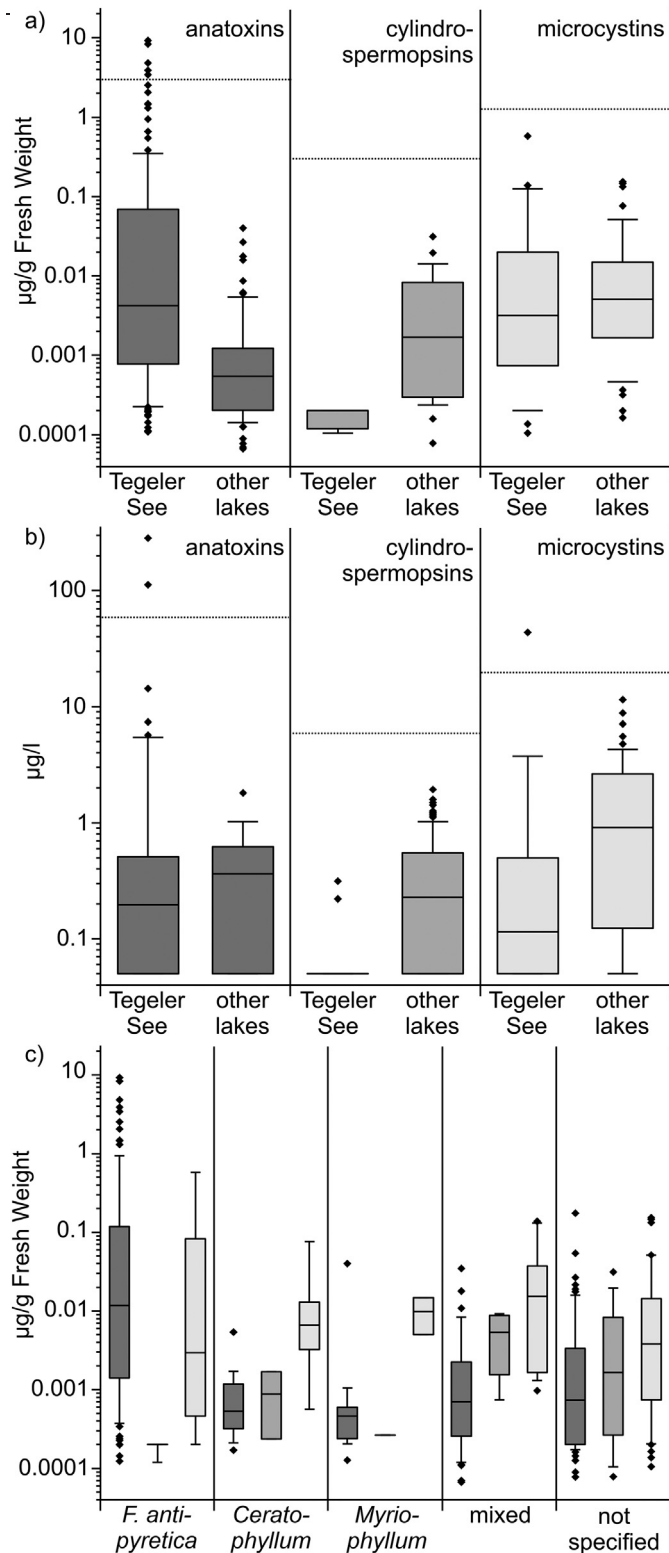
Table 2

Summary of samples positive for individual cyanobacterial toxins or toxin group in case of microcystins (MCs). Lake water refers to water samples taken in close proximity to macrophyte samples. Note that in individual samples multiple variants of a toxin group can occur.

Sample type	N	ATX	dhATX	HTX	CYN	dCYN	MCs
Lake water	463						
>LOD[a]		31	50	3	184	28	68
>1 μg/l		5	10	0	5	0	26
Macrophytes	398						
>LOD[b]		161	161	70	32	15	69
>0.1 μg/g f.w.		5	27	0	0	0	6

[a] LOD: limit of detection of approx. 0.05 μg/l; for details see Materials and methods section.

[b] LOD for macrophyte samples of approx. 0.0001 μg/g fresh weight.

Fig. 2. Distribution of cyanotoxin contents of macrophyte samples and concentrations in associated water samples taken in Berlin lakes in 2017–2021. a) contents in macrophytes [μg/g f.w.] by sample origin from Tegeler See and other lakes combined, b) concentrations in water in proximity to macrophyte samples [μg/l], c) contents in dominant macrophyte taxa [μg/g f.w.]. Anatoxins: ATX, dhATX, and HTX; cylindrospermopsins: CYN and dCYN; microcystins: quantified congeners given in the method section. The color code for toxin groups in c) is the same as in a) and b). Dotted lines in a) and b) indicate critical concentrations as given in Chorus and Welker (2021b) or discussed below, respectively. For lakes combined as "other lakes" see Table 1 and Fig. 1. Vertical bars in boxes: median; boxes: 50 % percentiles; whiskers 90 % percentiles.

slightly to about 10 % in a number of samples. In seven samples only HTX was detected but only on a low level of maximally 0.0004 μg/g f.w. Equally, the proportions of individual variants differ in the strains reflecting the year of isolation. While strain 18-026/2 produced ca. 80 % ATX and 20 % dhATX, strains 19-054/8 and A05/B5 produced only dhATX (Fig. 6).

Toxin analyses of single filaments from field samples suggested a high share of almost 100 % anatoxin producing genotypes in the population (45 out of 46 analysed filaments). The anatoxin pattern of filaments reflected well the share of variants in field samples (data not shown), but also showed a wide range of anatoxin contents from limit of detection to up to 4 μg/mm^3 biovolume (with a few outliers above, Fig. 7). In single filaments of three cultured strains the variability of measured anatoxin contents was much less and on average significantly lower compared to filaments from field samples. The lowest toxin contents were measured for strain 18-026/8 with an average of 0.2 μg/mm^3, while strains 19-054/2 and A05/B5 had average anatoxins contents of 0.4 μg/mm^3 and 0.3 μg/mm^3, respectively.

4. Discussion

4.1. Cyanobacteria and toxins associated with macrophytes

Microcystins and cylindrospermopsins were detected in macrophyte samples in relatively low amounts. Presumably, the toxins were produced by planktonic cyanobacteria that became mechanically entangled with macrophytes when both came in contact at the drift line. In contrast, anatoxin producing cyanobacteria appear to be effectively associated with macrophytes, in particular with the water moss *Fontinalis antipyretica*. In samples of other macrophyte species (*Ceratophyllum* sp., *Myriophyllum* sp., *Najas marina*, *Potamogeton* sp.) anatoxins were detected only at low levels. Therefore, the following will focus on samples of *Fontinalis antipyretica* from Tegeler See where this species was primarily collected.

In Tegeler See, *F. antipyretica* grows in dense meadows in greater depths down to the lower border of the euphotic zone (Hilt et al., 2010) but proliferates also at shallow, near-shore sites. The nature of the association of *Microcoleus* sp. with *F. antipyretica* is still poorly understood. Filaments of *Microcoleus* sp. are only loosely attached to the moss and moderate shaking suspended the cyanobacteria. Further, the occasional detection of elevated concentrations of anatoxins in water samples taken close to heavily colonized macrophytes indicate that the producing filaments are not firmly attached and are released by moderate hydrodynamic forces.

True symbiotic cyanobacteria-moss relationships have been largely described for nitrogen-fixing cyanobacteria forming heterocytes (Nostocales) and terrestrial mosses (Adams and Duggan, 2008) that can substantially contribute to the nitrogen-budget of ecosystems (Rousk et al., 2013). More general, associations of *Microcoleus* sp. and other Oscillatoriales with bryophytes have been reported, although it is not clear whether the moss only comprises a substrate for the cyanobacteria or a metabolic exchange occurs (Stanojković et al., 2022; Zhang et al., 2014). Other aquatic plants colonized by *Microcoleus* sp. include sea grass *Thalassia* (Stielow and Ballantine, 2003). Notably, complete *nif*-gene clusters coding for nitrogen fixation enzymes have been found in genomes of *M. chthonoplastes* (Bolhuis et al., 2010), suggesting that some *Microcoleus* strains may have the capacity to fix atmospheric nitrogen.

Microcoleus was found primarily associated with *F. antipyretica* and only occasionally in low amounts with other macrophytes in Tegeler See indicating that not only the physical substrate structure of submerged plants is of importance. Macrophytes, and especially mosses, are known to leak nutrients and organic metabolites both when alive and during senescence (Carpenter and Lodge, 1986), but some macrophytes like *Myriophyllum* may also deter epiphytic algae including cyanobacteria due to the high content of polyphenolic compounds (Bauer et al., 2009; Nakai et al., 2000). On the other hand, the substrate association may only reflect the low temperature optimum of *Microcoleus* of 15 °C which coincides with high amounts of detached perennial *Fontinalis* in spring. The growing season for other macrophytes and hence their availability as substrate sets in only later when the
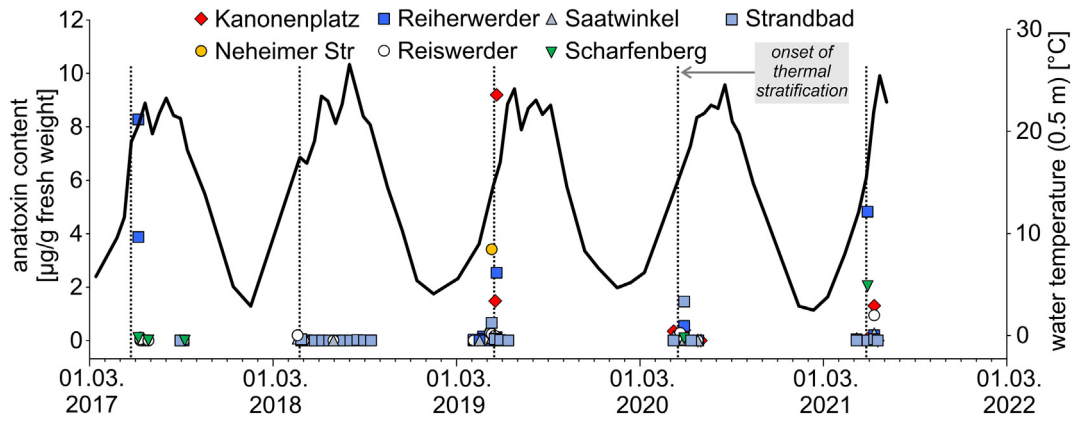
Fig. 3. Seasonal dynamics of anatoxin contents (ATX, dhATX, and HTX) of macrophyte samples in Tegeler See in 2017–2021 (left axis). Symbols represent individual sampling sites as given in Fig. 1. Epilimnetic water temperature was measured fortnightly in 0.5 m depth (black line, right axis). Dotted vertical lines indicate the onset of thermal stratification as inferred from temperature profiles obtained fortnightly by Berlin Senate Department for the Environment, Transport and Climate Protection.
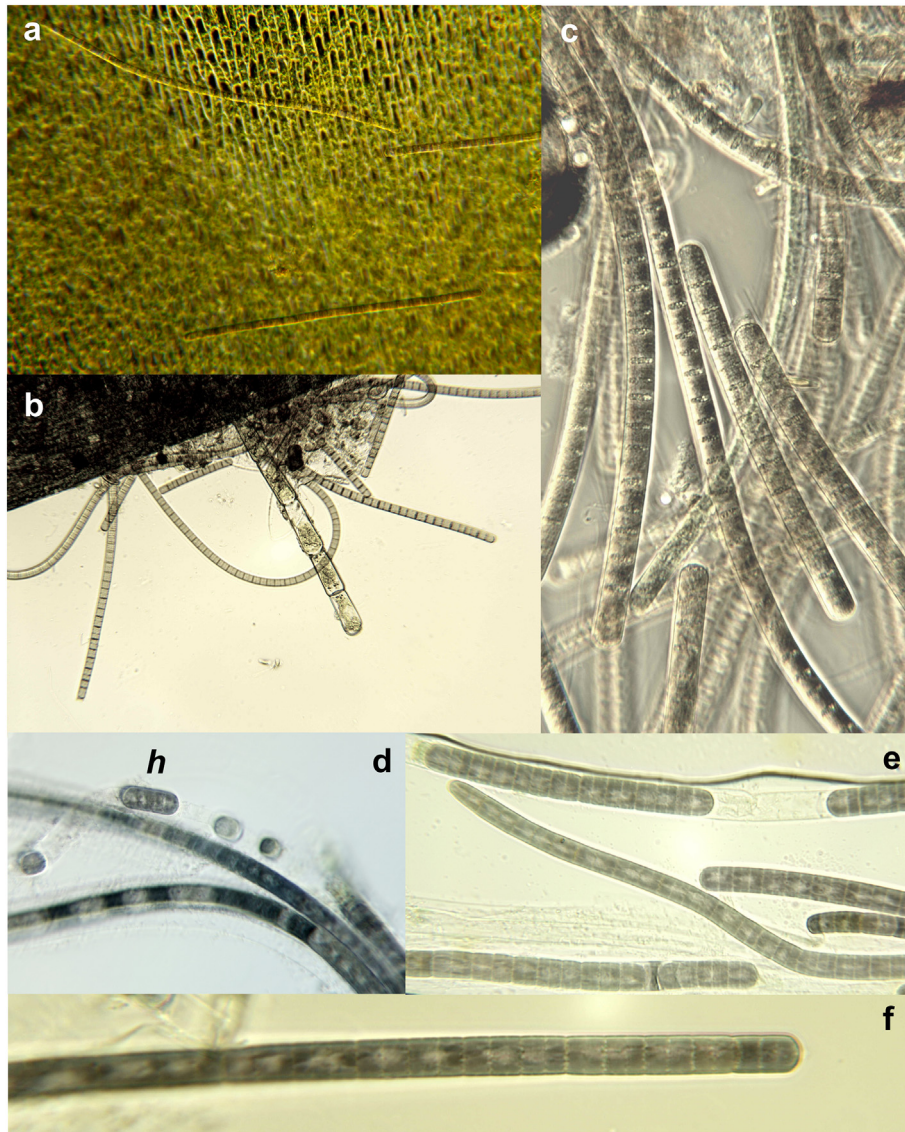

Fig. 4. Micrographs of *Microcoleus* sp. associated with *Fontinalis antipyretica* and *Microcoleus* sp. strains: a) *Microcoleus* trichomes on a vital *Fontinalis* leaf; b) *Microcoleus* trichomes attached to a senescent *Fontinalis* leaf; c) agglomeration of *Microcoleus* sp. filaments with typical banding in a macrophyte sample; d) strain 18-026/2 with a 4-cell hormogonium (*h*); e) strain 19-054/8 with partially empty sheaths; f) strain A05/B5.
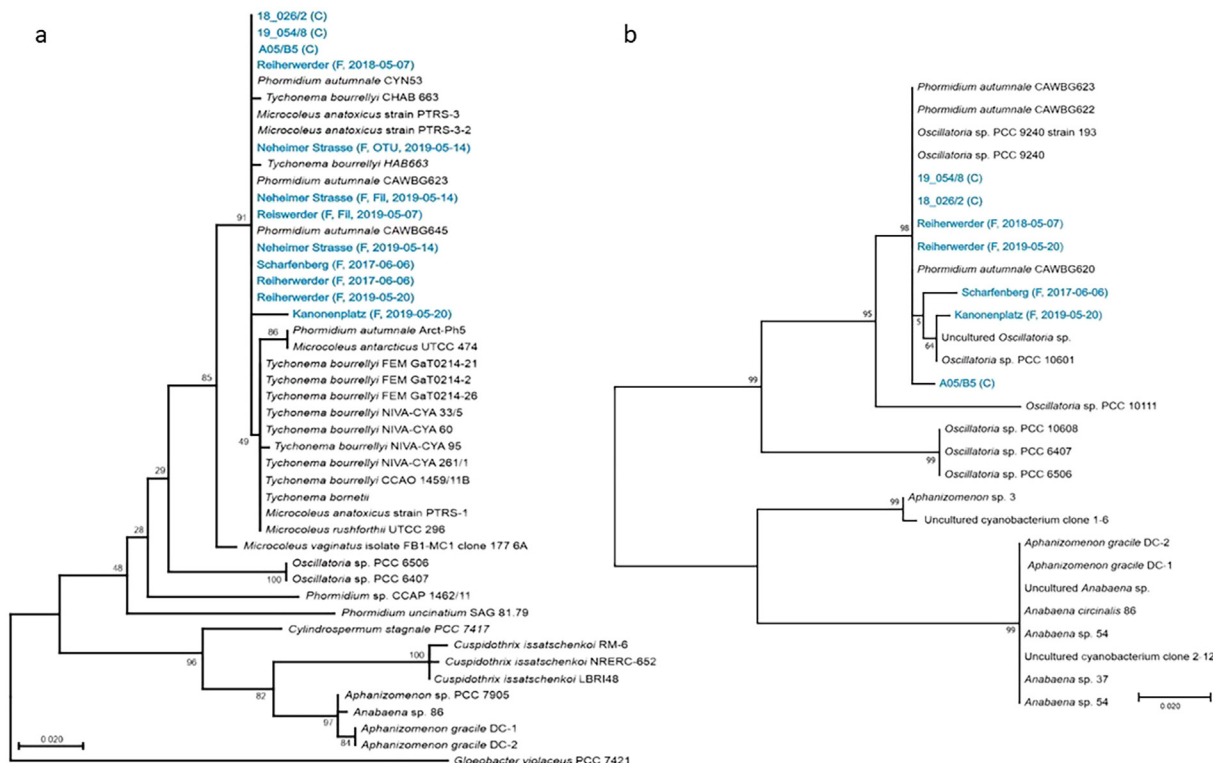
Fig. 5. Phylogenetic analysis of the partial 16S rRNA (a) and *anaC* (b) genes. In blue: sequences obtained in the present study, denoted by sampling site (F, see Fig. 1) or strain number (C). Note that not all strains possess *anaC* gene sequences and hence the *anaC* tree includes a lower number of sequences. *Gleobacter violaceus* PCC 7421 was used as an outgroup in a). Bootstrap values refer to 500 bootstrap replicates conducted in MEGA-X.

(epilimnetic) water temperatures exceeds 15 °C. Further studies will have to reveal if the lower abundance of *Microcoleus* associated with other macrophytes (e.g., *Myriophyllum*, *Ceratophyllum*) in summer is due to the low temperature prevalence of *Microcoleus* or due to substrate specificity.

The occurrence of anatoxin-producing *Microcoleus* associated with *F. antipyretica* showed a pronounced seasonal pattern. Highest anatoxin contents of macrophyte samples were measured in spring, when water temperatures were below 20 °C, around the onset of seasonal thermal stratification (Fig. 6). This is in accordance with the observation that cultivated strains isolated from Tegeler See show poor growth above temperatures of 15 °C (data not shown). The anatoxin variants produced by individual strains differed primarily in the ratio of ATX to dhATX. The strain isolated in 2018 (18-026/2) showed a similar ATX/dhATX ratio as was measured for most macrophyte samples in 2017/2018 whereas strains isolated in 2019 showed ratios similar to those found in macrophyte samples from this year. Strain diversity in *Microcoleus* (*Phormidium*) populations has

been reported by Wood et al. (2012), affecting absolute anatoxin content as well as variant shares in field populations (Wood and Puddick, 2017). Notably, the anatoxin contents of individual filaments were significantly lower in cultured strains compared to filaments isolated from field samples, indicating that anatoxin production may be elevated in *Fontinalis/ Microcoleus* associations.

4.2. Human exposure and risk assessment

While a large number of publications on cyanobacterial toxins emphasises the risk of exposure and adverse health effects, the number of reported cases of human illness is low considering the frequent exposure to potentially toxigenic cyanobacteria during recreational activity in natural waters (see, for example, Lavery et al. (2021)). In fact, compared to other toxins, cyanotoxins are responsible for only a very minor share of reported intoxications. In Germany, for example, suspected intoxications through plants
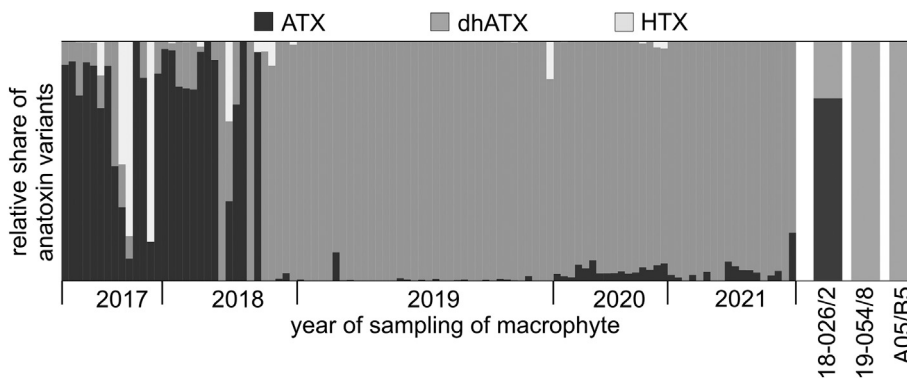

Fig. 6. Relative share of individual anatoxin variants detected in positive macrophyte samples from Tegeler See, arranged by years (only samples with anatoxin contents > 0.001 µg/g f.w.). The rightmost three columns represent anatoxin variants in three cultured strains isolated from samples in 2018 (18-026/2) and 2019 (19-054/8; A05/B5). Note that the absolute ATX-contents span several orders of magnitude and that for many samples individual variants were detected close to the limit of detection.
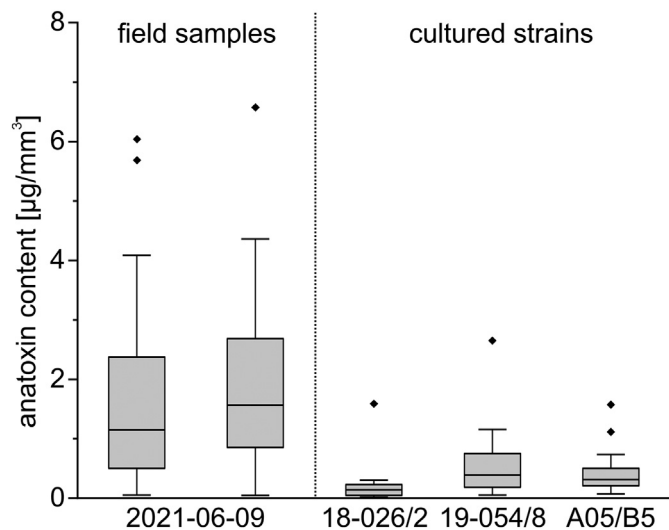
Fig. 7. Anatoxin (ATX, HTX, and dhATX) content per biovolume in *Microcoleus* sp. filaments isolated from macrophyte samples from Tegeler See (2021-06-09, Saatwinkel [left] and Kanonenplatz [right]) and cultured strains (18_026/2, 19_054/8, AO5/B5). For field samples 21–25 and for strains 10 filaments were isolated, measured, and analysed, respectively. Boxes and whiskers represent 50 % and 75 % percentiles, respectively.

and fungi (excluding tobacco products) were <20 % of total intoxications in children in 2002–2016 (Geith et al., 2018) and the share of illnesses thought to be caused by cyanobacteria was 0.25 % of total suspected plant & algae poisonings in 2021 (Stefanie Geith, Poison Emergency Call Centre Munich; pers. Communication). The death of dogs and other animals through cyanotoxin poisoning, however, necessitates a comprehensive risk assessment, irrespective of actual human case numbers and case severity.

The health based reference value proposed by WHO for recreational exposure to anatoxins (HBRV$_{recreation}$) is derived for intake of anatoxins contained in cyanobacterial cells suspended in water, i.e., planktonic cyanobacteria (WHO, 2020a). This approach is not directly applicable to macrophyte samples with associated toxic cyanobacteria. Therefore, we propose an approach that takes into account the particular exposure through macrophytes.

As with the HBRV$_{recreation}$, the point of departure is the NOAEL established for mice exposed to anatoxin by Fawell et al. (1999), 98 µg/kg body weight. The higher toxicity of dihydro-anatoxin compared to anatoxin reported by Puddick et al. (2021) cannot be taken as point of departure, because in their study they determined LD$_{50}$ instead of NOAEL by administering single doses orders of magnitude higher than doses in the experiments by Fawell et al. (1999). The combined uncertainty factor (UF = 100; 10 for intraspecies variation × 10 for interspecies variation) and the same body weight (b.w. = 15 kg for a child) can be taken as is but instead of a volume of 0.25 l of water incidentally taken up, an intake of macrophytes has to be estimated. In the absence of any data on macrophyte ingestion by children, we suggest that a child's fistful of plant material is a reasonable maximum amount, corresponding to a mass of 5 g of macrophytes (M).

With these numbers, an HBRV$_{macrophytes}$ computes as follows

$$HBRV_{macrophytes} = \frac{NOAEL \times b.w.}{UF \times M} = \frac{98 \times 15}{100 \times 5} = 2,94 \frac{\mu g}{g} \approx 3 \frac{\mu g}{g}$$

A similar calculation yields "Guideline Values" for recreational exposure through macrophytes for microcystins (GV$_{macrophytes}$ = 1.2 µg/g) and cylindrospermopsins (GV$_{macrophytes}$ = 0.3 µg/g). For respective points of departure and uncertainty factors see (WHO, 2020c) and (WHO, 2020b), respectively.

For microcystins and cylindrospermopsins, none of the 398 macrophyte samples toxin contents exceeded these "Guideline Values". The

HBRV$_{macrophytes}$ for anatoxins of 3 µg/g f.w. was exceeded in five samples (Fig. 2). Notably, when samples exceeding 3 µg/g f.w. were collected, other samples from the same site or nearby sites showed anatoxin contents orders of magnitude lower. The sample with the highest anatoxin content (9.2 µg/g f.w.) was taken on 20th May 2020 at the site "Kanonenplatz". The average of all four samples taken on the same date is 3.9 µg/g f.w. (0.12–9.2 µg/g f.w.) and the average of eight samples taken from 16th to 22nd in Tegeler See was 1.7 µg/g f.w. (0.001–9.2 µg/g f.w.). Exceedance occurred in 2.6 % of samples from Tegeler See (1.3 % of all macrophyte samples) and only during a narrow period in spring when water temperatures were below 20 °C. Fine-scale spatial variability of cyanotoxin content of benthic mats samples or of concentrations in water samples, respectively, is well documented (Ibelings et al., 2021; Miller et al., 2019; Wood et al., 2010). High spatial and temporal variability is a major challenge for risk assessment, in particular when relevant data such as toxin concentrations or contents are available only with substantial delay, during which the situation on site may have considerably changed. For planktonic cyanobacteria, short-term forecasting can be achieved by measurement of chlorophyll *a* as surrogate parameter (Qian et al., 2021) or be supported by remote sensing (Coffer et al., 2021). However, for benthic or macrophyte associated toxic cyanobacteria respective methods are still to be developed, although devices for spectrofluorometric detection have been designed to monitor benthic cyanobacteria in rivers (Echenique-Subiabre et al., 2016). In general, anatoxins produced by benthic cyanobacteria may be underreported in the literature because research was more focused on water blooms until recently (Christensen and Khan, 2020).

4.3. Dog exposure and risk assessment

Based on the maximum anatoxin contents measured in macrophyte samples, acute poisoning of dogs seems rather improbable. Toxicological data suggest an LD$_{50}$ in mice by oral administration of 5–8 mg/kg b.w. for ATX and 2.5 mg/kg b.w. for dhATX (Puddick et al., 2021). With a dog of 10 kg body weight, this would correspond to 25 mg dhATX. This amount of toxin would be contained in 250 g fresh weight of macrophytes (*F. antipyretica*) with 100 µg/g f.w. With lower toxin contents measured in the vast majority of samples ($\ll$ 1 µg/g f.w.) the mass of macrophytes theoretically needed to take up critical amounts of anatoxins increases to several kilogram. However, intake of macrophytes and lake water by dogs can be considered as significantly higher compared to children. While WHO assumes an intake of 0.25 l/day for a child of 15 kg body weight, Butler et al. (2012) suggest an intake of 0.255 l/kg b.w. and day for dogs (although uptake of 7.65 l by a dog of 30 kg appears improbable). Consequently, dog-specific guideline values as proposed by the Oregon Health Authority are 50-fold lower compared to those proposed for humans (Farrer et al., 2015). In this context, it is worth noting that dogs may be more susceptible to anatoxins than humans: the pharmacokinetics of other alkaloids such as theobromine in chocolate show significantly lower half-life times in dogs (Martinez et al., 2020) resulting in frequent consultations for "chocolate poisoning" in veterinary clinics (Cortinovis and Caloni, 2016; Weingart et al., 2021). Another aspect is that taste and odour substances co-produced with toxins (Gaget et al., 2022; Li et al., 2022) may attract dogs (Codd et al., 1992) and possibly lead to a selective intake of potentially toxic cyanobacteria. When dogs display symptoms, they need to be presented to a veterinarian as soon as possible (Bates, 2018).

5. Conclusions

Benthic, toxigenic cyanobacteria associated with submerged macrophytes may be a source of exposure of humans to cyanobacterial toxins, in particular anatoxins. Relatively high toxin contents of macrophyte samples were recorded only in samples of the water moss *Fontinalis antipyretica* and only during short periods in spring. The nature of the association of *Microcoleus* and *F. antipyretica* is unknown but an observed specificity of

this association suggests that to some degree it may be influenced a metabolic exchange between both taxa.

Although a high proportion of macrophyte samples was positive for anatoxins, toxin contents exceeding 3 μg/g fresh weight were measured in only a small fraction of samples. However, when focusing only on short periods of time in spring with ideal growth conditions (T < 20 °C), the proportion of samples exceeding 3 μg ATXs/g f.w. is substantially higher. This underlines the necessity to identify these short time periods of elevated risk and to provide appropriate recommendations.

Risk assessment based on established toxicological data suggests that human intoxication through the uptake of macrophytes is unlikely. Nonetheless, a cautious public health approach remains advisable until the spatial and temporal fluctuations in toxin occurrence are better understood. Regardless of this, the timely removal of accumulations of detached macrophytes from bathing sites is recommended to further minimize potential risks.

While for children the uptake of noxious amounts of anatoxins through toxigenic cyanobacteria associated with macrophytes is unlikely, dogs seem to be at substantially higher risk. Dog owners should therefore closely oversee their pets' behaviour, especially during critical periods in spring.

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CRediT authorship contribution statement

S.H., A.H., J.F., A.K.: conceptualization; J.F., A.H., M.W.: data curation; S.H., A.K.: Funding acquisition; J.F., A.H., J.T., E.D.: analyses; M.W., J.F.: data analysis & visualization; M.W., J.F.: writing original draft; all authors: review & editing.

Data availability

The authors do not have permission to share data.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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