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REPORT

Cylindrospermopsis raciborskii and Cylindrospermopsin in Lakes of the Berlin Area: Occurrence, Causes and Consequences

Project acronym: CYLIN

by

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Abstract (English)

Project name:	***Cylindrospermopsis raciborskii*** and ***Cylindrospermopsin*** **in Lakes of the Berlin Area: Occurrence, Causes and Consequences**
Duration:	**2/2004 to 1/2007**
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Cylindrospermopsis raciborskii, a cyanobacterium of tropical origin, can produce the toxin cylindrospermopsin (CYN). This originally tropical cyanobacterium (blue-green alga) had spread to the waters of the Berlin area. Cylindrospermopsin had been detected in two lakes in the area, but none of the *C. raciborskii* strains isolated here so far were found to produce the toxin.

The main objectives of the CYLIN project were therefore to analyze the distribution and regulation of *C. raciborskii* and cylindrospermopsin and to determine which cyanobacteria are producing this toxin in order to establish a basis to predict the further course of development of this species and the related health hazards for humans.

The CYLIN project was implemented as a three-part program. A screening program was first conducted in 2004 to test regional water bodies for the presence of cylindrospermopsin and potential CYN-producing cyanobacteria in order to obtain an overview of their distribution in the study region. A total of 142 regional water bodies were sampled once each in this qualitative analysis of cylindrospermopsin and cyanobacteria. The screening program was followed by a monitoring program designed to generate quantitative data on the concentrations of dissolved CYN, particulate CYN, cyanobacteria and target

environmental parameters at 20 selected lakes, which were sampled 3 times each. Furthermore, we investigated the seasonal dynamics of these parameters at two selected lakes in 2004 and 2005. Apart from this we isolated different cyanobacterial strains and conducted chemical and molecular biological analyses of CYN and CYN-coding genes, in order to identify CYN-producing cyanobacteria.

The results show that *C. raciborskii* and CYN are much more widespread than was previously assumed for the region. *C. raciborskii* was detected in 22 % of the investigated water bodies, and cylindrospermopsin in 52 %. Additionally, two other toxic cyanobacteria of tropical origin were found for the first time in the Berlin-Brandenburg region, *Anabaena bergii* and *Aphanizomenon aphanizomenoides*. The mean and maximum CYN concentrations were 1 µg L^{-1} and 12 µg L^{-1}, respectively. Since the particulate CYN fraction did not exceed 0.5 µg L^{-1}, the dissolved CYN fraction was found to be responsible for the high CYN concentrations. The proposed guideline value for cylindrospermopsin in drinking water (1 µg L^{-1}; Humpage and Falconer 2003) was exceeded 18 times at 8 different lakes. Although *Aphanizomenon flos-aquae* (Nostocales) has been unequivocally identified as a producer of cylindrospermopsin, the observed cylindrospermopsin concentrations cannot be attributed to this cyanobacterial species alone. *Aphanizomenon gracile* was also identified as a potential CYN-producing cyanobacterium.

Based on the findings of the CYLIN project, we recommend that cylindrospermopsin be included as in hazard analiysis for drinking and bathing water quality assessments. To identify risk conditions associated with this cyanotoxin, further investigations are needed to identify all cyanobacteria that produce cylindrospermopsin and to elucidate the mechanisms regulating the occurrence of CYN-producing cyanobacteria, CYN synthesis by these organisms, and CYN decomposition in aquatic ecosystems.

Our analysis of *C. raciborskii* population dynamics showed that its germination is temperature-dependent and its population growth light-dependent. Population size was determined by the time of germination, that is, the earlier the time of germination, the bigger the population. Based on these findings, it appears highly likely that the climate-related early rise in water temperatures over the course of the years has promoted the spread of this species to temperate regions.

Our hypothesis for the future course of cyanobacterial and cyanotoxin development in German waters is as follows: The combination of trophic decline and global warming works to the general benefit of cyanobacteria of the order Nostocales and leads to a shift in cyanobacterial species and toxin composition. This may ultimately lead to an increase in the incidence of neurotoxins as well as cylindrospermopsin.

Abstract (German)

Cylindrospermopsis raciborskii und Cylindrospermopsin in Gewässern der Berliner Region: Vorkommen, Ursachen, Auswirkungen (CYLIN)

Dauer: 2/2004 – 1/2007

Volumen: 470 000 €

Vertragspartner: Dr. C. Wiedner, Leibniz-Institut für Gewässerökologie und Binnenfischerei, Abteilung Limnologie Geschichteter Seen;
Prof. Dr. B. Nixdorf, Brandenburgische Technische Universität Cottbus, Lehrstuhl Gewässerschutz;
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Kontakt im KWB: Dr. Bodo Weigert

Cylindrospermopsis raciborskii ist ein Cyanobakterium (Blaualge) tropischen Ursprungs, das das Toxin Cylindrospermopsin (CYN) produzieren kann. Es hat sich bis in Gewässer der Berliner Region ausgebreitet. Dort wurde CYN in zwei Seen nachgewiesen, aber alle bisher analysierten Stämme von _C. raciborskii_ aus diesen Gewässern produzieren kein CYN.

Ziel des CYLIN-Projektes war es, die Verbreitung und Regulation von _C. raciborskii_ und CYN zu analysieren und die CYN-Produzenten zu ermitteln, um eine Basis zu schaffen, auf der die weitere Entwicklung dieser Art und die damit verbundene Gefährdung für den Menschen abgeschätzt werden kann.

Dies wurde in einem 3-Stufen-Untersuchungsprogramm umgesetzt: In einem Screening wurde ein Überblick zur Verbreitung von CYN und potenziellen CYN-Produzenten gewonnen (CYN und Cyanobakterien wurden 2004 in 142 Gewässern einmalig qualitativ erfasst). In einem Monitoring im Sommer 2005 wurden in 20 Seen je dreimal die Konzentrationen des gelösten und partikulären CYN sowie die Cyanobakterien und Umweltparameter quantitativ erfasst. Die saisonale Dynamik dieser Parameter wurde in 2 ausgewählten Gewässern in den Jahren 2004 - 2005 untersucht. Außerdem wurden für die Identifizierung der CYN-Produzenten verschiedene Cyanobakterienstämme isoliert, die chemisch und molekularbiologisch auf CYN und CYN-kodierende Gene analysiert wurden.

Die Ergebnisse zeigen, dass *C. raciborskii* und CYN deutlich weiter verbreitet sind als bisher bekannt war: *C. raciborskii* wurde in 22 % und CYN in 52 % der Seen qualitativ nachgewiesen. Außerdem wurden zwei weitere toxische Cyanobakterien tropischen Ursprungs, *Anabaena bergii* und *Aphanizomenon aphanizomenoides*, erstmals in der Region Berlin-Brandenburg nachgewiesen. Die Konzentrationen von CYN betrugen im Mittel 1 µg L^{-1} und maximal 12 µg L^{-1}, wobei die partikuläre Fraktion 0,5 µg L^{-1} nicht überschritt und hohe Konzentrationen durch die gelöste CYN-Fraktion bedingt waren. Ein empfohlener Leitwert für CYN im Trinkwasser von 1 µg L^{-1} (Humpage and Falconer, 2003) wurde insgesamt 18mal in 8 verschiedenen Seen überschritten. *Aphanizomenon flos-aquae* konnte eindeutig als CYN-Produzent identifiziert werden, allerdings lassen sich die CYN-Vorkommen nicht allein durch diese nostocale Art erklären. Als weiterer möglicher CYN-Produzent konnte *Aphanizomenon gracile* herausgearbeitet werden.

Aufgrund der Projektergebnisse wird empfohlen, CYN in die Gefährdungsanalyse für Trinkwasserversorgungen und für Badegewässer aufzunehmen. Um CYN-Analysen auf Risikosituationen eingrenzen zu können, ist die weitere Aufklärung der CYN-Produzenten, der Regulation ihres Vorkommens sowie ihrer Toxinproduktion und des CYN-Abbaus notwendig.

Die Analyse der Populationsdynamik von *C. raciborskii* ergab, dass ihre Keimung temperaturreguliert ist und das Populationswachstum durch Licht limitiert wird. Der Zeitpunkt der Keimung bestimmt die Populationsgröße (je früher desto größer). Ein klimatisch bedingter früherer Anstieg der Wassertemperaturen im Jahresverlauf hat daher mit großer Wahrscheinlichkeit die Ausbreitung dieser Art in die gemäßigten Breiten begünstigt.

Für die weitere Entwicklung von Cyanobakterien und Toxinen in deutschen Gewässern wurde folgende Hypothese aufgestellt: Die Kombination von Trophieminderung und globaler Erwärmung begünstigt generell nostocale Cyanobakterien und führt zu Verschiebungen in der Artenzusammensetzung und den Toxinvorkommen. Dabei ist neben CYN auch mit zunehmenden Vorkommen von Neurotoxinen zu rechnen.

Abstract (français)

Cylindrospermopsis raciborskii et Cylindrospermopsine dans les eaux superficielles de la région de Berlin: Persistance, causes et effets (CYLIN)

Durée :   2/2004 – 1/2007

Volume: 470 000 €

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Lehrstuhl Gewässerschutz [Université de technologie de Brandebourg,
chaire de Protection des Eaux];
Dr. Ingrid Chorus, Umweltbundesamt [office fédéral de l'environnement]

Contact au KWB :   Dr. Bodo Weigert

Cylindrospermopsis raciborskii est une cyanobactérie (algue bleue) d'origine tropicale qui est en mesure de produire la substance toxique cylindrospermopsine (CYN). Elle se développe depuis un certain temps dans les eaux de la région berlinoise. La CYN y a été décelée dans deux lacs, mais toutes les souches de C. raciborskii provenant des eaux berlinoises et analysées entre temps ne produisent pas la substance toxique CYN.

Le but du projet CYLIN a consisté à analyser la propagation et la régulation de la C. raciborskii et de la CYN et d'identifier les producteurs de CYN afin de créer une base permettant d'évaluer l'évolution de cette espèce et les risques qu'elle comporte pour les hommes.

Cet objectif a été mis en oeuvre par un programme d'analyse comprenant 3 volets : Un screening d'entrée a permis de faire le point sur la propagation de la CYN et de ses producteurs potentiels (les qualités de la CYN et des cyanobactéries ont été retenues en 2004 dans le cadre d'une action unique dans 142 eaux naturelles). Dans le cadre d'un monitoring qualitatif effectué en été 2005, les concentrations de la CYN dissoute et particulaire ainsi que des cyanobactéries et de certains paramètres environnementaux ont été mesurés à trois reprises dans 20 lacs. La dynamique saisonnière de ces paramètres a été étudiée entre 2004 et 2005 dans deux milieux aquatiques. Par ailleurs, diverses souches de cyanobactéries ont été isolées et analysées par des méthodes chimiques et de

biologie moléculaire pour capter la CYN et des gènes codés de CYN et identifier ainsi ses producteurs.

Les résultats montrent que la C. *raciborskii* et la CYN sont considérablement plus répandues qu'on le pensait jusqu'à présent. La C. raciborskii a pu être décelée qualitativement dans 22 % et la CYN dans 52 % des lacs. Par ailleurs, deux autres cyanobactéries toxiques d'origine tropicale – l'Anabaena bergii et l'Aphanizomenon aphanizomenoides ont été identifiées pour la première fois dans la région de Berlin-Brandebourg. Les concentrations de CYN étaient de 1 µg L-1 en moyenne et de 12 µg L-1 au maximum ; la fraction particulaire n'a pas dépassé 0,5 µg L-1, et les concentrations élevées étaient dues à la fraction de CYN dissoute. La valeur guide de CYN recommandée pour l'eau potable s'élevant à 1 µg L^{-1} (Humpage and Falconer, 2003) a été dépassée 18 fois dans 8 lacs différents. L'Aphanizomenon flos-aquae a pu être clairement identifiée en tant que producteur de CYN, toutefois, la présence de CYN ne se laisse pas expliquer par ce seul type nostocal. Un autre producteur potentiel de CYN a été identifié : l'Aphanizomenon gracile.

Compte tenu des résultats de ce projet, il est recommandé d'intégrer la CYN dans l'analyse des risques pour l'approvisionnement en eau potable ainsi que pour les eaux de baignade. Dans l'objectif de délimiter les analyses de la CYN à des situations de risques, il sera nécessaire d'approfondir les recherches sur ses producteurs, la régulation de leur présence et de leur production de toxines ainsi que sur la dégradation de la CYN.

L'analyse de la dynamique de la population de la C. raciborskii a révélé que sa germination est régulée par la température et que la croissance de la population est limitée par la lumière. Le moment de la germination détermine l'importance de la population (plus ce moment est précoce, plus la population est grande). Dans ce contexte, il est très probable qu'une augmentation des températures de l'eau intervenue plus tôt dans l'année et due au climat a favorisé la propagation de ces espèces dans les zones de climat tempéré.

Pour la propagation future des cyanobactéries et le développement des toxines dans les eaux allemandes, nous avons établi l'hypothèse suivante : La combinaison entre la réduction de la trophie et le réchauffement mondial favorise les cyanobactéries nostocales et provoque des changements dans la structure taxonomique et la présence de toxines. Dans cette hypothèse, il faut s'attendre, outre la CYN, à une croissance des concentrations de neurotoxines.

Acknowledgements

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Chapter 1

Introduction

Cylindrospermopsis raciborskii is a cyanobacterium that originally occurred in lakes and rivers of tropical and subtropical regions. Within the last few decades, it has spread to other parts of the world, out of the tropics into temperate climate zones. Taxonomically, *C. raciborskii* is a member of the order Nostocales. This cyanobacterium forms small filaments (trichomes) measuring on average 0.15 mm in length and 0.003 mm in diameter (Fig. 1). Like all Nostocales, *C. raciborskii* can produce vegetative cells as well as heterocysts for the fixation of molecular nitrogen and akinetes (resting cells) for survival in sediment during unfavorable growth conditions. *C. raciborskii* can produce potent toxins such as cylindrospermopsin (Fig. 1) and paralytic shellfish poisons. It is therefore important to carefully monitor the occurrence of this cyanobacterial species in water bodies used for drinking water or recreational purposes.

Fig. 1: *Cylindrospermopsis raciborskii* (left) and cylindrospermopsin (chemical formula, right).

Toxicity of cylindrospermopsin

The fact that *C. raciborskii* can be a health hazard for humans became acutely evident in the Palm Island incident in Australia in 1979. There, several cases of hepatoenteritis occurred in the local population due to contamination of drinking water with a cyanobacterium, which was later identified as *C. raciborskii* (Bourke et al. 1983). Epidemiological studies confirmed that a strain of *C. raciborskii* isolated from the drinking water reservoir produced serious

hepatotoxic effects in a mouse bioassay and was therefore responsible for the occurrences of hepatoenteritis (Hawkins et al. 1985). Cylindrospermopsin, the causative alkaloid hepatotoxin named after *Cylindrospermopsis raciborskii*, was later isolated from the implicated *C. raciborskii* strain (Ohtani et al. 1992).

The toxicity mechanism of cylindrospermopsin (CYN) still is not fully understood. One decisive aspect demonstrated in laboratory experiments (in vitro) was that CYN blocks protein synthesis rapidly, completely and irreversibly (Froscio et al. 2003). Experiments with laboratory animals (in vitro) showed that CYN induces damage to the liver, kidneys, lung, heart, stomach, adrenals, circulatory system and lymphatic system (Hawkins et al. 1985). Liver damage is dose-dependent and can be very severe, especially after exposure to acute doses (inducing injury within 1 to 2 days). Although CYN is generally classified as a hepatotoxin, it has a much wider range of effects. Renal toxicity is a prominent effect, as was clearly demonstrated in animals with subchronic exposure (periods of several weeks) to daily doses of CYN (Humpage and Falconer 2003). Genotoxicity of cylindrospermopsin has also been demonstrated in a number of studies (e.g., Humpage et al. 2005). Concerning its potential for carcinogenicity in humans, the International Agency for Research on Cancer (IARC) concluded in 2006 that the available data was insufficient to resolve this question. So far, only one study has shown evidence of an increased tumor incidence in living cells exposed to very high doses of CYN (Falconer and Humpage 2001).

Guideline values for CYN in drinking-water have not been established to date due to insufficiency of the available data, and the World Health Organization (WHO) will reassess the toxicity data as further studies emerge. In order to derive drinking-water guideline values for a toxin, the WHO requires toxicity data from at least two independent studies or sufficient data from suborganismic tests. The one available animal study following standard procedures is a subchronic exposure trial (10 to 11 weeks) in mice (Humpage and Falconer 2003); similar to the WHO guideline value for microcystin-LR, the authors recommend 1 µg L^{-1} as the drinking water guideline value for CYN. Drinking water guideline values have been established in Brazil (15 µg L^{-1}) and New Zealand (2 µg L^{-1}).

Occurrence of cylindrospermopsin and CYN-producing cyanobacteria

CYN has been detected in waters in tropical regions such as Australia (McGregor and Fabbro 2000, etc.) and Brazil (Carmichael et al. 2001) as well as in water bodies in North

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America (Burns et al. 2002) and Europe (Fastner et al. 2003, Manti et al. 2005, Quesada et al. 2006). In the six available studies that provide quantitative assessments of CYN in natural waters, CYN concentrations reportedly ranged from 1 to 20 µg L^{-1} with maximum concentrations of up to 100 µg L^{-1} (Manti et al. 2005, Quesada et al. 2006, Chiswell et al. 1999, McGregor and Fabbro 2000, Hoeger et al. 2004, Burns et al. 2002). The total CYN concentration may have been underestimated in some of these studies because the dissolved CYN fraction was not determined. On the whole, quantitative CYN concentration data suitable for assessment of the hazard potential of this toxin are still very scanty.

Further investigation is also needed to determine the identity of the cyanobacteria responsible for the production of cylindrospermopsin. The first *C. raciborskii* and cylindrospermopsin distribution studies revealed that the toxin is produced by other cyanobacterial species besides *C. raciborskii*. Those identified by the start of the CYLIN project were: *Umezakia natans* (Harada et al. 1994), *Aphanizomenon ovalisporum* (Banker et al. 1997), *Anabaena bergii* (Schembri et al. 2001), and *Raphidiopsis curvata* (Li et al. 2001a). Like *C. raciborskii*, these cyanobacterial species are also predominantly distributed in tropical and subtropical waters. Unlike *C. raciborskii*, however, they had not been detected in German waters before. *C. raciborskii* could not be implicated as the cause of the first occurrences of cylindrospermopsin at two German lakes (Melangsee and Langer See) because none of the *C. raciborskii* strains isolated from Melangsee to date produced the toxin (Fastner et al. 2003). Likewise, none of the *C. raciborskii* strains found in other European waters produce cylindrospermopsin (Bernard et al. 2003, Saker et al. 2003). These findings suggest that cyanobacterial species other than *C. raciborskii* produced the cylindrospermopsin detected in these waters or that *C. raciborskii* populations consist of both CYN-producing and non-producing genotypes. Another factor to consider is that the *C. raciborskii* strains found in Germany produce other toxic substances that have not yet been subjected to further analysis (Fastner et al. 2003). Therefore, further investigation of the spread and regulation of *C. raciborskii* is essential, even if this species is not the producer of cylindrospermopsin in regional waters.

Distribution and ecology of *C. raciborskii*

Cylindrospermopsis raciborskii (Woloszynká) Seenaya & Subba Raju was first discovered by Woloszynská (1912) in a lake in Java. This bloom-forming cyanobacterium has spread

from the tropics to temperate climate zones during the last few decades, as was first extensively documented by Padisák (1997). *C. raciborskii* was first sighted in Europe in 1938 at a lake in Greece (Skuja 1938). It was later detected in other European lakes, including Balaton in Hungary in 1970 (Padisák 1977), Lieps in Germany in 1990 (Krienitz and Hegewald 1996), the Old Danube (Alte Donau) in Austria in 1993 (Dokulil and Mayer 1996), the Scharmützelsee region in Germany in 1994 (Rücker et al. 1997, Wiedner and Nixdorf 1997), and a small lake north of Paris, France in 1994 (Couté et al. 1997).

Such drastic spread of a phytoplankton species has never been observed before. Climate change and ecotype selection have been proposed as potential causes of the invasive spread of *C. raciborskii*. Some investigators who did not detect any ecophysiological differences between *C. raciborskii* strains from the tropics and temperate zones conclude that climate change is the cause of spread (Briand et al. 2004). Others found strains that can survive at low temperatures, which is more suggestive of adaptation and selection mechanisms (Chonudomkul et al. 2004). The cause of *C. raciborskii* invasion is therefore a subject of debate that still cannot be resolved definitively.

The highest biomass levels of *C. raciborskii* occur in deep stratified lakes in tropical regions and in polymictic shallow lakes in temperate regions (Padisák 1997). According to Padisák, the reason for this difference in habitat preference is that, in temperate climate zones, the mean water temperatures of shallow lakes are higher than those of deep lakes; shallow lakes are therefore more suitable to the high-temperature preferences of this tropical species. *C. raciborskii* populations can thrive all year round in the tropics (Fabbro and Duivenvoorden 1996, Bouvy et al. 1999). In temperate latitudes, however, pelagic growth of *C. raciborskii* is limited to the summer months. In order to survive the winter, the species must form akinetes, from which it germinates again in the spring.

Studies have shown that akinetes of tropical *C. raciborskii* populations germinate at temperatures above 22 °C. Peak biomass values for pelagic populations were found under the following conditions: water temperature range 27 to 30 °C, high pH, high water column stability, long water residence times, and high global radiation (Branco and Senna 1994, Fabbro and Duivenvoorden 1996, Souza et al. 1998, Bouvy et al. 1999, McGregor and Fabbro 2000). In temperate latitudes, germination also occurs at temperatures above 22 °C (Górzo 1987, in Padisák 1997), and population peaks coincide with annual temperature peaks, but the start of pelagic growth occurs at relatively low temperatures in the 15 to 17 °C

range (Dokulil and Mayer 1996, Mischke 2003). Other investigators have postulated that *C. raciborskii* is a shade-tolerant species that benefits from low light intensity conditions, such as those occurring in eutrophic waters during the summer months (Dokulil and Mayer 1996, Padisák and Reynolds 1998), and from low concentrations of dissolved inorganic nitrogen and phosphorous (Mischke 2003, Briand et al. 2002a). Since most of the previous studies were only designed to characterize the conditions under which the populations occur, the actual mechanisms regulating *C. raciborskii* population dynamics and growth are still largely unclear. Consequently, it is not possible to predict the further course of development of *C. raciborskii* in our region at this time.

Based on the current data, the most northern occurrences of *C. raciborskii* are those in the lakes of the Scharmützelsee region (southeast of Berlin) and in Lake Lieps (north of Berlin). The extent of spread of the cyanobacterium in the region is, however, unclear. The Lieps sighting was based on an analysis of a single phytoplankton sample obtained in 1990. It is unclear whether *C. raciborskii* was there before or after this time or whether it occurs in neighboring lakes in the region. Further data from the lakes of the Scharmützelsee region show that the initial occurrence of *C. raciborskii* was not a transient event. Populations of this cyanobacterium have been found there regularly since 1994, and the *C. raciborskii* fraction sometimes comprises up to 20 % of the total phytoplankton biovolume (Wiedner and Nixdorf, 1997; Mischke 2003, Nixdorf et al. 2003). Although no "mass occurrence" of *C. raciborskii* has been observed in the region so far and although it has never occurred as the dominant phytoplankton species, its prevalence is remarkably high for recently invaded species.

In summary, we conclude that populations of the toxic cyanobacterial species *C. raciborskii* have become established in lakes in the Berlin area. Too little is known about the regional distribution of this new, toxic species to assess the potential health hazard it may pose in water bodies used for drinking water and recreational purposes. Further evidence clearly shows that CYN is present in lakes in the Berlin region, but the distribution and concentration of CYN and of the cyanobacteria that produce it are still unknown.

Chapter 2

Objectives

The foremost objective of the CYLIN project was to assess current and future hazard potential of the toxic cyanobacterium *Cylindrospermopsis raciborskii* and of the cyanobacterial toxin cylindrospermopsin in water bodies in the Berlin-Brandenburg region. Specifically, the three main work objectives of the CYLIN project were:

- To analyze the distribution of *C. raciborskii*, other potential CYN-producing cyanobacteria and CYN in regional water bodies with different trophic and morphological features and to characterize the lake types that provide suitable habitats for the CYN producers.
- To identify the mechanisms that regulate the seasonal dynamics of *C. raciborskii*, other potential CYN producers and CYN in order to predict the future course of development of CYN-producing cyanobacteria and CYN concentration in the study region.
- To determine which cyanobacteria produce CYN in the study region and to assess the variability of occurrence of the CYN producers.
- To characterize the frequency of CYN-occurrence and the concentrations at which CYN occurs in this northern region of Germany.

Chapter 3

Study design

Distribution of C. raciborskii, other potential cylindrospermopsin producers and cylindrospermopsin in the study region

Within in the scope of the CYLIN project, a three-part research program was used to investigate the spatial and temporal distribution of *C. raciborskii* and CYN in the study region (Fig. 2).
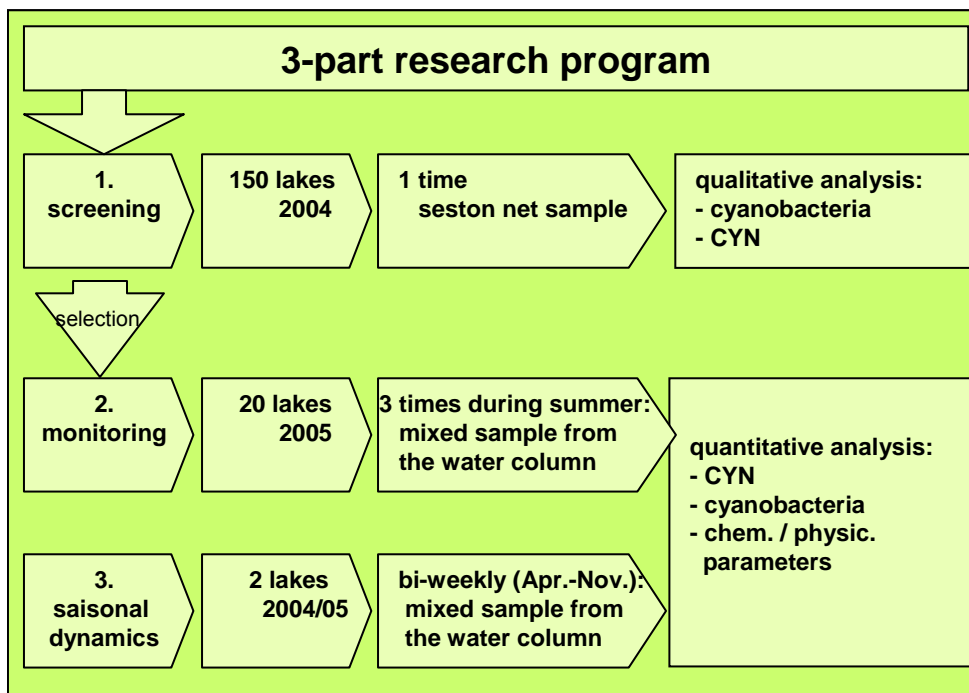
Fig. 2: Three-part research program of the CYLIN project.

Screening: The foremost objective of the screening program was to obtain a comprehensive overview of the spatial distribution of *C. raciborskii*, other potential CYN-producing cyanobacteria, and CYN. Water bodies throughout the entire study region and selected, representative lakes were included in the analysis. A large number of water bodies

had to be sampled for this purpose: 60 was the target number, and a total of 142 water bodies were actually sampled. Cyanobacterial species and CYN concentration were assessed from plankton net samples taken once from each water body.

Monitoring: Twenty lakes in which potential CYN-producing cyanobacteria or CYN had been identified by screening were selected for the monitoring program. Each of the lakes was sampled three times during the summer months. This generated the first quantitative data on dissolved and particulate CYN concentrations per volume water with which a risk assessment could be performed. Quantitative data on the biovolumes of the observed cyanobacterial species and relevant chemical and physical environmental parameters were also obtained. The overall data set was tested for correlations between CYN concentration and cyanobacterial biovolume and environmental parameters.

Seasonal dynamics: Quantitative measures of the variability of the seasonal dynamics of CYN concentration, potential CYN-producing cyanobacteria and environmental factors rounded off our comprehensive series of investigations. The quantitative seasonal data were analyzed to determine the mechanisms regulating CYN and cyanobacterial dynamics and to identify lake-specific correlations between CYN concentration and potential CYN-producing cyanobacteria.

Future course of C. raciborskii and cylindrospermopsin development

The results of the investigations of seasonal dynamics and regulatory mechanisms were used for forecasting purposes. In the case of *C. raciborskii* development, additional data sets from earlier study programs dating back to 1993 were also available for analysis. The serial data sets were analyzed for long-term trends and for factors that regulate *C. raciborskii* growth and population dynamics. Future development predictions were based on information derived from the analysis of regulatory mechanisms.

Identification of cylindrospermopsin-producing cyanobacteria

Strains of various cyanobacterial species of the order Nostocales (all of which are currently considered to be potential CYN producers) were studied using culture isolates obtained from samples collected from various lakes sampled in the scope of the screening program. The CYN content of all cultured strains was determined by chemical analysis, and the presence or absence of CYN-coding genes was determined by molecular biological analysis. A molecular biological method for detection of CYN-coding genes in individual filaments (trichomes) with which one could estimate the fractions CYN-producing genotypes within a given population had to be developed. Representative quantities of individual filaments of potential CYN-producing cyanobacteria were isolated from selected monitoring program samples and stored for later analysis.

Chapter 4
Results

The main results of the project are summarized in a monograph that is available in English and German:

Wiedner, C., Rücker, J., Weigert, B. (Hrsg.) 2007. *Cylindrospermopsis raciborskii* und Cylindrospermopsin in Gewässern der Berliner Region -Vorkommen, Ursachen, Auswirkungen. Schriftenreihe Kompetenzzentrum Wasser Berlin, Band 6, 92 S., ISBN 978-3-00-021363-2.

Wiedner, C., Rücker, J., Weigert, B. (eds.) 2007. *Cylindrospermopsis raciborskii* and Cylindrospermopsin in Lakes of the Berlin Area - Occurrence, Causes and Consequences. Kompetenzzentrum Wasser Berlin Publications Series, Volume, 89 p., ISBN 978-3-00-021364-9.

Copies (pdf-files) of these monographs can be downloaded at: www.kompetenz-wasser.de

Additionally, all results that were gained in this project are already published in international peer reviewed scientific journals or submitted to such journals (see list below). Overall a number of 10 publications / manuscripts resulted from the project. All publications / manuscripts are presented individually in Appendix A – J. The publications can be purchased from the individual journals. Manuscripts indicated as "submitted to.." are still in the peer reviewing process. These manuscripts have to be kept confidential and it is not allowed to distribute them until they reached the status of "in press".

List of publications / manuscripts originating from the CYLIN project

Fastner, J., Rücker, J., Stüken, A., Preußel, K., Nixdorf, B., Chorus, I., Köhler, A., Wiedner, C. 2007. Occurence of the Cyanobacterial Toxin Cylindrospermopsin in Germany. Environmental Toxicology 22 (1): 26-32.
(Apendix A)

Haande, S., Rohrlack, T., Ballot, A., Wiedner, C. Genetic characterisation of *Cylindrospermopsis raciborskii* isolates (Nostocales, Cyanobacteria) from Africa and Europe

(submitted to: Harmful Algae).

(Apendix B)

Preußel, K., Stüken, A., Wiedner, C., Chorus, I., Fastner, J. 2006. First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes. Toxicon 47: 156-162.

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(Apendix D)

Rücker, J., Stüken, A., Nixdorf, B., Wiedner, C. 2006. Distribution and regulation of the originally tropical cyanobacterium *Cylindrospermopsis raciborskii* at its northern limits. In: Rabitsch, W., Klingenstein, F., Essl, F. (Eds.): BfN-Skripten 184, Bundesamt für Naturschutz, Bonn: p. 229.

(Apendix E)

Stüken, A., Beck, M., Quesada, A., Sukenik, A., Dadheech, P., Wiedner, C. Phylogenetic position of the three cyanobacterial species *Anabaena bergii*, *Aphanizomenon ovalisporum* and *Aphanizomenon aphanizomenoides* (order Nostocales) (submitted to: Int. J. Syst. Evol. Microbiol.).

(Apendix F)

Stüken, A., Rücker, J., Endrulat, T., Preussel, K., Hemm, M., Nixdorf, B., Karsten, U., Wiedner, C. 2006. Distribution of three alien cyanobacterial species (Nostocales) in Northeast Germany: *Cylindrospermopsis raciborskii*, *Anabaena bergii* and *Aphanizomenon aphanizomenoides*. Phycologia 45: 696–703.

(Apendix G)

Tingwey, E.I., Rücker, J., Launhardt, A., Wiedner, C. Nixdorf, B. 2007. Germination of *Cylindrospermopsis raciborskii* and *Aphanizomenon* species under natural and experimental conditions. Deutsche Gesellschaft für Limnologie. Tagungsbericht 2006: 240-244.

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Wiedner, C., Rücker, J., Brüggemann, R., Nixdorf, B. 2007. Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. Oecologia,

152: 473-484.

(Apendix I)

Wiedner, C., Rücker, J., Stüken, A., Fastner, J., Chorus, I., Nixdorf, B. Seasonal dynamics of cylindrospermopsin and cyanobacteria in two German lakes (submitted to: FEMS Microbiol.).

(Apendix J)

Chapter 5

Conclusions - Hazard potential, guidelines and perspectives

Based on the findings of the CYLIN project, our preliminary assessment of the hazard potential of cylindrospermopsin in waters in the study region and issues requiring further investigation are summarized below.

5.1 Hazard potential of cylindrospermopsin

Because cylindrospermopsin is produced by at least one widespread indigenous species (see below), it is highly likely that the toxin first appeared in the study region decades ago. It can therefore be presumed that cylindrospermopsin might be the cause of several yet unexplained cases of cyanobacterial poisoning.

The guideline value 1 µg L^{-1} for cylindrospermopsin in drinking water, which was proposed by Humpage and Falconer (2003), can be used for a preliminary toxicological assessment of current CYN concentrations.

Basic information concerning guideline values:

Extrapolation and uncertainty factors with which guideline values are calculated must be taken into account when assessing toxin levels exceeding up to 10 times the guideline value. An uncertainty factor[1] of 100 to the highest "no observed adverse effect level" (NOAEL) determined in laboratory animals during exposure trials is generally used as the safety margin. Both Humpage and Falconer's proposal for cylindrospermopsin and for the preliminary WHO Guideline value for microcystin-LR in drinking-water include a further uncertainty factor of 10 to account for extrapolation from laboratory animals to humans and for extrapolation of data from part of the life cycle to lifetime exposure. This factor is

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[1] Product of a factor of 10 for extrapolation from animals to humans times a factor of 10 for susceptibility differences between individuals in a given population.

used because drinking-water guideline values are set at levels intended to be safe for daily lifetime consumption. The overall assessments of health hazards associated with toxin levels exceeding the guideline value must therefore be weighed against a background of extrapolation and uncertainty factors that, when multiplied, yield a total uncertainty factor of 1000. Transient human exposure to concentrations exceeding the guideline value only by a factor in the range of 10, as is the case with the data from our study, therefore do not imply an immediate health risk.

These considerations play an increasingly important role in defining action levels that trigger immediate responses to hazardous substances in drinking water management. Action levels can be higher than guideline values for lifetime exposure. They indicate whether drinking water contaminated with a hazardous substance poses an acute health hazard and whether measures must be taken immediately (e.g in extreme cases interruption of the water). As long as a temporary, transient exceedance of the safety limit remains below the action level and the affected population has been informed, the situation can be tolerated under certain conditions, e.g. if a remedial plan is submitted and implemented. This helps to focus efforts on eliminating the cause of the problem (e.g., reduction of eutrophication instead of more sophisticated drinking water treatment). Action levels are derived specifically for each individual substance in consideration of its mechanism of action. An action level for cylindrospermopsin does not yet exist.

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The proposed guideline value for CYN in drinking water (1 µg L^{-1}) was exceeded 18 times at lakes studied by us. In the study region, cylindrospermopsin now occurs just as frequently as the well-studied cyanobacterial toxin microcystin and at comparable concentration ranges. As explained below, an increase in CYN distribution and concentration can be expected in the future. These findings indicate that cylindrospermopsin may pose a health risk in certain situations.

Based on the current evidence, we recommend that cylindrospermopsin be included as a potential hazard in drinking and bathing water safety risk assessments. Data on the monitoring, risk assessment and toxin elimination available for microcystin cannot be transferred directly to cylindrospermopsin because of the need for further scientific investigation of the following major issues:

1. Unlike microcystin, cylindrospermopsin often occurs to a high share dissolved forming water. This poses a risk of breakthrough of cylindrospermopsin into the drinking water because simple particle removal-based methods of drinking water treatment (flocculation and filtration) eliminate the cell-bound, particulate CYN fraction but not the dissolved CYN fraction. Microbial decomposition of cylindrosper-mopsin may be slower than that of microcystin, which often decomposes within a few days. Data on the persistence and biological degradation of cylindrospermopsin is

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therefore required, especially for assessing the risk of a breakthrough of cylindrospermopsin into the drinking water.

2. The main producers of cylindrospermopsin and the conditions for their development have not been unequivocally determined. *Aphanizomenon flos-aquae* is a confirmed producer of cylindrospermopsin, but this cyanobacterial species is not the main producer of cylindrospermopsin in water bodies. *A. gracile*, also an indigenous cyanobacterial species, probably is one of the main producers, but unequivocal proof of this has not been demonstrated. Further cyanobacterial species must be targeted as potential producers of the cyanotoxin. Once they have been identified, suitable methods for detection of these organisms must be developed. Further investigation of the mechanisms that regulate the occurrence of the CYN-producing cyanobacteria is also essential. This will be discussed in the following section in conjunction with the population dynamics of *Cylindrospermopsis raciborskii.*

5.2 Hazard potential of *Cylindrospermopsis raciborskii*

C. raciborskii is a widespread cyanobacterium that occurs at relevant biomass levels. Although *C. raciborskii* has been ruled out as the main source of cylindrospermopsin occurrence, no "all-clear signal" can be given for this species for three reasons:

1. *Hazard potential:* In a predecessor project, *C. raciborskii* isolates were shown to induce significant toxicity that was not related to cylindrospermopsin. The resulting risk for humans still has not been determined. Further studies for structural determination of the toxin and extensive toxicity tests are therefore required.

2. *Relevant ecological issues: C. raciborskii* invasion can be expected to induce changes in our aquatic ecosystems. This will mainly affect biodiversity in that indigenous species will be supplanted. Relevant changes in biotic interactions and other processes cannot be excluded.

3. *Water management:* Water management measures are based on knowledge of (biotic) colonization patterns and processes in aquatic ecosystems (see above). Any changes in these parameters must be managed by adapting existing water purification and management measures accordingly. However, there is much less research data on

predicting the effects of trophic decline than on eutrophication, e.g., "What Vollenweider couldn't tell us" (Reynolds 1992).

5.3 Prognosis of further cyanobacteria development and toxin occurrence

The analyses of *C. raciborskii* population dynamics conducted in the present study provide fundamental new insights into the regulation of tropical species in temperate regions, the causes for its spread, and its further course of development.

Climate change (earlier rise in water temperatures over the course of the year) has promoted the establishment of *C. raciborskii* populations because it shifts the life cycle of this cyanobacterium to a period of improved growth conditions. If this trend in climate change continues, an increase in the size of *C. raciborskii* populations can be expected. Contrary to previous reports, we found that light, not temperature, is the growth-limiting factor for *C. raciborskii* growth during the vegetative phase. This finding should be considered in water management, because decreases in trophic level are always associated with an increase in water transparency and, thus, in light intensity. Trophic decline not only leads to a reduction of phosphorous concentrations but also, in many cases, to a reduction of nitrogen concentrations. As a nitrogen-fixing species, *C. raciborskii* would then have a competitive advantage over other phytoplankters.

If global warming continues, one can expect to see an increase in *C. raciborskii* populations and, in all probability, the further spread of this species within our region and further north. Two other neo-cyanobacteria (also of the order Nostocales) were also detected in our waters; their life cycle begins with the germination of akinetes, and they are also able to fix nitrogen. Consequently, a northward shift in the expansion of these species can generally be expected. Further studies should be conducted to determine whether the causes of spread of these species are the same as for *C. raciborskii*.

It must be assumed that the mechanisms that regulate *C. raciborskii* population dynamics, as determined in the present study, also apply to indigenous species of the order Nostocales. If the current trends in climate change continue and trophic state f water bodies declines further, then a general increase in species of the order Nostocales can be expected. The first signs of this have already been detected. This also applies to the *Aphanizomenon* species that produce cylindrospermopsin. An increase in

cylindrospermopsin concentrations in our waters is therefore likely. We emphasize that this is not an argument against trophic decline, as under more eutrophic conditions, other toxic cyanobacteria – largely the microcystin-producing species – prevail, and often with yet higher biovolumes and in consequence, very high levels of microcystin. Rather, this project outcome makes a case for developing a more comprehensive understanding of the factors that determine the occurrence of CYN-producing species and the levels at which to expect this toxin under given environmental conditions.

A Furthermore, a change in the species composition of cyanobacteria will ultimately lead to a change in occurrence also of other toxins, e.g neurotoxins potentially produced by some species of the order Nostocales, such as anatoxin and saxitoxin. This aspect must be included in hazard analysis..

Last but not least, we would like to emphasize that the CYLIN project outcomes on the current status and future course of toxic cyanobacterial development in the Berlin-Brandenburg region are likely to apply to similar water body types throughout the entire temperate climate zone, though local verification of this assumption would be desirable.

Chapter 6
Literature

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Appendix A
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Occurrence of the Cyanobacterial Toxin Cylindrospermopsin in Northeast Germany

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ABSTRACT: The frequent occurrence of the cyanobacterial toxin cylindrospermopsin (CYN) in the (sub)-tropics has been largely associated with cyanobacteria of the order Nostocales of tropical origin, in particular *Cylindrospermopsis raciborskii*. *C. raciborskii* is currently observed to spread northwards into temperate climatic zones. In addition, further cyanobacteria of the order Nostocales typically inhabiting water bodies in temperate regions are being identified as CYN-producers. Therefore, data on the distribution of CYN in temperate regions are necessary for a first assessment of potential risks due to CYN in water used for drinking and recreation. A total of 127 lakes situated in the north-eastern part of Germany were investigated in 2004 for the presence of the toxin CYN and the phytoplankton composition. The toxin could be detected in half of the lakes ($n = 63$) and in half of 165 samples ($n = 88$). Concentrations reached up to 73.2 μg CYN/g DW. CYN thus proved more widely distributed than previously demonstrated. The analyses of phytoplankton data suggest *Aphanizomenon* sp. and *Anabaena* sp. as important CYN producers in Germany, and confirm recent findings of *Aphanizomenon flos-aquae* as CYN-producing species frequently inhabiting water bodies in temperate climatic regions. The data shown here suggest that CYN may be an important cyanobacterial toxin in German water bodies and that further data are needed to assess this. © 2007 Wiley Periodicals, Inc. Environ Toxicol 22: 26–32, 2007.

Keywords: cylindrospermopsin; lakes; phytoplankton; *Cylindrospermopsis raciborskii*; *Aphanizomenon*; *Anabaena*

INTRODUCTION

Certain species of cyanobacteria are well-known for their biosynthesis of potent hepato- and neurotoxins, and knowl-

edge on e.g., their toxicity, production, and occurrence has enlarged enormously during the last decades (for a review see Chorus and Bartram, 1999). Cyanobacterial toxins can pose a risk for the health of animals and humans, when present in hazardous concentrations in water bodies used as drinking water source or for recreational purposes. This is often the case under eutrophic conditions leading to massive proliferation of cyanobacteria and thus enhanced toxin concentration. Eutrophication as well as mass developments of (toxic) cyanobacteria are a global problem, and are

reflected by reports about human and animal intoxications from cyanobacteria world-wide (e.g., Kuiper-Goodman et al., 1999).

One incidence of human intoxication occurred in 1979 at Palm Island (Australia) where adults and children suffered from hepatoenteritis after being supplied with drinking-water from a reservoir infested with cyanobacteria (Bourke et al., 1983). The investigations following the incidence revealed the toxin cylindrospermopsin (CYN) produced by the cyanobacterium *Cylindrospermopsis raciborskii* blooming in the reservoir as the causative agent (Hawkins et al., 1985). CYN is an alkaloid toxin and shows primarily severe hepatotoxicity in mice, though kidneys, heart, thymus, spleen and intestine are also affected (Falconer et al., 1998). Mutagenicity of CYN was proven *in vitro* and strong evidence exists for its *in vivo* carcinogenicity (Humpage et al., 2000; Falconer and Humpage, 2001; Shen et al., 2002). Based on its toxicological characteristics a guideline value of 1 μg/L CYN in drinking water has been proposed (Humpage and Falconer, 2003).

Data on the distribution of CYN on a global scale are still scarce, though occurrence of CYN has been documented for all continents either through monitoring of field samples and/or by isolation of CYN-producing cyanobacteria. The analysis of field samples revealed frequent occurrence of CYN in Australia, most probably due to increased monitoring following the Palm Island incidence (e.g., McGreggor and Fabbro, 2000). CYN occurrence in other countries such as New Zealand (Stirling and Quilliam, 2001), North America (Burns et al., 2002), Brazil (Carmichael et al., 2001), Italy (Manti et al., 2005), and Germany (Fastner et al., 2003) is so far reported only sporadically.

The occurrence of CYN in Australian field samples could in most cases be clearly attributed to the presence of *C. raciborskii*, and numerous CYN-producing strains of this species have been isolated from Australian water bodies (Hawkins et al., 1997; Saker and Griffiths, 2000). CYN-producing strains of *C. raciborskii* were also found in New Zealand, Thailand and Japan (Li et al., 2001a; Wood and Stirling, 2003; Chonudomkul et al., 2004). In Europe and Brazil so far only *C. raciborskii* strains not producing CYN have been isolated, yet these strains contained paralytic shellfish poisons or so far unknown toxins (Lagos et al., 1999; Bernard et al., 2003; Fastner et al., 2003; Saker et al., 2003).

In addition to *C. raciborskii* other CYN-producing cyanobacteria have been isolated worldwide: *Umezakia natans* in Japan (Harada et al., 1994), *Aphanizomenon ovalisporum* in Australia and Israel (Banker et al., 1997; Shaw et al., 1999), *Aphanizomenon flos-aquae* in Germany (Preußel et al., 2006), *Anabaena bergii* in Australia (Schembri et al., 2001) and *Raphidiopsis curvata* in China (Li et al., 2001b). *Aphanizomenon* and *Anabaena* are common members in cyanobacteria dominated phytoplankton communities in temperate regions and especially the *Aphanizomenon* spe-

cies can form dense blooms under favorable conditions (e.g., Kann and Welch, 2005). Therefore, it is likely that CYN is more widely distributed than previously expected and that high concentrations may occur. Evidence for this is documented already for *Aphanizomenon ovalisporum* from Israel and Australia (Banker et al., 1997; Shaw et al., 1999).

Despite the fact that CYN production could not be demonstrated for European *C. raciborskii* so far, this species must still be considered as CYN-producer in Europe, since for other cyanotoxins distribution of producing and nonproducing genotypes is usually global. In this regard the increasingly frequent and occasionally abundant occurrence of *C. raciborskii* in European water bodies is of great importance (Manti et al., 2005; Stüken et al., 2006).

CYN has been detected for the first time in Germany in 2000 in two lakes which was also the first record of this toxin in Europe (Fastner et al., 2003). In consequence this initial screening for CYN was conducted in 2004 to assess the distribution of CYN in a larger area. A total of 127 lakes situated in the northeastern part of Germany were investigated for the presence of CYN and their phytoplankton composition.

MATERIALS AND METHODS

Sampling Sites

The study was conducted in the lowland region of Northeast Germany (Fig. 2) with the sampling area extending from 51° 53′ 48″ N/14° 09′ 57″ E (South) to 54° 25′ 32″ N/ 12° 41′ 07″ E (North) and from 52° 44′ 29″ N/12° 15′ 23″ E (West) to 52° 04′ 32″ N/14° 28′ 55″ E (East). A total of 127 of the areas' more than 5000 natural and artificial water bodies were chosen, including a wide range of lake types differing in morphometry (maximum depth, volume, surface area), mixing regime (di- and polymictic) and trophic status (oligo- to hypertrophic).

Sampling

Lakes were sampled between June and September 2004. Around 110 lakes were sampled once, 14 twice (at the beginning and toward the end of the study period), and 3 monthly. Samples were taken at the deepest point of the lake, or alternatively at the middle of the lake. This was not possible at five sites where samples were taken either from bridges or piers.

At each sampling site, a phytoplankton net sample was taken by hauling the net vertically through the water column. Nets of 10 μm mesh size were used for oligotrophic and of 25 μm mesh size for meso- to hypertrophic water bodies. Two 50 mL subsamples of which one was preserved with formaldehyde (final concentration 4%) were taken and stored in opaque cool bags until arrival at the laboratory.
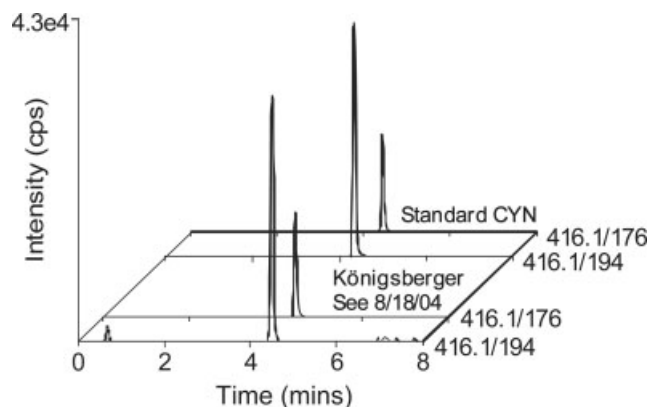
Fig. 1. LC-MS/MS ion chromatograms. Transitions 416/176 and 416/194 for standard CYN (top), and for lake Königsberger see 8/18/04 (bottom).

The subsamples for CYN analysis were frozen at −20°C after arrival in the laboratory.

Phytoplankton Analysis

The fresh phytoplankton material was analyzed within 48 h of sampling using light microscopes (Nikon OPTIPHOT-2 and Zeiss Axioscope) at 400–1000× magnifications.

Cyanobacteria were identified according to the following literature: Geitler, 1932; Huber-Pestalozzi, 1938; Komárek and Ettl, 1958; Horecká and Komárek, 1979; Hindák, 1992; Hindák, 2000.

Cylindrospermopsin Analysis

About 5 mg of lyophilized net-sample was extracted with 1 mL water according to Welker et al. (2002). CYN was determined in the aqueous extract by LC-MS/MS using an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Framingham, MA, USA) equipped with a turbo-ionspray interface.

The extract was separated using a Nova-Pak C_{18} column (150 × 4.6 mm^2, 5 μm particle size; Waters, USA) at 30°C and a flow rate of 0.8 mL min^{-1} with the following gradient program: 100% A for 1 min, ramped to 100% B in 5 min, held for 3 min and then to 100% A in 1 min and equilibrated for 7 min (solvent A: 1% methanol/deionized water, solvent B 60% methanol/deionized water, both solvents contained 5 mM ammonium acetate (Eaglesham et al., 1999).

The mass spectrometer was operated in the multiple reaction-monitoring mode (MRM) with a collision energy of 48 eV. For the determination of CYN the transitions m/z 416.1 (M+H^+) to 194 and 416.1/176 were monitored with a

dwell time of 0.2 s. Quantitation of CYN (purchased from Dr. A. Humpage, Australian Water Quality Centre, Salisbury, Australia) was achieved using the 416.1/194 transition with the 416.1/176 transition monitored as confirmation ion. The detection limit was 10 pg on column.

Map-Design

The map was designed with ArcView GIS 3.2 (ESRI) using geographical coordinates in Grad, Word Geodetic System 84 (WGS 84).

RESULTS AND DISCUSSION

CYN analysis by LC-MS/MS using the MRM mode enabled a highly selective analysis, an exemplary chromatogram of which is shown in Figure 1. Under the analytical conditions applied (i.e., amount of seston material extracted, injection volume etc.) a detection limit of around 0.1 μg/g DW for the crude extracts was achieved.

CYN was found to be widely distributed in water bodies situated in Northeastern Germany: 63 of 127 lakes sampled and 88 of 165 samples collected were CYN positive (Table I). CYN thus surprisingly seems to be as widespread as in Australian and North American water bodies (McGreggor and Fabbro, 2000; Burns et al., 2002). Furthermore, the frequency of CYN detection in German water bodies is similar to that of microcystin (Fastner et al., 1999), a cyanobacterial toxin which is so far considered to be the most frequent cyanotoxin worldwide. As with microcystin CYN was found to be evenly distributed across the study area with no obvious regions of increased occurrence (Fig. 2).

The CYN concentrations detected in the field samples ranged up to maximally 73.2 μg CYN/g DW with a median of 2.3 μg CYN/g DW (Fig. 3, Table II). Grouping samples into different concentration classes revealed that most samples contained between 1 and 10 μg CYN/g DW ($n = 43$) followed by the group with concentrations <1 μg CYN/g DW ($n = 29$) and those >10 μg CYN/g DW ($n = 16$) (Fig. 3, Table II). Overall, the concentrations are similar to those detected in the two lakes investigated in 2000 (Fastner et al., 2003), but are low compared to microcystin concentrations typically found in German seston samples (Fastner et al., 1999). In contrast, strains of *C. raciborskii*, *Aph.*

TABLE I. Summary statistics of water bodies and samples analyzed for CYN; number and share of CYN positive lakes/samples

	Total Number (n)	CYN Positive (n)	CYN Positive (%)
Water bodies	127	63	50
Samples	165	88	53

samples more than one genus and in case of *Anabaena* and *Aphanizomenon* usually more than one species could be found. However, species identification was not possible unequivocally in all cases. Thus, grouping of samples was performed on a genus level except for *C. raciborskii*.

Evaluation of the data revealed that the majority ($n = 61$) of the samples was composed of a combination of different *Aphanizomenon* and *Anabaena* species, 14 of *Aphanizomenon* species and *C. raciborskii*, and 26 samples of a mixture of *Aphanizomenon* and *Anabaena* species and *C. raciborskii* (Table II). CYN was detected in about half of these samples with the exception of the group comprising *Aphanizomenon*, *Anabaena,* and *C. raciborskii* with around 90% of the samples being CYN positive (Table II). In all these groups CYN concentrations were mostly below 10 μg/g DW.

The other samples showed either single occurrence of *Anabaena* sp. ($n = 10$) or *Aphanizomenon* sp. ($n = 19$) with half of them being CYN positive (Table II). This indicates that both *Aphanizomenon* and *Anabaena* species must be considered as prominent CYN-producers in Germany. This is substantiated by recent findings of Preußel et al. (2006) who showed *Aphanizomenon flos-aquae* isolated from two German lakes included in this study to produce CYN. Other known CYN producing species from these genera are *Aphanizomenon ovalisporum* (Banker et al., 1997; Shaw et al., 1999) and *Anabaena bergii* (Schembri et al., 2001). As most of the CYN producing cyanobacteria identified to date belong to the order Nostocales, it is likely that more species of Nostocales will be identified as CYN producers in the future. However, as other cyanotoxins such as microcystins and anatoxin-(a) have been found in species of different orders such as Chroococcales, Oscillatoriales, and Nostocales, and CYN has also been detected in *Umezakia natans* from the order Stigonematales (Harada et al., 1994), it can not be excluded that other orders may also comprise CYN-producing species responsible for CYN production in the field samples investigated. Thus far, however, CYN

Fig. 2. Study area including all water bodies sampled. Open circles: CYN not detected, filled circles: CYN detected.

ovalisporum, and *Aph. flos-aquae* were found to contain about 0.5–6 mg of CYN/g DW (Banker et al., 1997; Hawkins et al., 1997; Preußel et al., 2006). The reason for this discrepancy can be both a low abundance of CYN-producing species in the lakes, or—as in field populations usually toxin-producing and nonproducing genotypes coexist—low numbers of CYN producing genotypes or low levels of CYN produced by dominant genotypes. Another possible explanation is that not all of the CYN present in the lakes has been subjected to analysis, as culture and field studies showed that up to 95% of CYN can be extracellular (Chiswell et al., 1999; Shaw et al., 1999) and sampling with a plankton net excludes the liquid phase of water samples.

Phytoplankton analysis in this study concentrated primarily on the order Nostocales in which CYN producers have been identified previously. Numerous different species of *Anabaena*, *Aphanizomenon*, *Raphidiopsis,* and *C. raciborskii* were identified in the lakes investigated. In most

Fig. 3. CYN concentrations in decreasing order in the field samples as determined by LC-MS/MS analysis. $N =$ number of samples within each of three different concentration classes (> 10, 1–10, < 1 μg/g DW).

TABLE II. Summary of lakes and samples analyzed for CYN

	Total No. (n)	Samples (n) Consisting of Different Assemblages of Nostocales (Genera)											
		Ap/An	Ap/An/Cr	Ap	Ap/Cr	An	Ap/An/Ra	Ap/An/Cr/Ra	Ap/Cr/Ra	Ra	An/Ra	Ap/Ra	No/Nos
Total no. (n)	165	61	26	19	14	10	7	6	2	1	1	1	17
CYN n.d.	77	34	2	8	5	5	2	1	1	1	1	0	17
CYN Pos. [μg/g DW]													
>10[a]	16	3	5	3	2	0	0	2	0	0	0	1	0
1–10	43	9	16	4	5	2	4	2	1	0	0	0	0
<1	29	15	3	4	2	3	1	1	0	0	0	0	0

Total number of samples in different concentration categories and number of samples with a certain composition of cyanobacterial genera. Ap, *Aphanizomenon* sp.; An, *Anabaena* sp.; Cr, *Cylindrospermopsis raciborskii*; Ra, *Raphidiopsis*; Nos, Nostocales.

[a] Max concentration was 73.2 μg/g DW.

could not be detected in any of 17 samples in which cyanobacteria of the order Nostocales were absent (Table II).

Samples with *Raphidiopsis* in combination with the other genera were rarely observed, and sole occurrence of this genus was observed only once (Table II).

The role of *C. raciborskii* as CYN-producer in Europe is still uncertain. *C. raciborskii* strains isolated from lakes containing CYN in 2000 did not synthesize CYN (Fastner et al., 2003), and this applies also for other European strains (Saker et al., 2003; Bernard et al., 2003). Manti et al. (2005) reported the occurrence of CYN in two Italian lakes du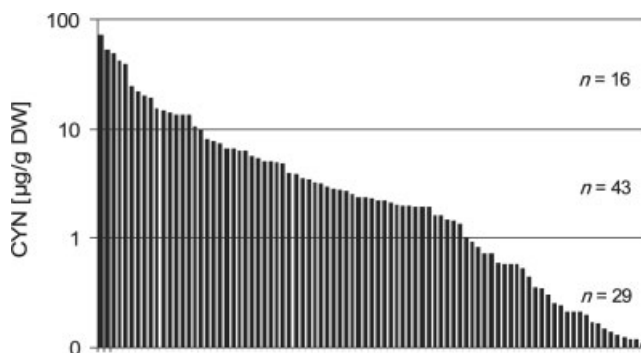ring the presence of *C. raciborskii*, but did not verify the producer organism by culture studies. As in none of the lakes studied here and containing CYN, *C. raciborskii* was the only potential CYN producer, these results show no indication of whether or not this species includes any CYN-producing genotypes in Germany. To clarify this question more strains of this species will have to be isolated and/or single filaments from the lake directly subjected to PCR analysis.

In conclusion, the high frequency of CYN detection in the investigated lakes and the indication of *Aphanizomenon* and *Anabaena* as CYN producers suggest that the occurrence of CYN should be given a greater attention in Germany and also in Europe. This study is a first step toward hazard analysis and risk assessment. While the water bodies included are sporadically or frequently used for bathing or fisheries, none of them are used as drinking-water reservoirs. Furthermore, the concentrations given here are related to biomass determined from net samples, while volumetric data are necessary for a risk assessment.

However, high CYN concentrations in the biomass of culture strains isolated from water-bodies included in this study, i.e., up to 6000 μg/g DW (Preußel et al., 2006) indicate a potential for high CYN concentrations if these genotypes attain high cell densities. CYN concentrations from <1 to several 100 μg/L reported from Australia, North America, and Italy show that in these settings CYN concentration during blooms of *C. raciborskii* and *Aphanizomenon*

ovalisporum are likely to exceed the proposed guideline value of 1 μg/L (Humpage and Falconer, 2003). As *Aphanizomenon* and *Anabaena* are often part of phytoplankton communities in temperate water bodies and may form blooms under favorable conditions, it is likely that also in German water bodies concentrations may be well above this proposed level. Quantitative data on intra- and extracellular CYN concentrations are therefore the important next step toward characterizing the CYN hazard.

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1 **Genetic characterisation of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria)**

2 **isolates from Africa and Europe.**

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18 Running title: Characterisation of *Cylindrospermopsis raciborskii* isolates

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27 **Abstract**

28 The invasive cyanobacterium *Cylindrospermopsis raciborskii* is increasingly spreading to

29 temperate freshwater habitats world wide and is of major concern due to its ability to produce

30 potent toxins. It is therefore important to understand the mechanisms behind the dispersal of

31 this species. Different hypotheses have been proposed to explain the phylogeography and

32 mechanisms underlying the recent expansion of *C. raciborskii* into temperate latitudes, but

33 there is still no conclusive evidence whether the obvious ecological success of *C. raciborskii*

34 is due to selection mechanisms, physiological tolerance, climatic change or radiation after the

35 last ice age. In this study, new isolates of *C. raciborskii* from Europe and Africa were

36 genetically characterized by sequencing the ITS1, PC-IGS, *nifH* and *rpoC1* genes and

37 compared to corresponding sequences of *C. raciborskii* available in GenBank in order to test

38 different phylogeographical hypotheses. The strains were also morphologically examined and

39 screened for production of the hepatotoxic cylindrospermopsin (CYN). We clearly

40 demonstrate that there are phylogenetic, morphological and toxicological differences between

41 the isolated strains. The phylogenetic analyses revealed a clustering of the strains due to

42 geographic origin. The ITS1 and *nifH* genes separated into American, European and

43 Australian-African groups, whereas the PC-IGS and *rpoC1* separated into American and

44 European/Australian/African groups. Our findings do not strongly support any of the existing

45 hypotheses on the phylogeography of *C. raciborskii*, and most likely a combination of these

46 hypotheses is the best approach to understand the evolution and dispersal of this species.

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52   Introduction

53   *Cylindrospermopsis raciborskii* (Woloszyńska) Seenayya et Subba Raju 1972 (Order

54   Nostocales) is a filamentous, heterocystous freshwater cyanobacterium commonly found in

55   tropical to subtropical climate regions. The species was first identified in a sample from a

56   pond in Java (Woloszyńska, 1912) and initially described as a tropical cyanobacterium

57   (Geitler and Ruttner, 1936), but is now increasingly expanding to temperate latitudes,

58   becoming prevalent in temperate freshwater habitats worldwide (for a review see Padisàk,

59   1997; Krienitz and Hegewald, 1996; Rücker *et al.*, 1997; Chapman and Schelske, 1997;

60   Druart and Briand, 2002; Wood and Stirling, 2003; Hamilton et al., 2005). In some

61   geographical areas, *C. raciborskii* is recorded as rapidly expanding (Stüken et al., 2006), and

62   it is regarded as an invasive species (Chapman and Schelske, 1997).

63   The increased proliferation of *C. raciborskii* is of particular concern due to the ability

64   of some strains to produce various toxins including different variants of the hepatotoxic

65   alkaloid cylindrospermopsin (CYN; Hawkins et al., 1985; Li et al., 2001; Saker and Neilan,

66   2001). CYN has been implicated in a severe incident of human illness (Bourke et al., 1983;

67   Hawkins et al., 1985) and caused cattle mortality (Saker et al., 1999). CYN producing strains

68   of *C. raciborskii* have been found in Australia and Asia (Li et al., 2001; Hawkins et al., 1997;

69   Saker et al., 1999), whereas no CYN production has been found in strains isolated from

70   Europe, America and Africa (Saker et al., 2003; Fastner et al., 2003; Valério et al., 2005).

71   However, Brazilian strains of *C. raciborskii* have been reported to produce paralytic shellfish

72   poisoning (PSP) toxin (Lagos et al., 1999), many of the European strains are found to be

73   hepatotoxic in the mouse bioassay (Bernard et al., 2003; Saker et al., 2003; Fastner et al.,

74   2003), and recently, Mohamed (2007) reported that Egyptian strains of *C. raciborskii* showed

75   hepatotoxic effects in the mouse bioassay. *C. raciborskii* can pose a significant threat to

76   animal and human health, and it is important to understand the mechanisms of its currently

77   increasing expansion and dispersal.

78   Different hypotheses have been proposed to explain the phylogeography of the

79   proliferating *C. raciborskii*: (i) there is a primary evolutionary centre in the deep lakes in

80   tropical Africa and a dispersal to the Australian, Eurasian and American continents (Padisàk,

81   1997), (ii) there is a secondary evolutionary centre in Australia with a more recent

82   colonization to the Eurasian and subsequent radiation to the European continent (Padisàk,

83   1997), or (iii) There is a recent colonization across the American and European continents

84   from warm refuge areas within the continents (Gugger et al., 2005). Further, different

85   hypotheses to explain the mechanisms underlying the recent expansion of *C. raciborskii* into

86   temperate latitudes have been suggested: (i) climatic change increases water temperatures and

87   allows strains of tropical origin to spread to temperate habitats, (ii) the species has ability to

88   tolerate a wide range of climatic conditions, or (iii) selected ecotypes with lower temperature

89   and light requirements are spreading to temperate areas (Briand et al., 2004).

90   Ecophysiological studies with strains of *C. raciborskii* from different tropical and temperate

91   areas revealed that all isolates had very similar light requirements for growth (Briand et al.,

92   2004) suggesting that the species is able to colonize temperate habitats due to the ability to

93   tolerate different environmental conditions in combination with global warming (Briand et al.,

94   2004). Wiedner et al. (2007) designed a model based on studies of the long time population

95   dynamics of *C. raciborskii* in two German water bodies and found that an earlier rise in water

96   temperature associated with climatic change has promoted the proliferation into temperate

97   areas. Chonudomkul et al. (2004) found different strains of *C. raciborskii* from Japan and

98   Thailand to be either adapted or not-adapted to low temperatures, concluding that ecotype

99   selection plays an important role in the dispersal of this species.

100   Molecular phylogeny has become a powerful tool in elucidating evolutionary patterns,

101   and a number of genes can be used as genetic markers to reveal the degree of genetic

102   similarity and distribution among cyanobacterial populations. Strains of *C. raciborskii*

103   originating from Australia, Germany, Hungary, Brazil and the US were compared based on

104   genetic analyses of the *nifH* (dinitrogenase reductase) gene (Dyble et al., 2002) and by the

105   16S rDNA gene (Neilan et al., 2003), both separating the strains in three groups, American,

106   European and Australian. However, based on the phycocyanin intergenic spacer between the

107   bilin subunit genes *cpc*B and *cpc*A (PC-IGS), the strains separated in two groups, American

108   and European-Australian (Dyble et al., 2002). Recently, Gugger et al. (2005) studied the

109   genetic diversity among strains of *Cylindrospermopsis* sp. isolated from Europe, America,

110   Australia and Africa by sequencing the 16S internal transcribed spacer (ITS) of the ribosomal

111   operon. The strains separated according to geographic origin into American, European and

112   Australian-African groups. The studies concerning the phylogeny of *C. raciborskii* have used

113   many of the same strains although using different genetic markers, e.g. the same German,

114   Hungarian, Portuguese, Australian, Brazilian and US strains have been used by Dyble et al.

115   (2002) and Neilan et al. (2003), and Gugger et al. (2005) have used the same German and

116   Hungarian strains as Neilan et al. (2003) and the same Australian strains as Wilson et al.

117   (2000) (Table 1). More information on new strains of *C. raciborskii* is therefore of major

118   importance to understand the phylogeography of this invasive species.

119   Therefore, we have isolated new strains of *C. raciborskii* from Europe and Africa,

120   areas highly relevant to bring more insight to the phylogeography of this invasive species.

121   The European strains were isolated from Lake Zierkersee in Northern Germany, regarded as

122   the species northernmost European habitat, and these strains therefore represent the invasive

123   population of *C. raciborskii*. The African strains were isolated from two tropical water bodies

124   in Uganda, Kazinga Channel between Lakes George and Albert and Murchison Bay in Lake

125   Victoria, representing the area considered to be the possible evolutionary centre of the species

126   *C. raciborskii*. The new isolates of *C. raciborskii* were genetically characterised with respect

127   to ITS1, PC-IGS, *nifH* and *rpoC1* (RNA polymerase) genes and compared to corresponding

128   sequences of *C. raciborskii* available in the GenBank. We complemented the study with

129   morphological analyses of the strains, and also screened the strains for production of CYN,

130   both by checking for the presence of the genes for encoding the CYN biosyntheses, peptide

131   synthase (PS) and polyketide synthetase (PKS) gene fragments, and chemically by analysing

132   the strains with Liquid Chromatography - Mass Spectrometry (LC-MS/MS).

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134

135   **Materials and methods**

136

137   *Strain isolation and culture conditions*

138   The *Cylindrospermopsis* sp. strains were isolated from water samples from Ugandan

139   and German fresh water bodies (Table 2). The cyanobacterial strains were isolated either by

140   capillary isolation, agar plate spreading or by serial dilution, and placed in capped tubes

141   containing 10 mL culture medium. The growth medium used for isolation and cultivation of

142   all Ugandan *Cylindrospermopsis* strains was the Z8 medium (Kotai, 1972). The German

143   *Cylindrospermopsis* strains were isolated and grown in BG-11 medium (Rippka et al., 1979).

144   All strains included in this study were maintained at 20 °C and illuminated under a 12h:12h

145   light-dark cycle with an average photon flux density of 20 μmol photons $m^{-2}s^{-1}$.

146   Morphological observations were made using a Nikon Optiphot 2 light microscope (Nikon,

147   Tokyo, Japan).

148

149   *DNA extraction*

150   Fifty millilitres of culture material were centrifuged and total genomic DNA was

151   extracted and purified using Dynabeads DNA DIRECT System (Invitrogen/Dynal Biotech,

152   Oslo, Norway) according to the manufacturer's manual.

153

154   *PCR amplification and sequencing*

155   PCR amplification was performed using the Taq PCR Core Kit (Qiagen GmbH,

156   Hilden, Germany) in a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, USA). For

157   the amplification of PC-IGS, the primers PCβ-F and PCα-R (Neilan et al., 1995) were used,

158   the ITS1 was amplified using the primers 322 and 340 for ITS rDNA (Iteman et al., 2000),

159   and the *nifH* and *rpoC1* genes were amplified using the primers *nifH*f and *nifH*r and *rpoC1*f

160   and *rpoC1*r respectively (Gugger et al., 2005). PCR was carried out in a final volume of 50

161   µL containing 5 µL of 10× PCR reaction buffer, 5 µL of 25 mM MgCl$_2$, 5 µL of 2 mM dNTP,

162   1 µL of 10 µM of each primer, 0.2 µL of 5 U *Taq* DNA polymerase and 1 µL DNA and

163   water. The amplification of PC-IGS was performed with an initial denaturation step of 94 ºC

164   for 5 min, followed by 30 cycles of 94 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 1 min,

165   followed by a final extension step at 72 ºC for 10 min. The amplifications of ITS1, *nifH* and

166   *rpoC1* were performed with an initial denaturation step at 97 ºC for 5 min, followed by 30

167   cycles of 97 ºC for 15 sec, 48 ºC for 30 sec and 72 ºC for 1 min, and then a final extension

168   step at 72 ºC for 5 min. The PCR products were separated by 1.5 % agarose gel

169   electrophoresis in TAE buffer according to standard protocols (Sambrook et al., 1989). For

170   photo documentation an AlphaImager 2200 MultiImage Light Cabinet (Biozyme Diagnostik

171   GmbH, Oldendorf, Germany) was used.

172   PCR products for sequencing were purified through Qiaquick PCR purification

173   columns (Qiagen, Hilden, Germany) and DNA was redissolved in elution buffer according to

174   the manufacturer's protocol. The amplification of the ITS1 gave three products which were

175   separated by agarose gel electrophoresis. The short and middle band represented the ITS1-S

176   and ITS1-L respectively and were excised from the gel and subsequently purified through

177   Qiaquick PCR purification columns. The ITS1-S and ITS-L were cloned separately in the

178   pGEM-T vector (Promega, Madison, WI, USA) according to the manufacturer protocol. The

179   recombinant plasmids were purified using Wizard Plus SV Minipreps DNA purification

180   system (Promega, Madison, WI, USA). Four microlitres of purified DNA were sequenced

181   with Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems,

182   Darmstadt, Germany) as described in the user guide of the kit. Both strands were sequenced

183   with an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, Darmstadt, Germany.

184   The sequencing of the recombinant plasmids was performed using the primers M13f and

185   M13r binding to sites in the vector. The other PCR-products were sequenced using the same

186   primers as for the amplification.

187

188   *Sequence alignment and phylogenetic analysis*

189   The DNA sequences of *C. raciborskii* were aligned using the BioEdit sequence

190   alignment Editor Version 5.0.6 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the

191   sequence alignment was corrected manually. Gaps, highly variable and ambiguous positions,

192   where a proper alignment was impossible, were excluded from the alignments. For ITS1-L

193   the alignment consisted of 540 bp, the *nifH* comprised 297 bp, the *rpoC1* comprised 380 bp,

194   and in the case of PC-IGS the alignment consisted of 523 bp.

195   MrModeltest (Nylander, 2002) based on Modeltest (Posada and Crandall, 1998) was

196   used to find the best substitution model for each alignment. Phylogenetic trees were

197   constructed by using the maximum likelihood method in PHYML (Guindon and Gascuel,

198   2003), by the neighbour joining method on Jukes and Cantor using PAUP* 4.0 (Swofford,

199   2002) and by MrBayes v3.0 (Huelsenbeck and Ronquist, 2001). In the PHYML-analyses,

200   both proportion of invariable sites and the gamma distribution parameter were estimated. The

201   number of categories was set to 4. Different models and parameter values were favoured for

202   the different genetic markers; ITS1-L: GTR+G model of substitution, gamma shape

203   parameter: 0.477; *nifH*: HKY model of substitution, transition/transversion ratio: 4.711;

204   *rpoC1*: HKY+G model of substitution, gamma shape parameter: 0.878, transition/transversion

205   ratio: 4.560; PC-IGS: K2P+G model of substitution, gamma shape parameter: 0.012,

206   transition/transversion ratio: 4.654. Node support was statistically evaluated with bootstrap-

207   analysis (number of replicates =1000). In the MrBayes analyses the gamma distribution of site

208   rates was approximated using four rate categories with equal probability. Other prior settings

209   were set to default values. The Markov chain Monte-Carlo (MCMC) chains lasted for

210   10,000,000 generations, and trees were saved each 100 generations. After burn-in, which was

211   based on visual inspection of the stationary phase of the MCMC chains, the remaining trees

212   were used for calculating the consensus tree and posterior probability values. In the case of

213   MrBayes, the parameter values for the best substitution model were imported from

214   MrModeltest. Sequences of *Anabaena* sp. were used as outgroups in the phylogenetic

215   analyses; ITS1-L, strain BC-Ana-0026 (AJ496732), *nifH*, strain PCC 9109 (AY768419),

216   *rpoC1*, strain ANA118C (AF159373) and PC-IGS, strain PCC 9109 (AY768473). The

217   sequence data were submitted to the EMBL database and the accession numbers are listed in

218   Table 2.

219

220   *PCR amplification of PS and PKS gene fragments*

221   The peptide synthase (PS) gene was amplified using the primers M13 and M14

222   (Schembri et al., 2001) and the polyketide synthetase (PKS) gene was amplified using the

223   primers M4 and K18 (Fergusson and Saint, 2003). Both analysis were carried out in a final

224   volume of 20 µL reaction mixture containing 2 µL of 10× PCR reaction buffer, 0.8 µL of 25

225 mM MgCl$_2$ (Qiagen, Hilden, Germany), 0.4 µL of 2 mM dNTP, 1 µL of 10 µM of each

226 primer, 0.2 µL of 5 U *Taq* DNA polymerase (Qiagen, Hilden, Germany) and 0.5 µL DNA and

227 water. The initial denaturation step was 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 10

228 sec, 55 ºC for 20 sec and 72 ºC for 1 min, and then a final extension step at 72 ºC for 7 min.

229 The PCR products were separated by 1.5 % agarose gel electrophoresis in TAE buffer

230 according to standard protocols (Sambrook et al., 1989). The etidiumbromide stained gels

231 were visualized and photographed under UV illumination in an AlphaImager 2200

232 MultiImage Light Cabinet (Biozyme Diagnostik GmbH, Oldendorf, Germany) Standard DNA

233 fragments (Peqlab, Erlangen, Germany) were used as size markers.

234

235 *Screening for cylindrospermopsin and deoxy-cylindrospermopsin by LC-MS/MS*

236 To extract cylindrospermopsins from *C. raciborskii*, we adapted an already established

237 method that uses biomass collected on filters as basis (Rohrlack et al., 2003). In contrast to

238 the original method, deionized water was applied as extraction agent. Briefly, *C. raciborskii*

239 cultures were filtered individually through 25 mm GF/C filters which, after lyophilisation,

240 were extracted with water in a two step procedure. All extracts were centrifuged at 12000 x g

241 for 10 minutes to remove particles.

242   The screening of extracts was done using liquid chromatography with mass

243 spectrometric detection (LC-MS/MS). The instrumental setup included a Waters Acquity

244 UPLC System equipped with a Waters Atlantis C18 column (2.1 x 150 mm, 5 µm particle

245 size) and directly coupled to a Quattro Premier XE tandem quadrupole MS/MS detector. The

246 UPLC system was set to deliver a linear gradient from 1 % to 25 % MeOH in water, both

247 containing 0.1 % formic acid, within 5 minutes at a flow rate of 0.25 mL min^{-1}. The column

248 and auto sampler temperatures were 20 and 4 ºC, respectively. The MS/MS detector was run

249 in positive electrospray multiple reaction monitoring mode with the ions 416.1 and 400.1 Da

250    $[M+H]^+$ (cylindrospermopsin, deoxy-cylindrospermopsin) as parents and the typical

251   indicative fragments 176.1 and 194.1 Da as daughters. The cone voltage was 50 V, the

252   collision energy 50 eV and the dwell time 0.1 seconds. Other settings included a source

253   temperature of 120ºC, a desolvation temperature of 350 ºC, a drying gas flow rate at 800 l

254   hour^{-1}, a gas flow at the cone of 50 l hour^{-1}, and standard voltages and energies suggested by

255   the manufacturer for the ESI+ mode. Nitrogen continuously delivered by a nitrogen generator

256   (Parker Blaston, model NG 11) served as drying, nebulising and cone gas. The entire system

257   was calibrated and tested using a CYN standard purchased from Sigma-Aldrich. The

258   detection limit for the LC-MS/MS method was found to be 0.1 ng CYN per mL extract at an

259   injection volume of 25 μL.

260

261

262   **Results**

263

264   *Strain morphology*

265   All strains studied shared the common morphological traits corresponding to the description

266   of *C. raciborskii* (Horecká and Komárek, 1979). The strains isolated from Ugandan water

267   bodies had flexuous trichomes, terminal heterocysts and lacked akinets. The exception was

268   the strain NIVA-CYA 506 which had a mix between flexuous and straight trichomes. All the

269   strains isolated from Germany had straight trichomes, terminal heterocysts and possessed

270   akinets (Fig. 1, Table 2).

271

272   *Phylogenetic analyses*

273   The amplification of the ITS1 rDNA region with the primers 322 and 340 revealed

274   polymorphism both in number and length of the obtained amplicons. Two of the amplified

275 products corresponded to the short and long ITS1 with flanking regions, ranging from 395

276 (ITS1-S) and 589-590 (ITS-L) respectively. The ITS1-L sequences contained two tRNA

277 genes and had more informative sites than the ITS1-S. Variation within the ITS-L sequences

278 reflected a distinct geographic grouping of the isolates. Our isolates from Europe were

279 genetically identical and the African strains were >99.4% similar to each other and differed

280 from the European sequences by 3%. The maximum likelihood tree including the strains

281 sequenced in this study and sequences obtained from the GenBank showed a distinct

282 clustering of *C. raciborskii* based on geographic origin supported by moderate to high

283 bootstrap values (Fig. 2). Neighbor-joining analysis and the MrBayes inference gave trees

284 congruent with the maximum likelihood tree. The African strains formed a cluster with the

285 strains from Australia, the European strains formed a second cluster, and the American strains

286 formed a third cluster (Fig. 2).

287 The sequence similarity of the amplified *nifH* gene was 100% both within the African

288 isolates and within the European isolates of *C. raciborskii*. The sequences from the two

289 different continents were >99.3% similar to each other. The maximum likelihood analyses

290 comparing our sequences with sequences obtained from the GenBank, confirmed a distinct

291 clustering of sequences from different geographical regions but with moderate bootstrap

292 values (Fig. 3). The three phylogenetic approaches produced similar tree topology. We found

293 the same clustering pattern for *nifH* as for ITS1-L.

294 For the *rpoC1* gene the degree of sequences similarity from Africa and Europe were

295 high (>98.7%). The phylogenetic analyses with our sequences together with the sequences

296 obtained from the GenBank did not show a separation of the sequences due to geographic

297 origin (data not shown). The three phylogenetic approaches produced similar tree topology.

298 Only the America strains were clearly separated from the strains from other geographical

299 areas.

300   For the amplified phycocyanin operon (PC-IGS) sequences of *C. raciborskii* isolates,

301   the European sequences were 100% identical and the African isolates were >99.6% similar to

302   each other. The sequences from the two different continents only differed by 0.04%. The

303   phylogenetic analysis comparing our strains with strains obtained from the GenBank did not

304   separate the European, Australian and African isolates, although the isolates from Africa

305   formed a sub cluster within cluster I, supported by moderate to high bootstrap values (Fig. 4).

306   The American strains were separated into two clusters, America I and II. The Brazilian strains

307   and the strains Florida G and F formed two sub clusters within America I, whereas the strains

308   Florida D and I were delineating early, forming a distinct cluster (America II). Neighbor-

309   joining analysis and the MrBayes inference gave trees congruent with the maximum

310   likelihood tree.

311

312   *Toxin analysis*

313   The possible existence of PKS and PS gene fragments implied in the cylindrospermopsin

314   production was checked by PCR. The strain *C. raciborskii* CR 3 (Saker and Griffith, 2000)

315   was used as a positive control and the *C. raciborskii* strain CYLI 19 (Fastner et al., 2003) was

316   applied as a negative control. None of the isolated strains from Africa and Europe yielded any

317   PCR product, showing that the strains lacked the cylindrospermopsin synthetase gene

318   fragments.

319   All *C. raciborskii* strains were tested for the occurrence of cylindrospermopsin and

320   deoxy-cylindrospermopsin by LC-MS/MS. No traces of these compounds were found in any

321   of the strains, although a sensitive method was used that could detect cylindrospermopsin at

322   nanogram level.

323

324

Discussion

325

326   The results from this study clearly demonstrate that there are phylogenetic,

327 morphological and toxicological differences between the populations of *C. raciborskii* from

328 different geographical regions. The phylogenetic analysis of the ITS1 and *nifH* loci of our

329 sequences together with sequences obtained from the GenBank showed that the *C. raciborskii*

330 strains from the same continent were more closely related to each other than the strains

331 originating from different continents. A distinct clustering of an African-Australian group,

332 one European group and an American group was found, hence consistent with earlier findings

333 with *nifH* and ITS1 and 16S rDNA (Dyble et al., 2002; Neilan et al., 2003; Gugger et al.,

334 2005). The African strains from Uganda clustered with the strains from Senegal, having

335 identical *nifH* sequences and a sequence similarity of 98.9% for ITS1-L. In contrast, the

336 *rpoC1* gene only separated the American strains from the *C. raciborskii* strains from other

337 geographical regions, confirming the same clustering pattern for *rpoC1* found by Gugger et

338 al. (2005). In comparison with the other genetic markers, PC-IGS showed a different

339 clustering pattern of the *C. raciborskii* strains. The American strains formed two clusters,

340 displaying a higher genetic variation within the isolates from America. The other strains

341 formed one cluster, with a sub cluster containing the African strains, thus indicating a slight

342 delineation of the strains from this continent. Dyble et al. (2002), originally sequencing the

343 mentioned American strains with PC-IGS, described a separation of the sequences from the

344 American strains into a distinct cluster, although the phylogenetic tree clearly show the same

345 clustering pattern as shown in this study. With exception of PC-IGS, all the genetic markers

346 used in the phylogenetic studies on *C. raciborskii* first delineate a distinct cluster containing

347 the American strains. This would indicate an early evolutionary splitting of the American

348 population of *C. raciborskii* from the other populations. Thus, PC-IGS interestingly shows a

349 different clustering pattern of the *C. raciborskii* strains in comparison with the other genetic

350   markers. Although PC-IGS successfully has been used to differentiate other cyanobacterial

351   species from different geographical regions (Bolch et al., 1996), it seems like the ITS1-L is

352   the most appropriate tool for the phylogenetic analysis of *C. raciborskii*. The widely used 16S

353   ribosomal DNA (rDNA) gene displays low intragenic variability and is unsuitable for

354   studying the relationship within species level (e.g. Neilan, 1997; Case et al., 2007).

355   Our *C. raciborskii* strains from Germany and Uganda possessed two morphotypes,

356   having straight or flexuous trichomes, and separated morphologically with respect to

357   geographic origin. The wide distribution of *C. raciborskii* coincides with a large degree of

358   morphological variation (Komárek and Kling, 1991; Cronberg and Komárek, 2004). Straight,

359   curved and flexuous morphotypes occur in most parts of the world (Chapman and Schelske,

360   1997; Saker et al., 1999; Berger et al., 2006), though only straight morphotypes have been

361   observed in Europe (e.g. Padisák, 1997; Couté et al., 1997; Stüken et al., 2006, and this

362   study). The German strains produced akinetes, whereas no akinete production was observed

363   in the Ugandan isolates. In general, akinets are rarely observed among *C. raciborskii* in

364   tropical areas where the strains can persist in its vegetative form throughout the year (Saker et

365   al., 2003). Strains growing in temperate areas, on the other hand, are more likely to develop

366   akinetes as an adaptation to lower growth temperatures and ability to survive during winter

367   periods.

368   CYN could not be detected in any of the nine *C. raciborskii* strains isolated from

369   Uganda and Germany. The method used for analyses of CYN by LC-MS was very sensitive

370   with a detection limit of 0.1 ng CYN per mL extract and thus, even trace elements of CYN

371   could have been detected when present. Further, the PCR conducted for the PS or PKS gene

372   fragments did not yield amplicons for any of the strains, confirming the results of the LC-MS

373   analysis. We are therefore confident that the strains are non-CYN producers, confirming

374   earlier findings that no CYN producing strains of *C. raciborskii* have been isolated from

375   European waters (Fastner et al., 2003; Valério et al., 2005). Less is known about the toxicity

376   of *C. raciborskii* in African water bodies. Berger et al. (2006) have isolated strains of *C.*

377   *raciborskii* from Lake Guiers in Senegal, but none of the strains isolated were CYN

378   producing and they all lacked the PKS/PS-genes. Strains of *C. raciborskii* from Egypt showed

379   hepatotoxic effects in mouse bioassays (Mohamed, 2007). Our strains isolated from Ugandan

380   water bodies are not CYN producers, and to our knowledge no CYN producing strains of *C.*

381   *raciborskii* have yet been isolated from African water bodies. There are, however, a limited

382   number of strains isolated from African water bodies, and more work need to be done in order

383   to reveal the possible presence of CYN producing strains of *C. raciborskii* on this vast

384   continent.

385   Our findings do not strongly support any of the existing hypotheses on the

386   phylogeography of *C. raciborskii*, and a combination of these hypotheses is therefore most

387   likely the best approach to understand the evolution and dispersal of this species. Gugger et

388   al. (2005) suggest that the recently observed proliferation of *C. raciborskii* in temperate areas

389   comes from a colonisation from warm refuge areas on each continent rather than a

390   colonisation from main evolutionary centres in Africa and Australia as proposed by Padisák

391   (1997). The multiple glaciations and climatic and environmental fluctuations during the

392   Pleistocene are commonly believed to have structured the present populations by forcing

393   species to adjust the distribution areas according to their adaptive ability. A radiation of *C.*

394   *raciborskii* from warm refuge areas within the continents would require a warming

395   environment. Studies have found that European strains have the same temperature (Saker and

396   Griffiths, 2000; Briand et al., 2004; Chonudomkul et al., 2004) and light (Briand et al. 2004)

397   demands for optimal growth as tropical strains. This is in agreement with the requirement of a

398   warmer environment in temperate areas for this species to proliferate. On the other hand,

399   Chonudomkul et al. (2004) showed that strains of *C. raciborskii* from Thailand could be

400 separated into adaptive and non-adaptive groups to low temperature and that *C. raciborskii*

401 strains from Japan were low temperature adaptive, hence, concluding that different strains

402 have different genotypes that enable the population as a whole to adapt to temperature

403 variations. Chonudomkul et al. (2004) suggested that the dispersal of *C. raciborskii* could be

404 explained through selection mechanisms of ecotypes with different temperature tolerance. The

405 observation that temperate strains produce more akinetes under culture conditions than

406 tropical strains (Saker et al., 2003) supports this latter theory. The *C. raciborskii* strains also

407 differ morphologically and toxicologically according to geographical origin, but these

408 differences are not always in agreement with the phylogeography. The morphotypes do not

409 coincide with the phylogeny as the straight Australian morphotype clusters with the straight

410 Australian and flexuous African morphotype for all genetic markers. Previous genetic

411 comparisons of the different morphotypes of *C. raciborskii* have shown contradictory results.

412 Saker et al. (1999) found coiled and straight morphotypes of Australian isolates of *C.*

413 *raciborskii* to be nearly genetically identical based on 16 S rDNA, *nifH* and PC-IGS

414 sequences (Saker et al., 1999; Dyble et al., 2002). The coiled and straight morphotypes of *C.*

415 *raciborskii* from Florida were, however, genetically separated based on PC-IGS and *nifH*

416 (Dyble et al., 2002). There is also not a clear relationship between the phylogeny and

417 toxicology. The African non-CYN producing strains clusters with the Australian CYN-

418 producing strains for all genetic markers, and the Brazilian PSP producing strains clusters

419 together with strains from the US which neither produce CYN nor PSP. Although the genetic

420 similarity supports a recent invasion of *C. raciborskii* from Africa to the Australian continent,

421 the toxicological data undermine this hypothesis. However, the lack of conformity between

422 phylogeny, morphology and toxicology gives indication of a radiation of *C. raciborskii* within

423 the continents, rather than a recent exchange between continents.

424   Although no CYN-producing strains of *C. raciborskii* are found in Europe, recent

425   studies reveal that CYN is produced by other species of cyanobacteria (for a review see Spoof

426   et al., 2006). It is therefore worth considering weather horizontal gene transfer or

427   recombination events may play a role in the distribution of CYN-producers among

428   cyanobacteria. The occurrence of horizontal gene transfer and recombination events has been

429   shown to occur within the Microcystin (*Mcy*) operon (Christiansen et al., 2003; Mikalsen et

430   al., 2003; Kurmayer and Gumpenberger, 2006) and is thought to play a role in the sporadic

431   distribution of microcystin producers among cyanobacteria. Similar patterns may be found for

432   the CYN syntethase genes and can explain the conflict between toxicology and phylogeny.

433   This would again support the theory that the species have been exchanged between

434   continents.

435   *C. raciborskii* shows a surprisingly low degree of diversity in comparison with other

436   groups of cyanobacteria. Studies on *Microcystis aeruginosa* (e.g. Bittencourt-Oliveira et al.,

437   2001; Wilson et al., 2005) and *Planktothrix* sp. (e.g. Mbedi et al., 2005; Kurmayer and

438   Gumpenberger, 2006) have revealed that even the genetic variation within a population in a

439   single water body is considerable. In general, the spreading of cyanobacteria within and

440   between continents is most likely a dynamic process enabling a continuous changing of the

441   genotype composition of a population. A higher degree of diversity among the population of

442   *C. raciborskii* in tropical areas is likely, and more extensive studies and isolation of new

443   strains will probably reveal a higher degree of diversity within the tropical populations. To

444   obtain a clear relationship between genetic variation and geography, several new strains need

445   to be isolated from different parts of the world, and it is indeed necessary to use a polyphasic

446   approach including phylogenetic, morphological and toxicological analyses.

447   In conclusion, there is still uncertain evidence whether the obvious ecological success

448   of *C. raciborskii* is due to selection mechanisms (Chonudomkul et al., 2004), physiological

449 tolerance (Briand et al., 2004), climatic change (Wiedner et al., 2007) or radiation after the

450 last ice age (Gugger et al., 2005). Most likely, a combination of coexisting mechanisms

451 enables *C. raciborskii* to proliferate into temperate areas, thus underline the complexity of the

452 phylogeography of this species.

453

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Tables

Table 1 Gene accession numbers, morphology and toxicology of *Cylindrospermopsis raciborskii* strains available in the GenBank

Strain	Origin	ITS1-L	*rpoC1*	*nifH*	PC-IGS	Morphology	CYN	Reference
PMC98.14	France	AJ582268	AJ582286	AJ582094		NA	NA	Gugger et al., 2005
PMC99.12	France	AJ582273	AJ582287	AJ582095		NA	NA	Gugger et al., 2005
PMC114.02	France	AJ582279		AJ582096		NA	NA	Gugger et al., 2005
Germany 1	Germany	AJ582281	AJ582288	AF426776	AF426797	Straight	-[b]	Gugger et al., 2005, Dyble et al., 2002,[e]
Germany 2	Germany			AF426777	AF426798	Straight	-[b]	Dyble et al., 2002,[e]
Bal 5	Hungary	AJ582280		AF426769	AF426790	Straight	-[b]	Gugger et al., 2005, Dyble et al., 2002,[e]
Bal 6	Hungary			AF426770	AF426791	Straight	-[b]	Dyble et al., 2002,[e]
Caia	Portugal			AF426773	AF426794	Straight	-[b]	Dyble et al., 2002,[e]
4799	Portugal			AF426767	AF426786	Straight	-[b]	Dyble et al., 2002,[e]
Marau 1	Portugal			AF426779	AF426801	Straight	-[b]	Dyble et al., 2002,[e]
CYP-030B	Australia	AJ582277	AJ582089			NA	NA	Gugger et al., 2005
CYP-023	Australia	AJ582278				Coiled/ Straight	NA	Gugger et al., 2005
CYP-026J	Australia	AJ582269	AJ582090			Straight	NA	Gugger et al., 2005,[c]
Aqc	Australia			AF426782	AF426788	Coiled	-	Dyble et al., 2002,[e]
Aqs	Australia			AF426783	AF426789	Straight	-	Dyble et al., 2002,
Sdc	Australia			AF426780	AF426803	Coiled	+[b]	Dyble et al., 2002,[e]
Sds	Australia			AF426781	AF426804	Straight	+[b]	Dyble et al., 2002,[e]
Mk	Australia				AF426802	Straight	-	Dyble et al., 2002
Goon	Australia				AF426799	Straight	+[b]	Dyble et al., 2002,[e]
LJ	Australia			AF426778	AF426800	Straight	-[b]	Dyble et al., 2002,[e]
AWT205	Australia			AF426768	AF426787	Straight	+[b]	Dyble et al., 2002,[e]
PMC115.02[a]	Senegal	AJ582272	AJ582093	AJ582101		Straight	-	Gugger et al., 2005,[f]
PMC117.02	Senegal	AJ582271	AJ582092	AJ582100		Flexuous	-	Gugger et al., 2005,[f]

PMC118.02	Senegal	AJ582270	AJ582091	AJ582099		Flexuous	-	Gugger et al., 2005, [f]
Florida D	USA			AF426774	AF426795	Coiled	NA	Dyble et al., 2002, [e]
Florida F	USA			AF426784		Straight	NA	Dyble et al., 2002, [e]
Florida G	USA			AF426775	AF426796	Straight	NA	Dyble et al., 2002, [e]
Florida I	USA			AF426785	AY078438	Coiled	NA	Dyble et al., 2002, [e]
PMC99.06	Mexico	AJ582282		AJ582097		NA	-	Gugger et al., 2005
PMC99.08	Mexico		AJ582289	AJ582098		NA	-	Gugger et al., 2005
PMC00.01	Brazil	AJ582283				NA	NA	Gugger et al., 2005
ITEP-A3	Brazil	AJ582284				NA	NA	Gugger et al., 2005
ITEP-018	Brazil	AJ582276				NA	NA	Gugger et al., 2005
Brazil1	Brazil			AF426771	AF426792	Straight	- [c]	Dyble et al., 2002, [d,e]
Brazil2	Brazil			AF426772	AF426793	Straight	- [c]	Dyble et al., 2002, [d,e]
Crescent2	USA			AY553317		NA	NA	Dyble et al., unpublished
FLCultureE3	USA			AY553318		NA	NA	Dyble et al., unpublished
FLCultureL5	USA			AY553319		NA	NA	Dyble et al., unpublished
Newnans4	USA			AY553320		NA	NA	Dyble et al., unpublished

[a]*Cylindrospermopsis Africana*
[b]Toxicity confirmed by mouse bioassay
[c]Producing PSP
[d]Wilson et al., 2000, Consensus sequence *rpoC1* (AF159371)
[e]Neilan et al., 2003, Saker et al., 1999, Hawkins et al., 1997, 16S rDNA (AF516724-AF516746, AF067818-AF067819, AF092504)
[f]Berger et al., 2006

Table 2 Origins, morphologies, sequenced genes and accession numbers, and information on toxin production of the *Cylindrospermopsis*

raciborskii isolates used in this study.

Strain	Geographic Origin	Morphology	Accession numbers				PS/PKS gene fragments	CYN production
			ITS1-L	*nifH*	*rpoC1*	PC-IGS		
NIVA-CYA 506	Kazinga Channel, Uganda	flexuous /straight	AM502068	AM502059	AM502050	AM502041	-/-	-
NIVA-CYA 507	Kazinga Channel, Uganda	flexuous	AM502069	AM502060	AM502051	AM502042	-/-	-
NIVA-CYA 508	Kazinga Channel, Uganda	flexuous	AM502070	AM502061	AM502052	AM502043	-/-	-
NIVA-CYA 509	Kazinga Channel, Uganda	flexuous	AM502071	AM502062	AM502053	AM502044	-/-	-
NIVA-CYA 510	Kazinga Channel, Uganda	flexuous	AM502072	AM502063	AM502054	AM502045	-/-	-
NIVA-CYA 511	Lake Victoria, Uganda	flexuous	AM502073	AM502064	AM502055	AM502046	-/-	-
ZIE05CR	Zierker See, Germany	straight	AM502074	AM502065	AM502056	AM502047	-/-	-
ZIE11CR	Zierker See, Germany	straight	AM502075	AM502066	AM502057	AM502048	-/-	-
ZIE13CR	Zierker See, Germany	straight	AM502076	AM502067	AM502058	AM502049	-/-	-

Figure captions

Fig. 1: Isolated *Cylindrospermopsis raciborskii* strains with different morphotypes: A) NIVA-CYA 506 with flexuous trichomes and B) ZIE11CR with straight trichomes. The scale bars indicate 10 μm.

Fig. 2: Maximum likelihood tree of *Cylindrospermopsis raciborskii* inferred from the ITS1-L region. Strains from this study are marked in bold. Support values are given in the following order: maximum likelihood bootstrap proportions/neighbour-joining bootstrap proportions/posterior probabilities. Only support values >50% are given.

Fig. 3: Maximum likelihood tree of *Cylindrospermopsis raciborskii* inferred from the *nifH* gene region. Strains from this study are marked in bold. Support values are given in the following order: maximum likelihood bootstrap proportions/neighbour-joining bootstrap proportions/posterior probabilities. Only support values >50% are given.

Fig. 4: Maximum likelihood tree of *Cylindrospermopsis raciborskii* inferred from the PC-IGS region within the phycocyanin operon. Strains from this study are marked in bold. Support values are given in the following order: maximum maximum likelihood bootstrap proportions/neighbour-joining bootstrap proportions/posterior probabilities. Only support values >50% are given.

Fig. 1

Fig. 2

Uganda (NIVA-CYA 509)
Uganda (NIVA-CYA 510)
Uganda (NIVA-CYA 508)
Uganda (NIVA-CYA 507)
Uganda (NIVA-CYA 506)
Uganda (NIVA-CYA 511)
Senegal (PMC 117.02) AJ582271
Senegal (PMC 115.02) AJ582272
Senegal (PMC 118.02) AJ582270
Australia (CYP-023) AJ582278
Australia (CYP-030B) AJ582277
Australia (CYP-026J) AJ582269

AFRICA

AUSTRALIA

Germany (ZIE05CR)
Germany (ZIE11CR)
Germany (ZIE13CR)
Hungary (ACT-9502) AJ582280
Germany (CYLI53) AJ582281
France (PMC 99.12) AJ582273
France (PMC114.02) AJ582279
France (PMC 98.14) AJ582268

EUROPE

Mexico Brazil (PMC 99.06) AJ582282
Brazil (ITEP-A3) AJ582284
Brazil (PMC00.01) AJ582283
Brazil (ITEP-018) AJ582276

AMERICA

75/67/67
-/75/64
76/-/100
76/97/98
-/-/62
78/88/57
87/80/91
60/65/74
69/90/90
87/97/-
66/85/97
AJ496732

0.01

Fig. 3

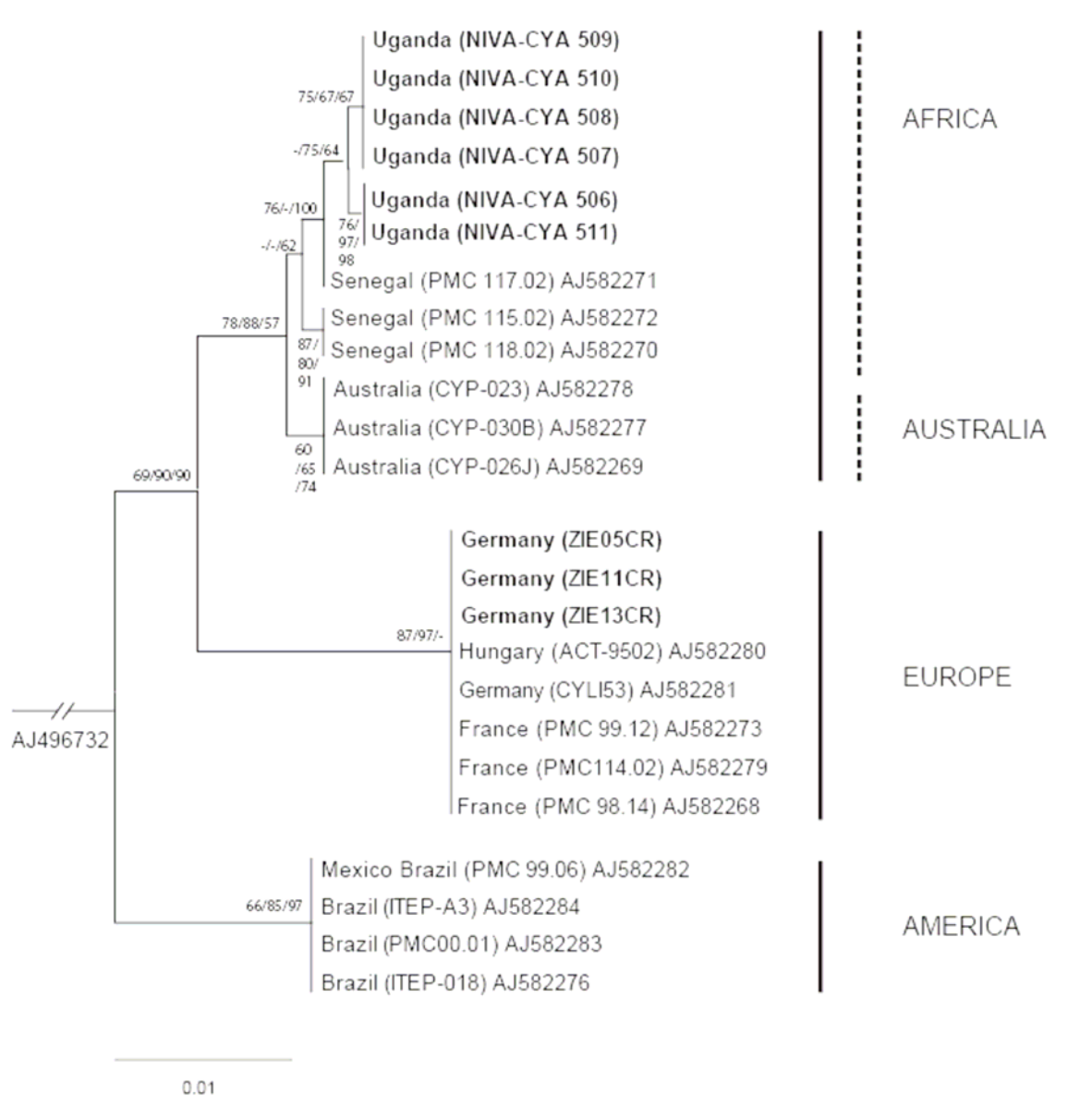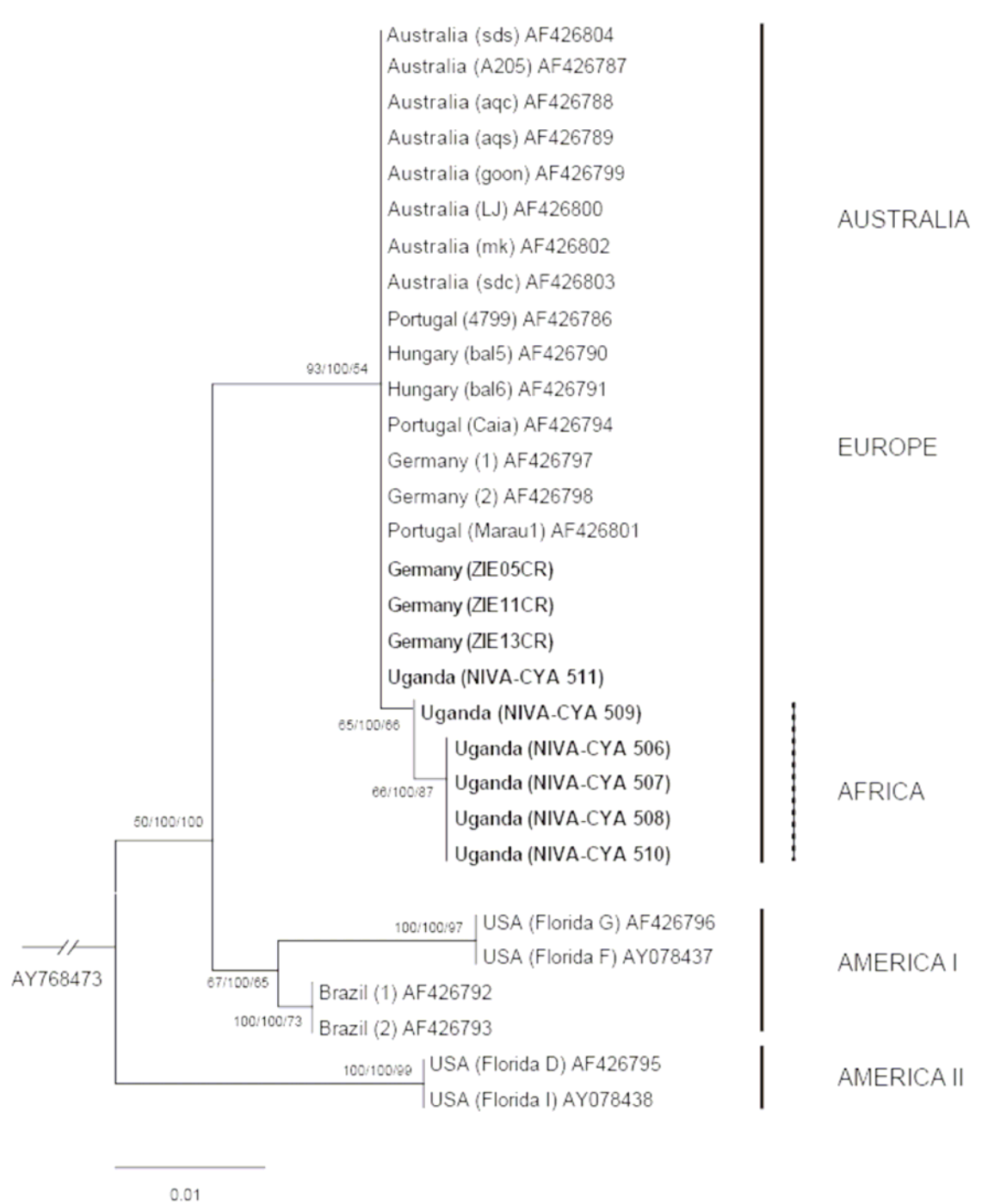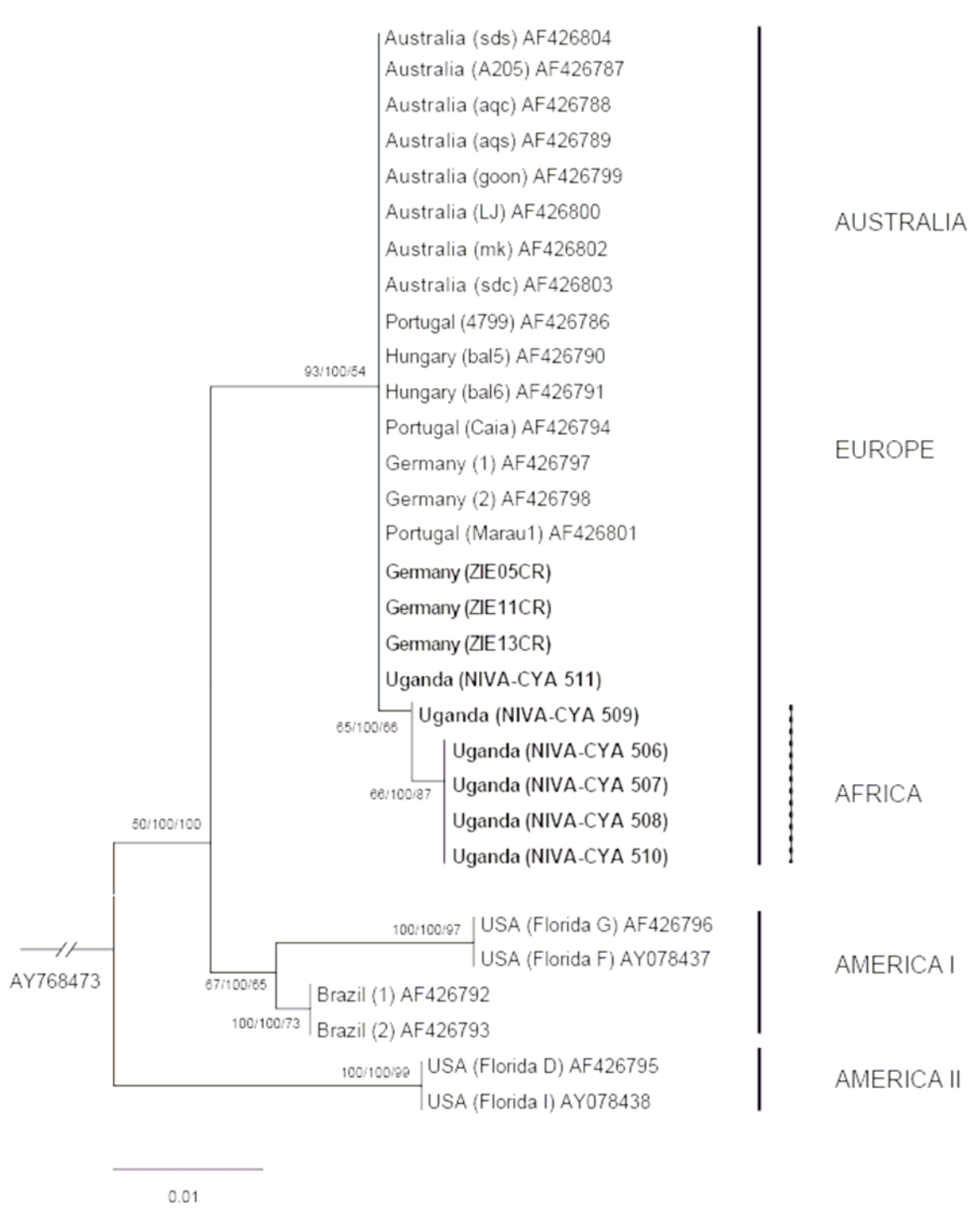**Fig. 4**

First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes

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Abstract

Three single-filament isolates of *Aphanizomenon flos-aquae* from two German lakes were found to produce remarkable amounts of the cyanobacterial hepatotoxin cylindrospermopsin (CYN). CYN-synthesis of the strains were evidenced both by LC-MS/MS analysis and detection of PCR products of gene fragments which are implicated in the biosynthesis of the toxin. The strains contain CYN in the range of 2.3–6.6 mg g^{-1} of cellular dry weight. To our knowledge this is the first report of CYN in *A. flos-aquae*.
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Keywords: Cylindrospermopsin; *Aphanizomenon flos-aquae*; Cyanobacteria; Germany

1. Introduction

Mass developments of cyanobacteria mainly caused by eutrophication of aquatic ecosystems are a worldwide problem. Besides the ecologically important suppression of other planktonic species cyanobacterial blooms hold a high risk for human and animal health due to the ability of several species to produce potent toxins. Human intoxications after consumption of water containing cyanobacteria or cyanotoxins are reported globally (overview in Chorus and Bartram, 1999), and cyanobacteria related death of waterfowl (Krienitz et al., 2003), cattle and domestic animals (overview in Briand et al., 2003) are documented even more widely.

One of the causative toxins is the alkaloid cylindrospermopsin (CYN) (Ohtani et al., 1992). As first report the toxin produced by the cyanobacterium *Cylindrospermopsis raciborskii* was found to be involved in a poisoning incident at Palm Island (Australia) in 1979, where 148 aborigines, mainly children, fell ill with gastroenteritic symptoms (Bourke et al., 1983; Hawkins et al., 1985). Furthermore, CYN was also detected in *Umezakia natans* (Harada et al., 1994), *Aphanizomenon ovalisporum* (Banker et al., 1997), *Raphidiopsis curvata* (Li et al., 2001a) and in *Anabaena bergii* (Schembri et al., 2001). CYN is described as potent hepatotoxin with additional affection of kidneys, heart, thymus, spleen and intestine (Hawkins et al., 1997; Falconer et al., 1998), whose damage is chiefly caused by inhibition of protein synthesis (Terao et al., 1994). Furthermore, CYN-induced mutagenicity by DNA strand breaking and chromosome loss during cell division was proven in vitro (Humpage et al., 2000; Shen et al., 2002). Strong evidence for in vivo carcinogenicity was demonstrated by Falconer and

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Humpage (2001). In consequence of the various effects on mammalian organ systems Humpage and Falconer (2003) propose a guideline value of $1 \, \mu g \, l^{-1}$ CYN in drinking water, based on the determined no observed adverse effect level (NOAEL) of $30 \, \mu g \, kg^{-1} \, day^{-1}$.

This limit value became important due to the frequent and massive occurrence of the CYN-producing species, especially of *C. raciborskii* and *A. ovalisporum* in water-bodies used as drinking water reservoir or for recreation, particularly in subtropical and tropical regions as Australia (Fabbro and Duivenvoorden, 1996; McGregor and Fabbro, 2000; Saker and Griffith, 2001), Brazil (Bouvy et al., 2000), Florida (Chapman and Schelske, 1997; Burns et al., 2002; St Amand, 2002) and Israel (Banker et al., 1997).

Besides the evidenced CYN-synthesis by the above-mentioned species, a number of CYN findings in freshwaters could not clearly relate to the occurring cyanobacterial species (Stirling and Quilliam, 2001; Carmichael et al., 2001; Fastner et al., 2003). For example, Fastner et al. (2003) detected CYN in two German lakes as first report of the toxin in Europe but isolated *C. raciborskii* strains from these lakes did not produce CYN. The isolated genotypes missed the genes for encoding CYN biosynthesis. It is still open whether isolation procedure favored non-producing strains under the given growth conditions or *C. raciborskii* genotypes occurring in these water-bodies generally did not contain the biosynthesis apparatus. Based on the fact that other known CYN-producing species were not found in these two lakes, related co-occurring species of the nostocales genera *Aphanizomenon*, *Anabaena* and *Raphidiopsis* were additionally discussed as possible CYN-producers.

Thus, in addition to the arising demand for *C. raciborskii* and CYN monitoring programs in Germany, for estimation of this toxin's human health risk potential, the potential of species other than *C. raciborskii* to produce CYN needed clarification.

2. Materials and methods

2.1. Isolation and strain cultivation

Net samples (25 μm) were taken from the shallow hypertrophic lake Melangsee (Eastern Brandenburg) in June 2004 and from 13 m-deep polytrophic lake Heiliger See (Potsdam) in August 2004.

Phytoplankton from Melangsee was diluted 1:30 (v/v) with nitrogen-free slightly modified Z8 medium (Zehnder and Gorham, 1960) and pre-incubated for 2 weeks at 20 °C, $65 \, \mu E \, m^{-2} \, s^{-1}$ and 12 h/12 h light dark cycle.

The enrichment culture from Melangsee and fresh material from Heiliger See were washed twice with sterile nitrogen containing culture medium. Afterwards single filaments were isolated using ultra-thin Pasteur pipettes and transferred to 96-well plates, filled with 300 μl medium per well. Plates were incubated analogous to the enrichment culture. Successfully isolated strains were transferred into 5 ml-reaction tubes and in 50 ml Erlenmeyer flasks for cultivation under the same conditions. All equipment and nutrient solutions were sterile.

Strains 10E6 and 10E9 could be obtained from Melangsee, strain 22D11 from Heiliger See. All strains were clonal but non-axenic.

2.2. Analysis of cylindrospermopsin by LC-MS/MS

Cyanobacterial material for CYN analysis was harvested from the late-exponential growth phase. For detection of total CYN (intra- and extracellular), the cultural solution was freeze-dried completely without any separation of the cells. About 5 mg of lyophilized culture material was extracted with water according to Welker et al. (2002). CYN was determined in the aqueous extract by LC-MS/MS using an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Framingham, MA) equipped with a turbo-ionspray interface.

The chromatographic separation was achieved using a Nova-Pak C_{18} (150×4.6 mm, 5 μm particle size; Waters, USA) at 30 °C and a flow rate of $0.8 \, ml \, min^{-1}$ with the following gradient programme: 100% A for 1 min, ramped to 100% B in 5 minutes, held for 3 min and then to 100% A in 1 min and equilibrated for 7 min (solvent A: 1% methanol/deionized water, solvent B: 60% methanol/de-ionized water, both solvents contained 5 mM ammonium acetate) (Eaglesham et al., 1999).

The mass spectrometer was operated in the multiple reaction-monitoring mode (MRM) with a collision energy of 48 eV. For the determination of CYN the transitions m/z 416.1 ($M+H^+$) to 194 and 416.1/176 were monitored with a dwell time of 0.2 s. Quantitation of CYN (purchased from Dr A. Humpage, Australian Water Quality Centre, Salisbury, Australia) was achieved using the 416.1/194 transition with the 416.1/176 transition monitored as confirmation ion. Using a 10 μl injection volume the limit of detection was less than $0.1 \, \mu g \, l^{-1}$.

2.3. Molecular analysis

All PCR-reactions were performed on a PTC-200 Peletier thermal cycler (MJ Research, Watertown/ USA) and were carried out in a final volume of 20 μl reaction mixture containing 0.5 μl culture material harvested at the late-exponential growth phase, $1 \times$ PCR-buffer (Qiagen, Hilden/Germany), 2.5 mM $MgCl_2$, 20 pmol of each M13 and M14 primers (Schembri et al., 2001, synthesized by Metabion, Martinsried, Germany), 200 μM of each dNTP, and 1 U Qiataq DNA Polymerase (Qiagen, Hilden/ Germany). Thermal cycling conditions were: 94 °C for 5 min; 30 cycles of 94 °C for 10 s, 55 °C for 20 s, and 72 °C for 60 s; and a final extension step of 72 °C for 7 min.

The amplified PCR products were visualized on 1.5% agarose gels stained with ethidium bromide and
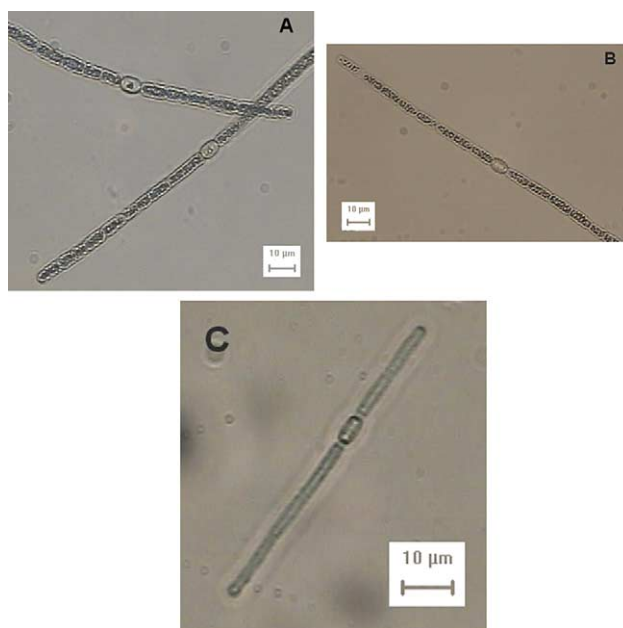
Fig. 1. Isolated *Aphanizomenon flos-aquae* strains with different morphotypes: strains 22D11 (A) and 10E9 (B) with gas vesicles; strain 10E6 (C) represents a gas vesicle-free genotype.

photographed under UV transillumination. Standard DNA fragments (Peqlab, Erlangen, Germany) were used as size markers.

3. Results

3.1. Strain characterization

The isolated strains could be identified as *Aphanizomenon flos-aquae*. Fig. 1A and B show typical gas vesicle containing filaments of the strains 10E9 and 22D11. They grow in homogenous cultural solutions with tendency to accumulate at the surface.

Strain 10E6 is a gas vesicle-free clone (Fig. 1C) and grows mainly benthically.

3.2. Cylindrospermopsin content of the isolates

CYN production of all strains could be proved by detection of CYN mass signals and their typical fragment transitions as shown for strain 22D11 in comparison to the toxin standard in Fig. 2.

Strain 22D11 contained 6.6 mg g^{-1} dry wt, 10E6 2.3 mg g^{-1} dry wt, and 10E9 3.2 mg g^{-1} dry wt CYN.

3.3. Molecular detection of putative CYN synthetase gene fragments

Fig. 3 shows amplified peptide synthetase gene fragments 597 bp, which are linked to CYN-synthesis in

the positive control *C. raciborskii* strain CR 3 (Saker and Griffith, 2000) (lane 1) as well as in our three *A. flos-aquae* isolates (lanes 2–4). As negative control *C. raciborskii* strain CYLI 19 (Fastner et al., 2003) was applied. In this case no PCR product was found (lane 5).

4. Discussion

The presence of CYN in surface waters and reservoirs worldwide is regarded as potential risk for human health due to its potent hepatotoxicity and the assumed carcinogenicity. The identified CYN-producing cyanobacterial species have different regional importance. In Australia (Saker et al., 1999) and North America (Burns et al., 2002), *C. raciborskii* blooms were held responsible as main producer of detected CYN. In New Zealand and Asia, several occurrences of *C. raciborskii* and related CYN are documented (Wood and Stirling, 2003; Li et al., 2001b; Chonudomkul et al., 2004). Reports of CYN-producing *A. ovalisporum* blooms are common for Australia (Shaw et al., 1999) and Israel where they cause huge problems for drinking water supply (Gophen et al., 1999). In particular cases, occurrences of CYN-containing *U. natans* and *R. curvata* in Asia are described (Harada et al., 1994; Li et al., 2001a). In Europe, CYN was found in two German lakes (Fastner et al., 2003) as sole report up to now, however, without identification of the producer.

As a result of our ongoing investigations we introduce *A. flos-aquae* as CYN-producing species for the first time, as far as we know and at the same time the first isolation of CYN-producing cyanobacterial strains from European
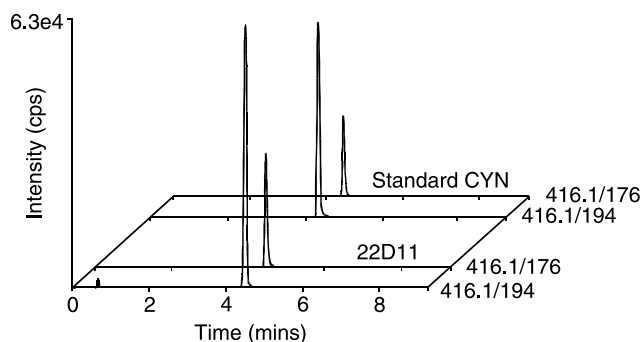
Fig. 2. LC-MS/MS ion chromatograms. Transitions 416/176 and 416/194 for standard CYN (top), and for *Aphanizomenon flos-aquae* 22D11 (bottom).

freshwaters. *A. flos-aquae* occurs frequently in aquatic ecosystems of the Northern hemisphere with high potential to form seasonal blooms in freshwaters as well as in marine systems (Leland and Berkas, 1998; Tsujimura et al., 2001; Willén and Mattsson, 1997; Stal et al., 2003). The species may grow independently of dissolved nitrogen resources in water-bodies by autonomous fixation of atmospheric nitrogen in specialized heterocysts. Furthermore, *A. flos-aquae* is able to survive unfavorable growth conditions, especially low temperatures, by formation of akinets. Germination and resuspension of these resting cells provide the inoculum for the development of proximate populations.

Our isolated strains contained total CYN in the range 2.3–6.6 mg g^{-1} dry wt. These concentrations are comparable with published data of ≤ 4.6 mg g^{-1} dry wt, respectively, 1 mg g^{-1} dry wt for *C. raciborskii* strains (Saker and Griffith, 2000; Li et al., 2001b) as well as with 1.3 mg g^{-1} dry wt deoxy-CYN measured in *R. curvata* (Li et al., 2001a). However, Saker and Griffith (2000) presented an extreme variability in CYN contents of different isolates and sensitivity of toxin production to temperature. Therefore, the variability and impact of environmental factors on CYN-production in *A. flos-aquae* should also be tested.

The discovery of CYN in *A. flos-aquae* is not very astonishing since this taxon is known to produce a variety of toxic and bioactive compounds. Different neurotoxic alkaloids—so called paralytic shellfish poisons (PSPs) were found with high diversity in toxin profiles in this species (Adelman et al., 1982; Mahmood and Carmichael, 1986; Pereira et al., 2000; Ferreira et al., 2001). Furthermore, lipids with negative effects to fish embryo larval development (Papendorf et al., 1997) as well as carboxypeptidase-A inhibitors (Murakami et al., 2000) are produced by *A. flos-aquae*. Uncharacterized secondary metabolites were described to cause massive hepatic and pulmonary lesions in mice (Unterdal et al., 1999) and may have antibacterial properties (Ostensvik et al., 1998). In addition, Torokne et al. (2001) demonstrated high allergenic potential of *Aphanizomenon* extracts.

Most such cyanobacterial secondary metabolites are synthesized by large multienzyme complexes—polyketide synthetases (PKS) and/or non-ribosomal peptide synthetases (NRPS). Integrated NRPS/PKS systems are completely described for various cyanobacterial genera like *Microcystis* (Tillet et al., 2000), *Planktothrix* (Christiansen et al., 2003), *Anabaena* (Rouiainen et al., 2000, 2004), *Lyngbya* (Chang et al., 2002), *Nostoc* (Hoffmann et al., 2003) and *Nodularia* (Moffitt and Neilan, 2004) and are responsible for a manifold array of compounds. NRPS genes were also detected in *Aphanizomenon (flos-aquae)* in certain studies (Neilan et al., 1999; Christiansen et al., 2001; Kodama et al., 2002) but without evidence for specific metabolites.

The biosynthetic pathway of CYN is not completely elucidated up to know. However, first biosynthesis steps realized by a PKS were obtained from feeding experiments with radioactive isotopes in *C. raciborskii* (Burgoyne et al., 2000). Subsequently, Schembri et al. (2001) identified genes, which are implicated in CYN biosynthesis in *C. raciborskii* and *A. bergii* as well as Shalev-Alon et al. (2002) in *A. ovalisporum*. Further analyses of Fergusson and Saint (2003) confirmed the proposed direct link between PKS and NRPS modules.

Our results suggest a similar biosynthetic pathway of CYN also in *A. flos-aquae*. We show the presence of a NRPS fragment, which is putatively incorporated in the CYN
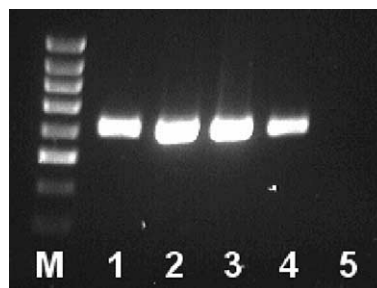

Fig. 3. PCR products amplified with M13/M14 primer set. Positive control CR3 (lane 1), 22D11 (2), 10E9 (3), 10E6 (4), negative control Cyli19 (5).

synthetase cluster. The fragment was amplified by using the primer set M13/M14 (Schembri et al., 2001; Fergusson and Saint, 2003), which can also be applied in *C. raciborskii, A. ovalisporum* and *A. bergii* for detection of CYN synthetase. However, the recommended application of primer set M4/K18 (Fergusson and Saint, 2003) for detection of the PKS component in the CYN-synthetase did not work in our *A. flos-aquae* strains. Probably, PKS sequences in *A. flos-aquae* differ slightly from those of other CYN-producing species. Such divergences in the structure and sequence of PKS/NRPS gene cluster for one and the same secondary metabolite in different cyanobacterial genera have been demonstrated for the hepatotoxic peptide microcystin in *Microcystis* (Tillet et al., 2000), *Planktothrix* (Christiansen et al., 2003; Mbed; et al., 2005) and *Anabaena* (Rouhiainen et al., 2004). Investigations of the phylogenetic evolution of CYN synthetase in the different species could contribute to understanding their origin and distribution either by horizontal gene transfer between the species or by dissemination from a common CYN-producing ancestor.

The possible occurrence of CYN in *A. flos-aquae* should be considered in the assessment of massive *A. flos-aquae* blooms due to associated possible risk of human exposure to high concentrations. Furthermore, consequences may arise for improving the surveillance of *A. flos-aquae* harvested for human dietary supplements, e.g. from Klamath Lake (Carmichael et al., 2000). Screening programs targeting a first overview of the share of CYN-producing genotypes (strains) in natural populations and in total phytoplankton would be an important basis for hazard assessment. This would need to be followed with research investigating the variability of CYN-concentrations in temperate aquatic ecosystems.

Acknowledgements

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Concentrations of particulate and dissolved cylindrospermopsin in 21 *Aphanizomenon*-dominated temperate lakes ☆

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Abstract

The cyanobacterial toxin cylindrospermopsin (CYN) is widely distributed in German lakes, but volumetric data for risk assessment are lacking and it is unclear which cyanobacterial species produce CYN in Europe. We therefore analyzed CYN concentration and cyanobacterial composition of 21 German lakes in 2005. CYN was detected in 19 lakes (102 of 115 samples). In total, 45 samples contained particulate CYN only, and 57 contained both dissolved and particulate CYN. The concentrations were $0.002–0.484 \,\mu g \, L^{-1}$ for particulate CYN and $0.08–11.75 \,\mu g \, L^{-1}$ for dissolved CYN with a maximum of $12.1 \,\mu g \, L^{-1}$ total CYN. A drinking-water guideline value of $1 \,\mu g \, L^{-1}$ proposed by Humpage and Falconer [2003. Oral toxicity of the cyanobacterial toxin CYN in male Swiss albino mice: determination of no observed adverse effect level for deriving a drinking water guideline value. Environ. Toxicol. 18, 94–103] was exceeded in 18 samples from eight lakes due to high concentrations of dissolved CYN. CYN occurrence in the German lakes could not be ascribed to the three known CYN-producing species *Cylindrospermopsis raciborskii*, *Anabaena bergii* and *Aphanizomenon flos-aquae*, which were detected in some lakes in low abundances. The highest correlation coefficients were observed between particulate CYN and the native *Aphanizomenon gracile*. It occurred in 98 CYN-positive samples, was the most abundant Nostocales and was the only Nostocales in five samples. This indicates that *A. gracile* is a potential CYN producer in German lakes.
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1. Introduction

Cyanobacteria can produce a wide range of bioactive and toxic substances which can be harmful to wildlife and humans (e.g. Carmichael and Falconer, 1993; Chorus and Bartram, 1999). One of these toxins is cylindrospermopsin (CYN), a

tricyclic alkaloid that was first detected in an Australian strain of *Cylindrospermopsis raciborskii* (Ohtani et al., 1992). CYN is a potent hepatotoxin; it can cause damage to the kidney, lungs and heart and is genotoxic. CYN is also suspected to be carcinogenic, as recently reviewed by Falconer and Humpage (2006). The toxicity of CYN to humans first became evident in the "Palm Island Mystery Disease" in Queensland, Australia in 1979. In this incident, local inhabitants supplied with drinking water from a reservoir where CYN-producing *C. raciborskii* bloomed contracted hepatoenteritis (Bourke et al., 1983; Hawkins et al., 1985). Combined effects of CYN and microcystin were implicated in the Brazilian dialysis clinic tragedy (Carmichael et al., 2001). Due to the toxicity of CYN, a drinking-water guideline value of $1\,\mu g\,L^{-1}$ has been proposed by Humpage and Falconer (2003).

CYN is widely distributed in tropical and subtropical freshwaters, e.g., in Australia (McGregor and Fabbro, 2000) and Florida (Burns et al., 2002), but is also found in temperate regions such as Europe (Fastner et al., 2003, 2007; Manti et al., 2005; Quesada et al., 2006). Species from at least six genera of freshwater cyanobacteria are known to produce CYN. Beside *C. raciborskii*, these are *Anabaena bergii* from Australia (Schembri et al., 2001), *Aphanizomenon ovalisporum* from Australia, Israel and Spain (Shaw et al., 1999; Banker et al., 1997; Quesada et al., 2006), *Umezakia natans* from Japan (Harada et al., 1994) and *Raphidiopsis curvata* from China (Li et al., 2001). *Aphanizomenon flos-aquae* from Germany (Preußel et al., 2006) and *Anabaena lapponica* from Finland (Spoof et al., 2006) were recently identified as CYN producers from northern European habitats. All of these species except *U. natans* (Stigonematales) belong to the order Nostocales.

The ability of cyanobacterial strains isolated from the temperate zone to produce CYN and the northwards spread of tropical CYN-producing species like *C. raciborskii* (Padisák, 1997) or *A. bergii* (Stüken et al., 2006) creates the necessity to conduct toxin screening in the northern hemisphere. Although CYN production by European strains of *C. raciborskii* could not be proved (Fastner et al., 2003; Saker et al., 2003; Neilan et al., 2003; Bernard et al., 2003, etc.) and has not been tested in German *A. bergii* isolates so far, these species must be considered as potential CYN producers here, since the co-occurrence of toxin-producing and non-producing genotypes within a given population occurs with other cyanotoxins. Until now, only two large-scale qualitative screening studies on the distribution of CYN in natural waters of northern Europe have been published. Whereas Spoof et al. (2006) did not find CYN in 51 Finnish freshwater sites or in the Baltic; Fastner et al. (2007) found the cyanotoxin in 63 out of 127 German lakes but not at the four Baltic coastal sites studied. Thus, CYN is widely distributed in German lakes but volumetric data for risk assessment of CYN are lacking.

The aims of our study were: (i) to collect the first data on CYN concentrations (dissolved and particulate fractions) in German freshwaters, (ii) to analyze environmental conditions under which CYN is likely to occur and (iii) to identify the CYN-producing species in these waters. For this purpose, CYN, abiotic and morphometric parameters as well as cyanobacterial abundance and composition were studied in 21 lakes during summer 2005.

2. Materials and methods

2.1. Study site

Twenty-one lakes were chosen based on their seston dry-weight-related CYN concentrations (Fastner et al., 2007) and cyanobacterial composition (Stüken et al., 2006) as determined in a preliminary study conducted at 142 lakes in 2004. The selected lakes contained CYN and known CYN producers (at that time *C. raciborskii* and *A. bergii*) and differed with respect to lake morphometry (maximum depth, volume and surface area) and mixing regime (di- and polymictic). Limnological characteristics of the lakes are given in Table 1. The lakes studied are located in the lowlands of Northeast Germany, between 52°07′17″N and 53°27′07″N latitude and 12°48′09″E and 13°59′41″E longitude and are mainly used for recreation and fishery.

2.2. Sampling and analyses

The lakes were sampled fortnightly at their deepest point between June and September 2005 for a minimum 6-week period. Quantitative data collected in 2004 were additionally used for two lakes, Melangsee and Langer See. On each sampling date, we determined the Secchi depth (SD) and vertical profiles of water temperature, pH and oxygen saturation of the lakes at $0.5\,m$ intervals

Table 1

Morphometric characteristics, mixing regime and mean values of total phosphorus (TP), Secchi depth (SD), and total cylindrospermopsin (CYN) (data set for statistical analysis, see Fig. 1 and Table 2) for 21 lakes studied in 2005

Lake	Abbreviation	Max. depth (m)	Area (km^2)	Volume (10^6 m^3)	TP (µg L^{-1})	SD (m)	CYN$_{total}$ (µg L^{-1})
Polymictic lakes							
Petznicksee	PZ	2.0	0.72		41.6	0.83	0.01
Braminsee	BR	2.2	0.69		78.6	0.38	0.79
Melangsee 2004	M4	2.8	0.12	0.20	59.7	0.43	0.23
Melangsee 2005	M5	2.8	0.12	0.20	51.1	0.53	0.09
Vielitzsee	VI	3.0	1.11		98.3	0.43	0.00
Langer See 2004	L4	3.5	1.38	3.27	85.9	0.44	0.85
Langer See 2005	L5	3.5	1.38	3.27	75.8	0.57	0.13
Lieps	LI	3.8	4.31	9.70	107.0	0.25	**5.07**
Petersdorfer See	PE	3.8	0.24	0.45	37.7	0.50	0.02
Bützsee	BU	4.0	2.23		42.3	0.70	**2.39**
Rahmer See	RA	4.0	0.80		42.9	0.82	0.19
Moderfitzsee	MF	5.5	0.59		34.2	0.63	0.01
Kleiner Zeschsee	KZ	5.6	0.23	0.61	32.9	1.05	0.14
Kutzingsee	KU	6.0	0.32	0.78	87.3	0.33	0.52
Zermützelsee	ZE	7.0	1.25		70.2	0.77	**8.40**
Scharmützelsee, Northern bay[a]	SM	7.9	1.40	7.50	29.3	1.58	0.16
Dimictic lakes							
Stolpsee	ST	12.0	3.81		41.3	0.98	**4.31**
Großer Glubigsee	GL	13.0	0.58	2.66	17.2	1.02	0.00
Großer Plessower See	GP	13.4	3.22	20.80	33.0	2.03	**1.10**
Motzener See	MZ	13.5	2.06	12.70	47.9	1.73	0.00
Springsee	SP	18.0	0.59	5.86	19.9	0.96	0.01
Pätzer Vordersee	PA	18.5	1.65		25.4	1.40	0.00
Ruppiner See	RU	23.0	8.08	66.00	36.2	0.97	**5.29**

Mean total CYN concentrations above the guideline value of 1 µg L^{-1} for dinking water proposed by Humpage and Falconer (2003) are highlighted in bold print. Lakes Melangsee and Langer See were investigated in 2004 and 2005.

[a]Northern bay of Scharmützelsee can be considered as a separated water body (Christen, 2006).

using a multi-parameter probe (Hydrolab H20). Photosynthetically active radiation (PAR) was measured at half-meter intervals through the water column using two spherical quantum sensors (Li Cor SA 193). Mean PAR in the mixed water column (I_{mix}) was calculated according to Riley (1957; see also Nixdorf and Rücker, 2006). Mixed samples from the whole water column (polymictic lakes) or epilimnion (stratified lakes) were prepared by taking samples at half-meter intervals with a 2.3 L LIMNOS sampler. Aliquots of the mixed samples were analyzed to determine the concentrations of ammonia (NH$_4$-N), nitrate plus nitrite (NO$_{total}$-N), dissolved inorganic phosphorus (DIP), total phosphorus (TP), total nitrogen (TN) and chlorophyll *a* according to standard methods (DEV, 1976–1998).

2.3. Phytoplankton analysis

Phytoplankton composition and biovolume were estimated using an aliquot fixed with Lugol's solution and studied under an inverse microscope according to Utermöhl (1958) and Rott (1981). The following references were used for identification of cyanobacteria: Geitler, 1932; Huber-Pestalozzi, 1938; Komárek and Ettl, 1958; Horecká and Komárek, 1979; Hindák, 1992, 2000.

2.4. CYN analysis

Aliquots of the mixed water samples were filtered over 47 mm diameter glass fiber filters (Macherey & Nagel MN-85/70). The seston-covered filters and 200 ml of filtrate were stored at −20 °C until investigation. For analysis of particulate CYN (CYN$_{part}$), the filters were placed in Eppendorf tubes and extracted by adding 1.5 ml of water (Welker et al., 2002). The samples were sonicated for 10 min, shaken for 1 h and then centrifuged. The extraction was repeated once and the pooled supernatants were dried by vacuum centrifugation. The dried extracts were stored at −20 °C until analysis.

Prior to analysis, the dried extracts were dissolved in 1 ml of water and filtered (0.45 μm). Dissolved CYN (CYN_{diss}) was analyzed directly in the filtrate, which has been additionally filtered over 0.45 μm to remove fine particles. CYN analysis was performed by LC-MS/MS. The method was described in detail in Fastner et al. (2007). The detection limit was 10 pg on column, i.e., 0.002 μg L^{-1} for particulate CYN and 0.05 μg L^{-1} for dissolved CYN, respectively, depending on the volume of the filtered sample and injection volume.

2.5. Statistical analysis

The study yielded 115 data sets. Some of the lakes were sampled for more than 6 weeks. In these cases, monthly means were calculated to ensure a comparable number of 3–4 data sets per lake for statistical analyses, resulting in 80 data sets in total (see Fig. 1). Correlations between the concentrations of different CYN fractions and the biovolume of cyanobacteria as well as environmental factors were analyzed by Spearman rank correlation analysis using SPSS 12.0 for Windows (SPSS Inc.).

3. Results

3.1. CYN concentrations

CYN was detected in 102 of 115 samples (89%), i.e., in 19 of 21 lakes (Fig. 1C). The highest concentration was 12.1 μg L^{-1} total CYN, and 18 samples from eight lakes exceeded the drinking-water guideline value of 1 μg L^{-1} CYN_{tot} proposed by Humpage and Falconer (2003). In 45 samples (39%), particulate CYN was the sole detectable toxin fraction, with values ranging from 0.002 to 0.194 μg L^{-1}. Dissolved CYN could not been detected in these samples, possibly due to the higher detection limit. Half of the samples (57) contained both dissolved (0.08–11.75 μg L^{-1} CYN_{diss}) and particulate CYN (0.002–0.484 μg L^{-1} CYN_{part}). In these cases, the dissolved CYN fraction comprised 24.3%–99.8% of CYN_{tot} (Fig. 1D).

3.2. Phytoplankton composition

Cyanobacteria were present in all samples and dominated the phytoplankton community in most of the lakes studied (Fig. 1A). Overall, 43 cyanobacterial species of 20 different genera were identified. Chroococcales and fine filamentous species of the order Oscillatoriales (*Limnothrix* sp., *Pseudanabaena* sp., *Leptolyngbya* sp.) are summarized in the following data analyses (Fig. 1A, Table 2).

Due to the absence of Stigonematales, the order Nostocales was the only group of pelagic cyanobacteria containing known potential CYN producers. The composition of Nostocales at the genus level was complex in most cases. Only 10 samples contained only one genus, which was either *Aphanizomenon* ($n = 9$) or *Anabaena* ($n = 1$). Only two Nostocales genera were present in 39 samples, but 64 contained three–five genera. *Aphanizomenon* was the most frequent genus among the Nostocales species in 112 of 115 samples and was the dominant Nostocales in 63%, as indicated by a mean fraction of >80% of total Nostocales biovolume (Fig. 1B). *Aphanizomenon gracile* was the most frequent species within this genus. It formed 80–100% of the total Nostocales biovolume and was the dominant species in 42% of samples. *A. gracile* was absent in only eight samples from three lakes. *A. flos-aquae*, on the other hand, was found in only 22 samples from 10 lakes. *A. bergii*, another known CYN producer, was present in 26 samples and comprised 64% maximum of the total Nostocales biovolume. *C. raciborskii*, the third known CYN producer, was found in 76 samples from 18 lakes. It reached fractions of more than 50% of the total Nostocales biovolume in only four samples, with a maximum fraction of 76%.

3.3. Data analysis

Selected Spearman rank correlation coefficients between the concentrations of different CYN fractions and environmental factors as well as cyanobacterial biovolumes are shown in Table 2. Among abiotic factors, the highest correlation coefficients were found with TP and SD. TP exhibited a significantly positive (0.44) and SD a significantly negative (−0.48) correlation to particulate CYN concentration. No correlations between CYN_{part} and morphometric parameters like lake volume, area or maximum depth were detected (data not shown). Regarding the relationship between CYN_{part} and cyanobacterial biovolumes, *Aphanizomenon* showed the highest positive correlation (0.6; $n = 78$) at the genus level. At the species level, this was *A. gracile* (0.53; $n = 74$), followed by *Planktothrix agardhii* (0.51; $n = 68$). Dissolved CYN and total CYN yielded generally lower
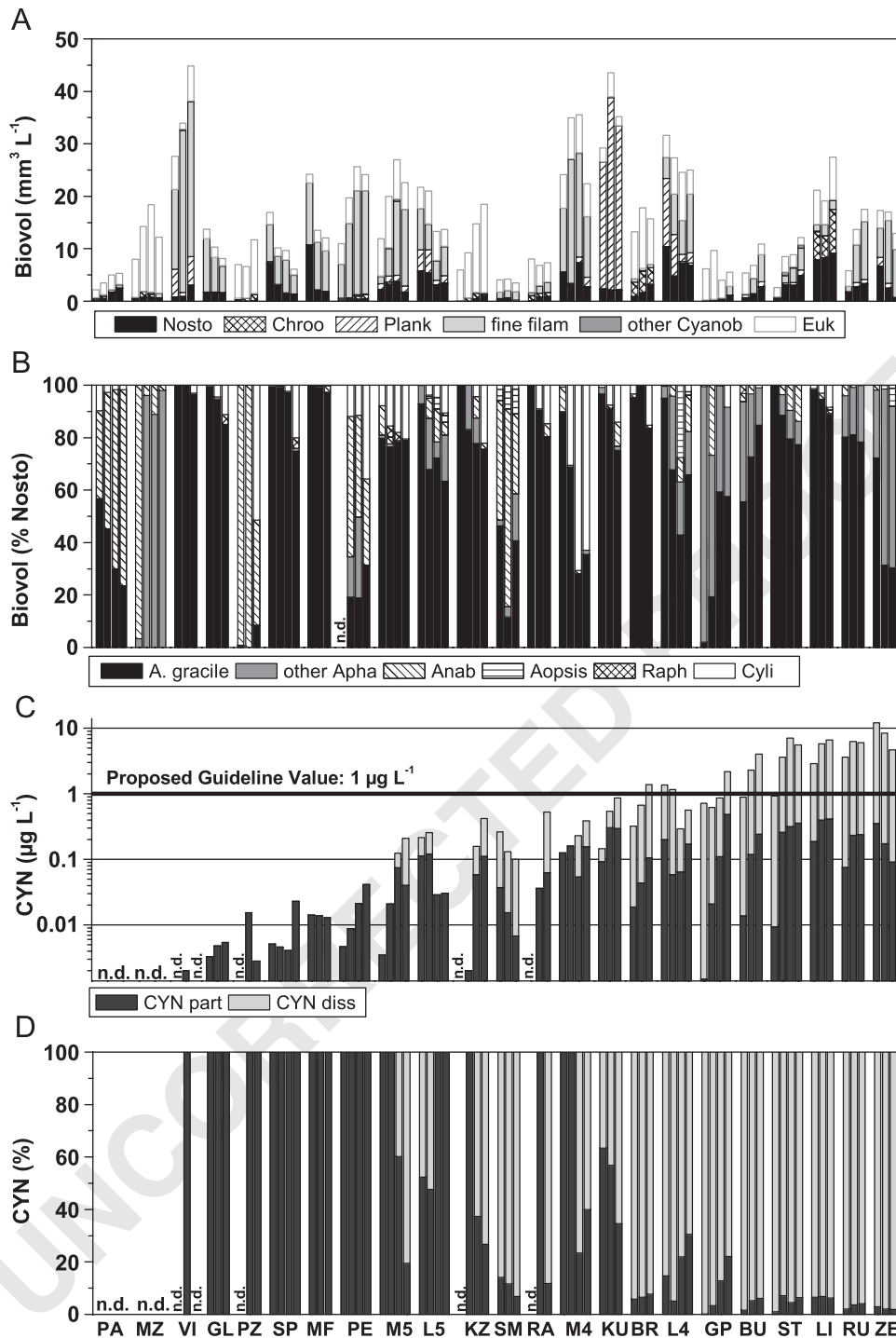
Fig. 1. Phytoplankton composition (A), fraction of different genera relative to total Nostocales biovolume (B) and concentration of particulate and dissolved cylindrospermopsin (CYN) on a logarithmic scale (C) and as a fraction of total CYN (D) in 21 lakes from June to September 2005 (data set for statistical analyses, i.e., fortnightly or monthly means, is presented here and sorted by mean of total CYN concentration). Nosto—Nostocales, Chroo—Chroococcales, Plank—*Planktothrix agardhii*, fine filam—fine filamentous cyanobacteria (*Limnothrix* spp., *Pseudanabaena* spp., *Leptolyngbya* spp.), other Cynob—other cyanobacteria, Euk—eukaryotic algae; *A. gracile—Aphanizomenon gracile*, Apha—other *Aphanizomenon* sp., Anab—*Anabaena* spp., Aopsis—*Anabaenopsis* spp., Raph—*Raphidiopsis mediterranea*, Cyli—*Cylindrospermopsis raciborskii*. For key to abbreviations of lake names and explanations see Table 1.

Table 2

Spearman rank correlation coefficients for particulate (part), dissolved (diss) and total cylindrospermopsin (CYN) and selected abiotic and biotic parameters

Parameter	n	CYN_{part} ($\mu g\,L^{-1}$)	CYN_{diss} ($\mu g\,L^{-1}$)	CYN_{total} ($\mu g\,L^{-1}$)
TP ($\mu g\,L^{-1}$)	80	0.441**	0.326**	0.346**
TN ($\mu g\,L^{-1}$)	80	0.246	0.200	0.230
DIP ($\mu g\,L^{-1}$)	80	0.020	−0.060	−0.081
NH$_4$-N ($\mu g\,L^{-1}$)	80	−0.219	−0.185	−0.231
NO$_{tot}$-N ($\mu g\,L^{-1}$)	80	0.106	0.088	0.096
Temperature (°C)	80	−0.180	−0.030	−0.114
Secchi depth (m)	79	−0.477**	−0.156	−0.275**
I_{mix} ($\mu E\,m^{-2}\,s^{-1}$)	80	−0.333**	−0.197	−0.193
Chlorophyll a ($\mu g\,L^{-1}$)	80	0.403**	0.071	0.158
Biovolumes of (mm^3 L^{-1}):				
Phytoplankton	80	0.351**	0.055	0.140
Cyanobacteria	80	0.441**	0.101	0.225
Nostocales	79	0.564**	0.321**	0.407**
Cylindrospermopsis raciborskii	49	0.431**	0.147	0.275*
Anabaena bergii	20	−0.064	−0.146	−0.100
Anabaena spp.	56	0.134	0.058	0.037
Anabaenopsis spp.	12	−0.217	−0.168	−0.224
Aphanizomenon spp.	78	0.597**	0.359**	0.460**
A. gracile	74	0.530**	0.261	0.348**
A. flos-aquae	22	−0.259	0.158	0.092
A. aphanizomenoides	7	−0.536	−0.204	−0.536
A. issatschenkoi	33	0.327	0.314	0.322
Aphanizomenon sp.	23	0.114	0.271	0.248
Raphidiopsis mediterranea	14	0.591	0.079	0.266
Planktothrix agardhii	68	0.506**	0.333**	0.371**
Fine filamentous Oscillatoriales[a]	78	0.162	−0.091	0.029
Chroococcales	80	−0.050	−0.148	−0.137
Other cyanobacteria	31	0.214	0.110	0.142

The significance level of $p < 0.01$ is indicated by **; n—number of cases included. Statistical analysis is based on 3–4 data sets per lake formed, in some cases, by calculating monthly means. TP—total phosphorus, TN—total nitrogen, DIP—dissolved inorganic phosphorus, NH$_4$-N—ammonia, NO$_{tot}$—nitrate plus nitrite, I_{mix}—mean photosynthetically active radiation in the mixed water column.

[a]For explanation see Fig. 1.

Spearman rank correlation coefficients than particulate CYN.

All data sets from CYN-positive samples were individually analyzed for the presence or absence of individual species of the order Nostocales. No Nostocales were detected in two samples (containing only small amounts of CYN$_{part}$); *A. gracile* was the only taxon of the order Nostocales in five of the CYN-positive samples; only *Anabaena* species occurred in one sample; 16 samples contained only taxa of the genera *Aphanizomenon* and *Anabaena*; in 13 samples, only *Aphanizomenon* species and *C. raciborskii* were found and three samples contained only *Aphanizomenon* and *Anabaenopsis* species.

4. Discussion

Our study provides the first volumetric data on concentrations of particulate and dissolved CYN in German freshwater lakes. The drinking-water guideline value of $1\,\mu g\,L^{-1}$ CYN$_{tot}$ proposed by Humpage and Falconer (2003) exceeded in 18 samples from eight different lakes. Concentrations up to $12.1\,\mu g\,L^{-1}$ total CYN are much lower than the maximum values reported from Australia or Florida (summarized in Table 3), but they are in a range which Falconer and Humpage (2006) refer to as the "more common concentrations of 1–$10\,\mu g\,L^{-1}$". The highest concentrations so far are reported to occur in surface scum (Shaw et al., 1999), aquaculture ponds (Saker and Eaglesham, 1999) and farm dams (Shaw et al., 2000). Excluding

Table 3
Overview of published data on cylindrospermopsin (CYN) concentration

Sampling site	Total CYN ($\mu g\,L^{-1}$)	Particulate CYN ($\mu g\,L^{-1}$)	Dissolved CYN ($\mu g\,L^{-1}$)	Organisms	Source
Europe					
115 samples from 21 lakes in NE German lowlands, June–Oct. 2005	0–12.1	0–0.5	0–11.8	*Aphanizomenon gracile, A. flos-aquae, C. raciborskii, Anabaena bergii*	This study
Samples from three Italian lakes, July–Oct. 2004	0–15	not det.	not det.	*C. raciborskii*	Manti et al. (2005)
Arcos Reservoir, South Spain, Aug–Sept. 2004	not det.	1.5–9.4	not det.	*Aphanizomenon ovalisporum*	Quesada et al. (2006)
Australia					
Hervey Bay area, Queensland, Nov. 1997–Jan. 1998	10–92	0.7–29	7–63	*C. raciborskii*	Chiswell et al. (1999)
183 samples from 15 reservoirs, Queensland, Oct. 1997–June 1999	0–80	not det.	not det.	*C. raciborskii*	McGregor and Fabbro (2000)
Raw water and treated water from a water treatment plant, Sept. 2001	1.17	not det.	20.5%		Hoeger et al. (2004)
Surface scum samples from two small shallow lakes, near Hervey Bay, QL, Feb., April, Oct., Nov. 1997	4–120	0–4	4–120	*Aphanizomenon ovalisporum* blooms, not *C. raciborskii*	Shaw et al. (1999)
Aquaculture pond, Queensland, Aug. 1997	589	550	39	*C. raciborskii*	Saker and Eaglesham (1999)
Farm dams, subtropical Australia	max. 800	not det.	not det.	*C. raciborskii*	Shaw et al. (2000)
Northern America					
Surface waters, FL, USA, 1999–2000	8.1–97.1	not det.	not det.	*C. raciborskii*	Burns et al. (2002)

not det.—not determined.

these artificial water bodies which are not used for production of drinking water or for recreation, there are only seven reports (including this study) providing volumetric data on natural waters (Table 3). Concerning the differentiation between dissolved and particulate CYN, the amount of quantitative data is even lower.

The dissolved CYN concentration may far exceed the particulate concentration. Since the particulate CYN concentration was low (between 0.002 and $0.484\,\mu g\,L^{-1}$), the dissolved CYN fraction alone was responsible for the values exceeding $1\,\mu g\,L^{-1}$. The proportion of dissolved CYN was more than 80% of CYN_{tot} in 31% of our samples. This corresponds to observations by Chiswell et al. (1999) and Shaw et al. (1999), who found 19% to almost 100% of total CYN dissolved. Chiswell et al. (1999) report that larger percentages of CYN were cell bound in younger cyanobacterial blooms, whereas a large

percentage of dissolved CYN was found in the water column in older blooms. The mechanisms of CYN release—active transport, leakage or release after break down of cells—still are not well studied, but in combination with the aforementioned mechanisms, the accumulation of dissolved CYN in the water column seems to be a result of the slow degradation of CYN in the water (Chiswell et al., 1999) as opposed to the faster degradation of other toxins like microcystin. High amounts and long persistence of dissolved CYN represent a high risk for human health since the toxin cannot be easily removed by simple drinking-water treatment methods such as flocculation, sedimentation and filtration. The decoupling of population growth, toxin release and toxin decomposition could be an explanation for the lower correlation coefficients of CYN_{diss} to cyanobacterial biovolume or abiotic parameters compared to CYN_{part} (Table 2).

After analyzing the abiotic and biotic conditions under which CYN is likely to occur, we found no significant correlations between the morphometric parameters of the lakes like area, volume or mean depth (data not shown) and CYN occurrence. Particulate CYN displayed positive correlations with total phosphorous, chlorophyll *a*, phytoplankton and cyanobacterial biovolume, and negative correlations with SD and I_{mix} (Table 2). However, the relatively low levels of correlation indicate that CYN occurrence is not confined to any specific type of lake. Hence, prediction of CYN concentrations based on morphometric or trophic parameters is not possible at present; however, the probability of occurrence of CYN is higher in eutrophic lakes than in other lakes. Exceptions to the general trend were observed, for example, Lake Vielitzsee (VI, Fig. 1), which was dominated by cyanobacteria but had almost no CYN, and Lake Großer Plessower See (GP), which was dominated by eukaryotic algae and had the highest mean SD (Table 1); CYN concentrations at GP were in the range of $1 \mu g\,L^{-1}$.

To achieve a better understanding of CYN occurrence and variability, it is necessary to find out which species are producing the CYN. Correlation analyses for particulate CYN and individual cyanobacterial genera or species showed highly significant correlations with the biovolumes of *Aphanizomenon* species in general and specifically with *A. gracile*, *P. agardhii* and *C. raciborskii*. However, at the species level, the correlation coefficients were relatively low. This suggests that the concentrations of particulate CYN varied per unit biovolume, indicating either that varying amounts of CYN were produced per unit biovolume or that CYN-producing and non-producing genotypes of single species occur and that the ratio of CYN-producing to CYN-non-producing genotypes varies. Secondly, more than one CYN producer may be present in a given sample.

In Europe, only two species have been proven to produce CYN: *A. lapponica* (Spoof et al., 2006) and *A. flos-aquae* (Preußel et al., 2006). *A. lapponica* was not present in any of our samples, and *A. flos-aquae* occurred in only 22 of 115 samples, 20 of which were CYN-positive. Consequently, if 102 samples contained CYN, the CYN producers in 82 samples remain unidentified. This means that there must be one or more other CYN producers in north European habitats. The highest correlation coefficient was found between CYN and *A. gracile*. Proof of CYN production by *A. gracile* has not been

demonstrated so far. Our suggestion that *A. gracile* produces CYN in German waters is supported by the fact that *A. gracile* was the only Nostocales species found in five of our field samples that contained CYN. No correlation was found between CYN and the biovolume of *A. flos-aquae*. The failure of correlation might be due to the low abundance of *A. flos-aquae*, especially CYN-producing genotypes, in the samples.

Furthermore, the fact that one CYN-positive sample contained *Anabaena* species (including *A. bergii*) as the only Nostocales strongly indicates *Anabaena* to be a CYN-producing genus. The significant correlation between CYN_{part} and *C. raciborskii* suggests that CYN-producing *C. raciborskii* strains may exist in German waters, but the low coefficient demonstrates that, if they do exist, they make up only a small part of the overall population. However, no CYN-producing *C. raciborskii* strain has been isolated from European waters to date (Fastner et al., 2003; Bernard et al., 2003; Saker et al., 2003). Also, since *C. raciborskii* was accompanied by other Nostocales in our samples, there was no proof that it was the source of CYN in the samples.

CYN_{part} correlated better with *P. agardhii* than with *C. raciborskii*. This could be by chance due to the co-occurrence of *Aphanizomenon* species and *P. agardhii* (correlation coefficient between both species 0.6). However, it might indicate that CYN-producing genotypes occur in German waters. If this were true, it could explain the occurrence of particulate CYN in two samples that contained only cyanobacteria of the order Oscillatoriales and Chroococcales but no Nostocales. However, these are speculations because no *Planktothrix* strains have yet been reported to be able to synthesize CYN. Seifert et al. (2007) found a benthic freshwater cyanobacterium of the order Oscillatoriales (*Lyngbya wollei*) that is able to produce CYN in Australian streams; this is the first indication that CYN might be also synthesized by non-heterocystous cyanobacteria.

In summary, our study demonstrates that CYN is widely distributed in German waters and that it has reached relevant concentrations close to or above $1 \mu g\,L^{-1}$, i.e., the value proposed by Humpage and Falconer (2003) as guideline value for drinking water. Therefore, we recommend the monitoring of CYN concentration in waters used for drinking water or recreational purposes, including lakes that appear to be clear and not dominated by cyano-

bacteria. Special attention should be placed on the dissolved fraction of CYN, which can contribute more than 99% of total CYN.

Our data show that, at this point, CYN concentrations cannot be predicted by any given specific environmental parameters or by the biovolume of certain cyanobacterial species. Our analyses identified the widespread native Nostocales species *A. gracile* as a new potential CYN producer in Europe. Even if proof of CYN production by this species can only be attained through strain isolation and genetic characterization, our data strongly suggest that CYN-producing cyanobacteria other than the currently known ones exist in German waters.

Q3 5. Uncited references

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Distribution and regulation of the originally tropical cyanobacterium *Cylindrospermopsis raciborskii* at its northern limits

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Cylindrospermopsis raciborskii is a filamentous freshwater cyanobacterium of tropical and subtropical origin that spread out up to northern temperate zone during the last decades. Belonging to the order Nostocales it can differentiate two types of non-vegetative cells: i) heterocysts, in which it can fix molecular nitrogen, and ii) akinetes, which are resting stages, that allow the species to pass-by unfavourable growth conditions in the sediment. In the tropics and subtropics, *C. raciborskii* is a perennial species, which often occurs in bloom densities, and which is prominent for the synthesis of various toxins including the potent hepatotoxin cylindrospermopsin (CYN) and neurotoxic paralytic shellfish poisoning toxins. In Europe, its northernmost populations were found in North German lakes. Here, it occurs only during the summer months and has not been observed to form mass developments. The highest documented biomass it attained was 23 % of the total phytoplankton biomass.

To investigate the spatial occurrence and relative frequency of *C. raciborskii*, we undertook a systematic survey, sampling 142 water bodies in the lowland region of Northeast Germany from June till September 2004. The cyanobacteria species present were analysed qualitatively and semi-quantitatively. *C. raciborskii* was a common species. It was present in 39 of the samples, of which 62 % were new findings. Further, we detected two additional thermophilic Nostocales that have not been reported from Germany before: *Anabaena bergii* and *Aphanizomenon aphanizomenoides*. These are the most northerly reports of both species so far. *A. bergii* occurred in 13 and *A. aphanizomenoides* in 19 of the 142 water bodies sampled.

To elucidate the regulation mechanisms of the population dynamics of *C. raciborskii* and its abiotic boundaries in the newly invaded habitats furthermore long-term data series (1993 - 2005) of two polymictic shallow lakes in the study area were analyzed. Population sizes largely varied between years without any distinct long-term trend. In the annual course, filaments of the species occurred in the pelagial at temperatures above 15 - 17 °C, i.e. akinetes must have been germinated at temperatures below 15 °C. Population growth started at high rates (1.5 - 2.8 d^{-1}) that declined continuously over the season. Growth rates were not significantly correlated with temperature but they were significantly positive correlated with the mean photosynthetic active radiation in the mixed water column (I_{mix}). At the time population starts growing, I_{mix} has exceeded its annual maximum, and growth rate declines with decreasing I_{mix}. The time of akinete germination is therefore of crucial importance for the success of the population: as earlier akinetes germinate the higher the growth rate will be, resulting in a greater population size, a higher number of akinetes produced, and vice versa. Therefore, we conclude that an earlier increase of the water temperature due to global warming is a major cause for the spreading of *C. raciborskii* into the temperate zone.

Distribution of three alien cyanobacterial species (Nostocales) in northeast Germany: *Cylindrospermopsis raciborskii, Anabaena bergii* and *Aphanizomenon aphanizomenoides*

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Cylindrospermopsis raciborskii is considered a cyanobacterium of tropical origin and an alien species to temperate waters. However, it has been detected as far north as northern Germany. While previous studies have shown that all isolated German *C. raciborskii* strains are hepatotoxic, little is known about the spatial occurrence and relative frequency of this species in temperate Germany. The aim of this study was to investigate the spatial distribution and relative frequency of *C. raciborskii* close to its northernmost distribution limit, to characterise the habitat in which it is most likely to occur in this climatic zone and to search for any other neocyanobacterial species that might be present in German waters but has so far been overlooked. One hundred forty-two water bodies in northeast Germany were sampled from June until September 2004. All cyanobacteria species were analysed qualitatively and semiquantitatively. Besides *C. raciborskii,* two additional neocyanobacterial species were detected: *Anabaena bergii* and *Aphanizomenon aphanizomenoides.* For both taxa, these findings represent their northernmost occurrence and their first report from German waters. *Cylindrospermopsis raciborskii* was present in 27%, *Anabaena bergii* in 9% and *Aphanizomenon aphanizomenoides* in 7% of the samples. The occurrence of each species was analysed in relation to maximum lake depth, Secchi depth, lake volume and lake surface area. All three species were present in a wide range of habitats, but *C. raciborskii* and *Anabaena bergii* occurred significantly more often in shallow, turbid waters than in deep, transparent water bodies. None of the parameters investigated were significantly correlated with the occurrence of *Aphanizomenon aphanizomenoides.* In conclusion, alien thermophilic cyanobacterial species are much more widely distributed in temperate Germany than previously known. The results are discussed with respect to the possible mechanisms that enable these organisms to expand northwards.

Key Words: *Anabaena bergii, Aphanizomenon aphanizomenoides, Cylindrospermopsis raciborskii,* Freshwater ecology, Cyanobacteria, Invasive species, Geographical distribution

INTRODUCTION

Little is known about the biogeography of freshwater cyanobacteria. For a long time it was believed that they were cosmopolitan species but it is now well established that most of them have either holarctic or pantropical distributions (Komárek 1994; Hoffmann 1996). Temperature and light are the environmental variables that vary most with latitude, and hence are the most important factors that determine the global distribution of freshwater cyanobacteria.

Cylindrospermopsis raciborskii (Woloszynká) Seenaya & Subba Raju 1972 (order Nostocales, formerly *Anabaena raciborskii* Woloszynká 1912 and *Anabaenopsis raciborskii* Elenkin 1923, Fig. 1a) is a filamentous freshwater cyanobacterium of tropical origin that does not seem to fit these presumptions. It increasingly expands to temperate latitudes (for a review see Padisák 1997; Saker *et al.* 2003; Wood & Stirling

2003; Hamilton *et al.* 2005; Manti *et al.* 2005) and is able to invade and sometimes even dominate the phytoplankton of temperate freshwater habitats (Chapman & Schelske 1997). Its current distribution reaches as far as northern Germany, where it has established populations (Wiedner *et al.* 2002; Mischke 2003; Nixdorf *et al.* 2003). Experimental comparisons of tropical and temperate *C. raciborskii* isolates revealed that all isolates have very similar light requirements for growth (Briand *et al.* 2004) and field studies disproved the idea that radiation conditions prevent *C. raciborskii* from colonizing temperate habitats (Dokulil & Teubner 2000; Mischke 2003). Similarly, laboratory studies showed that the optimal growth rate of *C. raciborskii* is between 25°C and 35°C independent of geographical origin (Saker & Griffiths 2000; Briand *et al.* 2004; Chonudomkul *et al.* 2004).

In the tropics and subtropics, *C. raciborskii* is a perennial species, which often occurs in bloom densities (e.g. Bouvy *et al.* 2000; Saker & Griffiths 2001), and which is prominent for the synthesis of various toxins including the potent hepato-

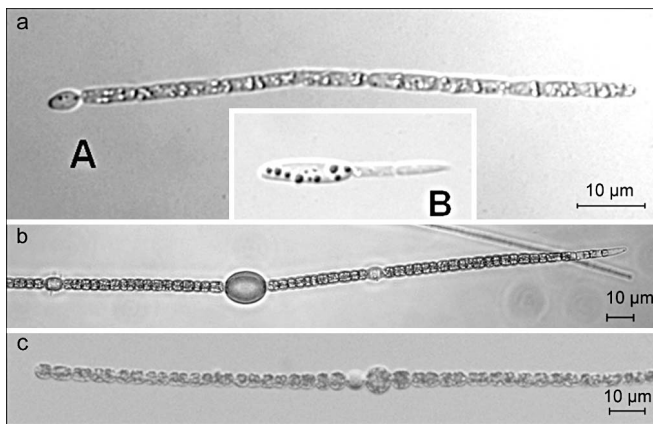* Corresponding author (stueken@igb-berlin.de).

Fig. 1. Photographs of three tropical cyanobacteria recorded in lakes of northeast Germany. (a) *Cylindrospermopsis raciborskii*: (A) filament with one flame-shaped heterocyst, (B) germinating akinete. (b) Filament of *Anabaena bergii* Ostenfeld with one akinete and two heterocysts. (c) Filament of *Aphanizomenon aphanizomenoides* with one heterocyst and one akinete.

Fig. 2. Overview and location of the study area.

toxin cylindrospermopsin (CYN; Ohtani *et al.* 1992; Li *et al.* 2001; Wood & Stirling 2003; Chonudomkul *et al.* 2004) and neurotoxic paralytic shellfish poisoning toxins (Lagos *et al.* 1999). In Germany, *C. raciborskii* occurs only during the warmer summer months and has not been observed to form blooms (Wiedner *et al.* 2002; Mischke 2003). However, it reached 24% of the total phytoplankton biomass in lake Scharmützelsee in 1999 (Nixdorf *et al.* 2003). All isolated German *C. raciborskii* strains are hepatotoxic; however, the toxin they produce has not yet been identified (Fastner *et al.* 2003).

Cylindrospermopsis raciborskii has been categorized as a neophyte (Mischke 2003), although cyanobacteria do not belong to the phylum Phyta or Viridiplantae, kingdom Eukaryota. They belong to the phylum Cyanobacteria, kingdom Eubacteria. Therefore, we suggest using the term neocyanobacteria as an analogue to the existing termini neophyta and neozoa.

The mechanisms that enable *C. raciborskii* to invade and to proliferate at high latitudes are still unresolved. There is controversial evidence whether its obvious ecological success is due to selection mechanisms (Chonudomkul *et al.* 2004), based on a wide physiological tolerance coupled with global warming (Briand *et al.* 2004), or due to radiation processes after the last glaciations (Gugger *et al.* 2005).

To evaluate the effects of neocyanobacterial species such as *C. raciborskii* on temperate ecosystems and to unravel the mechanisms that allow tropical cyanobacteria to invade and proliferate in temperate habitats, it is of prime importance to determine the extent of the problem. The data that exist on the distribution and frequency of *C. raciborskii* in Germany are limited to four well-studied lakes in the Scharmützelsee region (50 km southeast of Berlin, Germany; Wiedner *et al.* 2002; Mischke 2003; Nixdorf *et al.* 2003) and a single finding in lake Lieps in 1990 (100 km north of Berlin, Germany; Krienitz & Hegewald 1996).

Therefore, the aim of this study was to investigate for the first time the spatial distribution and relative frequency of *C. raciborskii* close to its northernmost occurrence, to characterise the habitat in which it is most likely to occur and to search
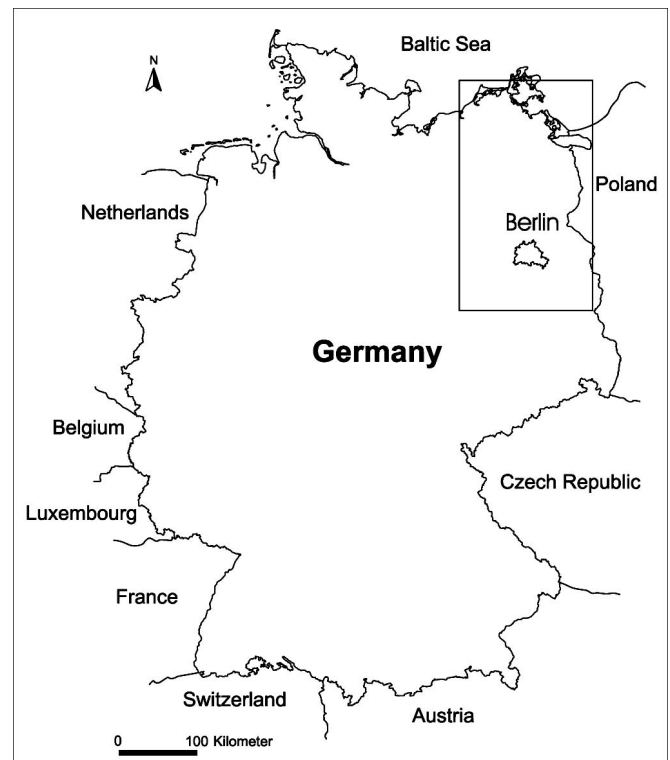
for any other neocyanobacterial species that might be present in German waters but has been overlooked so far.

MATERIAL AND METHODS

Sampling sites

The study area is located in the lowland region of northeast Germany (Fig. 2) and extends from 51°53′48″N, 14°09′57″E (South) to 54°25′32″N, 12°41′07″E (North) and from 52°44′29″N, 12°15′23″E (West) to 52°04′32″N, 14°28′55″E (East), an area that encompasses more than 5000 natural and artificial water bodies.

From the large number of water bodies, 142 sampling sites were chosen to include a wide range of lake types varying in morphometry (maximum depth, volume, surface area), mixing regime (dimictic and polymictic) and trophic status (oligotrophic to hypertrophic).

The morphometric data were extracted from a database at the chair of freshwater conservation of the Brandenburg Technical University of Cottbus, Germany (Nixdorf *et al.* 2004). The database includes morphometric data of all natural German lakes with a surface area $\geq$ 0.5 km^2. Data for smaller lakes were not always available.

Sampling

Lakes were sampled from the beginning of June until the end of September 2004. One hundred twenty-four lakes were sampled once, 15 were sampled twice (at the beginning and towards the end of the study period) and 3 were sampled monthly. Samples were taken at the deepest point of the lake, or

alternatively, at the middle of the lake. This was not possible at five sites; thus, samples were taken either from bridges or piers.

At each sampling site a series of phytoplankton net samples was taken by running the net vertically through the water column. Nets of 10-μm mesh size were used at oligotrophic and of 25-μm mesh size at mesotrophic to hypertrophic sites. Two 50-ml subsamples, of which one was preserved with formaldehyde (final concentration 4%), were taken and stored in opaque cool bags until arrival at the laboratory. The Secchi depth was determined using a white disc of 25 cm in diameter.

Phytoplankton analysis

The fresh phytoplankton material was analysed within 48 h of sampling using light microscopes (Nikon OPTIPHOT-2 and Zeiss Axioscope) at ×400–1000 magnification. For the measurement of cell dimensions photographs of formaldehyde-fixed samples (final concentration 4%) were taken with the digital microscope camera ColorView II (Olympus) on the Zeiss Axioscope and analysed with the AnalySIS 3.2 software package (Soft Imaging System).

Qualitative and semiquantitative analyses of all cyanobacteria were carried out. For the semiquantitative analysis, each species was assigned to one of the four following relative categories: absent (no filaments present), rare (only few filaments present, < 5% of all species), frequent (many filaments present, > 5%–< 50% of all species), or dominant (most prevalent filaments present, > 50% of all species). Cyanobacteria identification was carried out using the following literature: Geitler 1932; Huber-Pestalozzi 1938; Komárek & Ettl 1958; Horecká & Komárek 1979; Hindák 1992, 2000.

Additionally, sources of scientific data that have not been published in peer-reviewed journals were searched for reports of *C. raciborskii* and other neocyanobacteria species in Germany.

Map design

Maps were designed with ArcView GIS 3.2 (ESRI Inc.) using geographical coordinates in Grad, Word Geodetic System 84 (WGS 84). Background data were provided by the German Federal Environment Agency (Nixdorf *et al.* 2004) and included in a Microsoft Access 2002 database that was connected with the GIS (Hemm & Jönk 2004).

Statistical analysis

The following habitat characteristics were analysed: surface area (A), lake volume (V), maximum-depth (Z_{max}) and Secchi depth (Z_s).

Of those lakes that were sampled more than once, the data from the sampling date where *C. raciborskii* was most abundant were included in the analysis. The data were not normally distributed. Mann Whitney *U* tests were conducted to compare lakes in which each species occurred with those in which it did not. Spearman's rank-order correlations were carried out to test the interdependence of the variables. Statistical analysis was carried out using the software package SPSS 9.0 for Windows. Box plots were created using Microcal Origin 6.1.
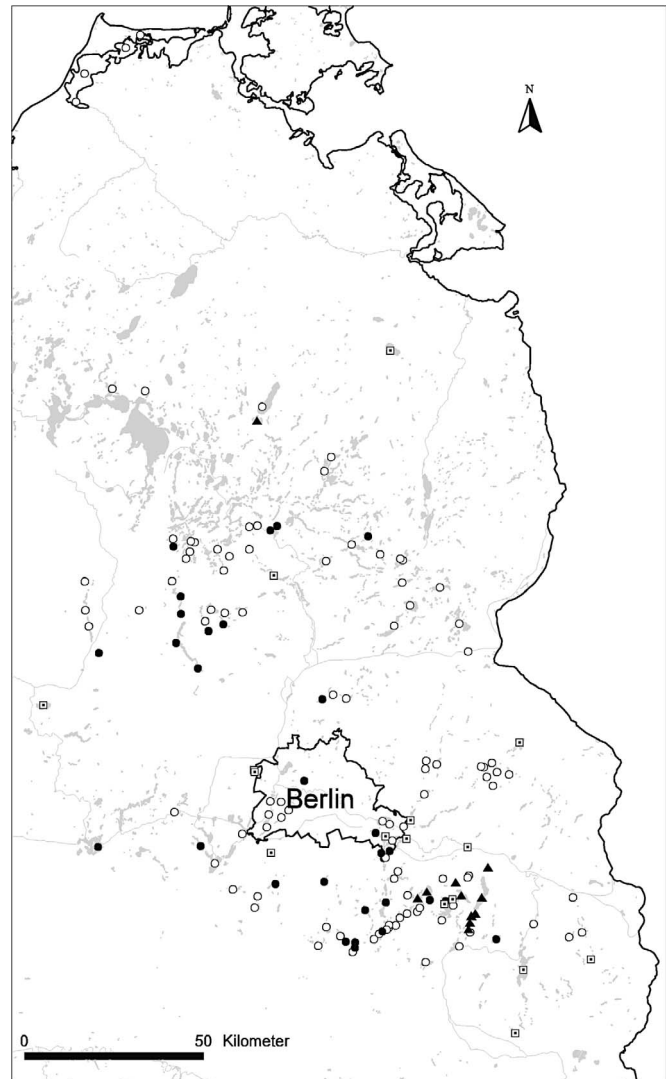
Fig. 3. Reports of *Cylindrospermopsis raciborskii*. Legend: ▲ *C. raciborskii* report prior to 2004; ○ lake sampled in 2004 but *C. raciborskii* not detected; ● lake sampled in 2004 and *C. raciborskii* detected; ⊡ report of *C. raciborskii* in non–peer-reviewed literature; ▬ borders; —— rivers; ▨ lakes.

RESULTS

Distribution of *C. raciborskii*

Cylindrospermopsis raciborskii is much more widely distributed in temperate waters than previously known (Fig. 3). It was detected in 39 of the 142 lakes sampled, and although it was never a dominant species it was considered frequent in 9 cases (Fig. 4). It was detected in all lakes previously published to contain *C. raciborskii* (Krienitz & Hegewald 1996; Wiedner *et al.* 2002; Nixdorf *et al.* 2003). The data survey of the non–peer-reviewed literature revealed 24 reports of *C. raciborskii* in Germany between 1994 and 2003 (Zippel 1996; Weithoff 1998; Mischke 2001; Täuscher 2003a, b, 2004a, b; Teubner *et al.* 2004). An additional nine reports were obtained through personal communications with U. Mischke and H. Täuscher. Of these 33 reports, 16 coincided with sampling sites of the present study, 10 of which were confirmed through the present

Fig. 4. Relative frequencies of *Cylindrospermopsis raciborskii, Anabaena bergii* and *Aphanizomenon aphanizomenoides* in the lake samples. A semiquantitative classification system was used.

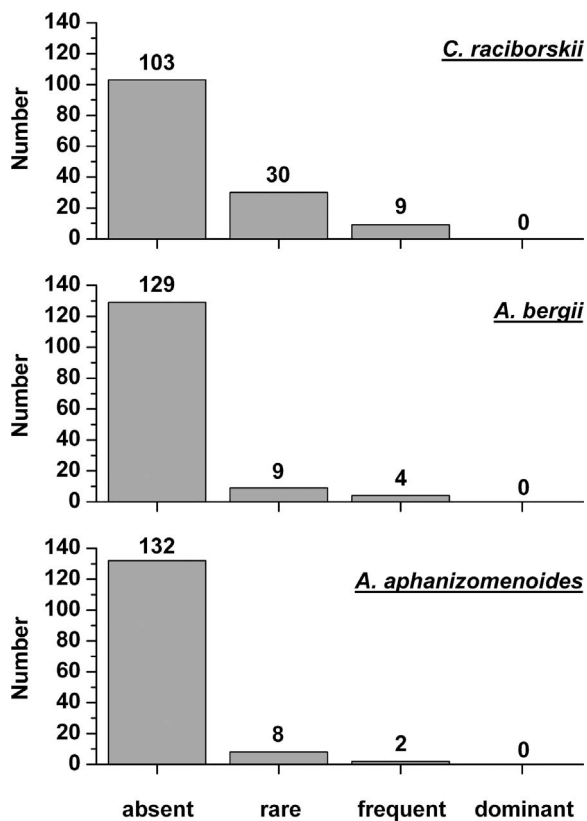**Fig. 5.** Comparison of maximum lake depth and Secchi depth between lakes in which *Cylindrospermopsis raciborskii, Anabaena bergii* and *Aphanizomenon aphanizomenoides* occurred and those in which they did not. Bars indicate maximum and minimum values, numbers above the bars indicate the number of sampling sites included. The box represents 95% of the values and is divided by the median.

study. The remaining 17 reports were additional findings. These 17 were not included in the habitat analysis due to a lack of accompanying data. In summary, *C. raciborskii* has been reported from 62 different water bodies in Germany.

Cylindrospermopsis raciborskii was distributed throughout the entire study area, and no distinct distributional pattern was observed (Fig. 3). It occurred in a wide range of habitats: in shallow and deep lakes, in lakes with relatively high and low Secchi depths (Fig. 5) and in lakes with a wide range of surface areas (range, 0.06–12.07 km^2) and volumes (range, 0.20–108.23 million m^3). However, the habitats in which *C. raciborskii* occurred differed significantly from those in which it did not. They were not as deep ($n = 132$, $Z = -2.871$, $P = 0.004$) and had a lower Secchi depth ($n = 136$, $Z = -3.861$, $P < 0.001$; Fig. 5). The variables Z_S and Z_{max} were highly correlated ($n = 127$, $r_S = 0.727$, $P < 0.001$). Volume and surface area had no significant effects on the occurrence of *C. raciborskii*.

Distribution of two as-yet-unreported cyanobacterial species in Germany

Two Nostocales species that until now have been unreported in Germany were detected during the present study: *Anabaena bergii* Ostenfeld 1908 (Fig. 1b) and *Aphanizomenon aphanizomenoides* (Forti) Horecká & Komárek 1979 (Fig. 1c).

Anabaena bergii was identified using the description provided by Hindák (2000). Synonyms are *Anabaena bergii* var. *minor* Kiselev and *Anabaena bergii* f. *minor* (Kiselev) Kosin-

skaja (Hindák 2000). The filaments were solitary, straight or slightly curved, usually slightly narrowing towards one or both ends, between 160 and 240 µm long and 4–6 µm wide. They had distinct, short, arrow-shaped apical cells, at least at one end of the filament. The filaments consisted of 32–57 vegetative cells that were barrel-shaped, generally shorter than wide and constricted at the cross-walls. They had one to three solitary spherical heterocysts with a diameter of 5–7 µm. Few filaments had akinetes. Akinetes were usually distant from the heterocyst, solitary, broadly oval to spherical and had a diameter of 11–12 µm.

Aphanizomenon aphanizomenoides was identified using the description provided by Horecká & Komárek (1979). Synonyms are *Aphanizomenon sphaericum* Kiselev and *Anabaena aphanizomenoides* Forti (Horecká & Komárek 1979). The filaments were solitary, straight and 140–270 µm long. Starting from the heterocysts, they gradually narrowed towards the ends. The apical cells were rounded, contrasting the distinctly arrow-shaped apical cells of *A. bergii*. The vegetative cells of *A. aphanizomenoides* were very variable in cell size and form, even within one filament. In general, the cells were barrel-shaped, rounded at the edges and shorter than wide. They were 4–6 µm wide and 3–6 µm long. The filaments had zero

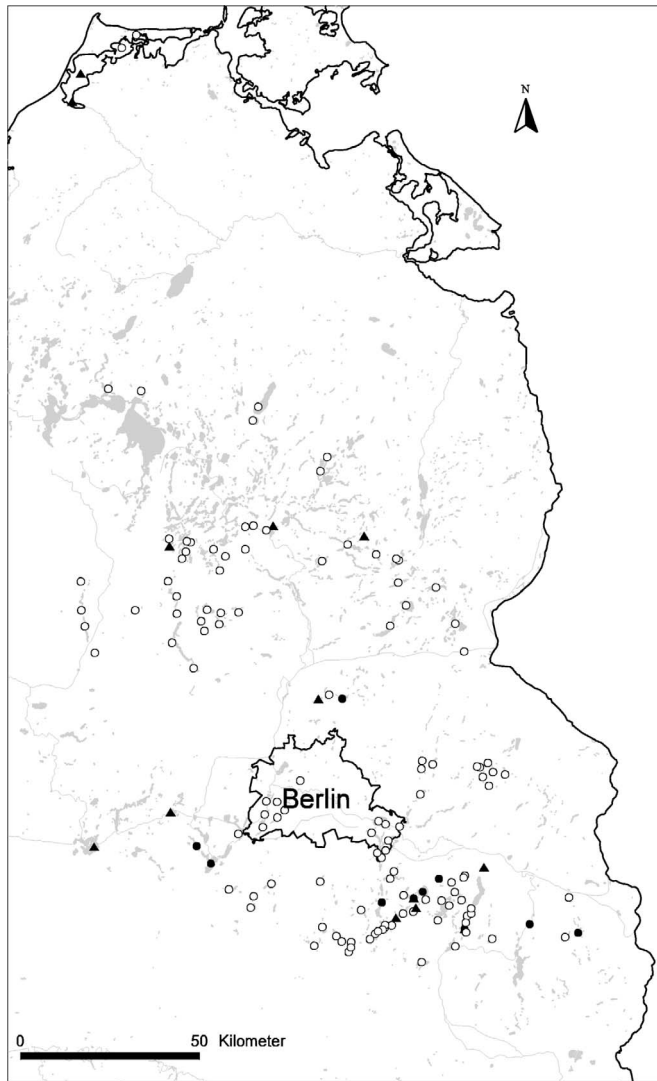**Fig. 6.** Reports of *Anabaena bergii* and *Aphanizomenon aphanizomenoides* during the present study. Legend: ○ lake sampled, but none detected; ▲ lake sampled and *A. bergii* detected; ● lake sampled and *A. aphanizomenoides* detected; ▬▬ borders; ─── rivers; ▨ lakes.

to three round- to oval-shaped heterocysts, each 5–6 μm wide and 5–6.5 μm long. Only filaments with young developing akinetes were present in the samples and thus, no description of the mature akinetes can be given here. All young akinetes observed were located directly next to the heterocysts. The akinetes were either solitary or two to three in a row, often one on each side of a heterocyst.

Anabaena bergii was detected in 13 out of 142 water bodies sampled, including 2 low-salinity sites (1–3 psu) of the Darss Zingst Bodden Chain, an innercoastal water system at the southern Baltic Sea coast. *Aphanizomenon aphanizomenoides* occurred at 10 freshwater locations (Fig. 6).

Anabaena bergii was considered to be present frequently in four cases and *A. aphanizomenoides* was considered to be present frequently in two cases. Neither of these species was observed to be the dominant species (Fig. 4). Like *C. raciborskii,* the lakes in which *A. bergii* occurred were significantly shallower than those in which it did not ($n = 132$, $Z = -2.590$, $P = 0.010$) and had a significantly lower Secchi

depth ($n = 136$, $Z = -2.281$, $P = 0.023$; Fig. 5). Neither the volume nor the surface area had significant effects on the occurrence of *A. bergii*. None of the parameters analysed had significant effects on the occurrence of *A. aphanizomenoides.*

DISCUSSION

Our results show that the originally tropical *C. raciborskii* is now a common cyanobacterial species in northeast Germany. It occurred in a wide range of habitats but was more likely to be encountered in shallow waters and in those with a high turbidity than in deep waters or in those with high transparency. However, the effects of depths and light climate could not be separated in this study. They were strongly correlated, suggesting that turbidity increased with shallowness.

It has previously been predicted that *C. raciborskii* would occur only in shallow lakes in temperate regions because these warm up quickly in early summer and thus provide the temperatures required for akinetes germination (Padisàk 1997). In the present study, *C. raciborskii* was detected at the centre of eight deep lakes ($Z_{max} > 10$ m), including the deep Scharmützelsee ($Z_{max} = 29.5$ m). These lakes are dimictic (i.e. thermally stratified in summer). It is possible that the *C. raciborskii* recruitment started in the shallower parts of the lakes and that the filaments were transported through the epilimnion to the centre once the summer stratification had set up. The conditions in the epilimnion of dimictic lakes are comparable to those in shallow polymictic lakes. Similar observations have been reported from the tropics, where *C. raciborskii* was most abundant in deep, stratified lakes (McGreggor & Fabbro, 2000).

In addition to *C. raciborskii* we detected two other neocyanobacteria species of the order Nostocales that have not been reported from Germany before: *Aphanizomenon aphanizomenoides* (Forti) Horecká & Komárek and *Anabaena bergii* Ostenfeld. Morphologically very similar species to *A. bergii* are *Anabaena minderi* Huber-Pestalozzi [= *Anabaena bergii* var. *limnetica* Couté & Preising (Hindák 2000)] and *Aphanizomenon ovalisporum* Forti. *Anabaena minderi* and *A. bergii* Ostenfeld (hereafter referred to as *A. bergii*) differ only in the size and form of their akinetes (Hindák 2000) and it has been suggested that *A. bergii* and *A. ovalisporum* might be morphotypes of the same species (Komárek & Ettl 1958). The same has been proposed for *A. minderi* and *A. ovalisporum* (Shaw *et al.* 1999; Fergusson & Saint 2000) and problems in attributing filaments from environmental samples to either species are very well documented (Bazzichelli & Abdelahad 1994). Considering these difficulties, it is possible that the species encountered during this study is elsewhere classified as *A. minderi* or *A. ovalisporum.* The latter is known to increase to bloom densities in Israel (Banker *et al.* 1997) and Australia (Shaw *et al.* 1999) and to produce the toxin CYN (Banker *et al.* 1997; Schembri *et al.* 2001). Strains of *A. bergii* have also been shown to synthesise CYN (Fergusson & Saint 2003). However, it is unclear whether strains of *A. bergii* Ostenfeld or of *A. bergii* var. *limnetica* Couté & Preising were analysed, and thus it remains uncertain which of the two species is capable of CYN synthesis. *Aphanizomenon aphanizomenoides* has also been shown to be toxic but the toxin it produces has not yet been identified (Hiripi *et al.* 1998).

The habitat preferences of *Anabaena bergii* observed in this study were similar to those of *C. raciborskii*. The lakes in which it occurred were significantly shallower and had a lower Secchi depth than those in which it did not. Neither of the parameters investigated had a significant effect on the distribution of *Aphanizomenon aphanizomenoides*.

Little is known about the distribution, physiology and ecology of both taxa. Both have predominantly been described from warmer climate zones such as the tropics, subtropics and southeastern Europe (Komárek & Ettl 1958; Horecká & Komárek 1979; Cirkaltindag *et al.* 1992). Within the last few years both species have also been reported from more temperate habitats: *A. aphanizomenoides* from the River Neuse estuary, North America (Moisander *et al.* 2002) and *Anabaena bergii* from Bratislava, Slovakia (Hindák & Hindáková 2003). However, to our knowledge, neither species has been reported from Germany or similar climatic zones before. In addition, these taxa have neither been mentioned in the relatively old but very comprehensive taxonomical guides (Geitler 1932; Hubert-Pestalozzi 1938; Komárek & Ettl 1958) nor in the more recent guides (Komarék 1999; Geissler & Kies 2003) that were assembled from the region investigated in this study. Thus, the data shown present the northernmost occurrence of *Aphanizomenon aphanizomenoides* and *Anabaena bergii*.

It is possible that these two thermophilic species are extending their distribution northwards like *C. raciborskii*. However, the lack of comprehensive data makes it impossible to draw an unambiguous conclusion, and the subsequent discussion will focus on *C. raciborskii*.

Three main theories exist to explain the mechanisms that enable the tropical *C. raciborskii* to proliferate in temperate regions: selection mechanisms (Chonudomkul *et al.* 2004), physiological tolerance coupled with global warming (Briand *et al.* 2004) and radiation after the last glaciations (Gugger *et al.* 2005). The second and third theories are similar in that they require a changing (i.e. warming) environment. They are supported by the finding that tropical and temperate isolates of *C. raciborskii* have the same optimal light (Briand *et al.* 2004) and temperature requirements for growth (Saker & Griffiths 2000; Briand *et al.* 2004; Chonudomkul *et al.* 2004). That they still occur at much lower water temperatures (Dokulil & Mayer 1996; Mischke 2003) can be interpreted as having a wide physiological tolerance. In contrast, Chonudomkul *et al.* (2004) found low temperature–adapted and -nonadapted *C. raciborskii* strains within a Thai population and only low temperature–adapted strains in a Japanese population. They concluded that different strains have different genotypes that enable the population as a whole to adapt to temperature variations and suggested that the proliferation of *C. raciborskii* in temperate regions can be explained through selection mechanisms. The observation that temperate isolates produce more akinetes under culture conditions than their tropical counterparts (Saker *et al.* 2003) supports this theory.

Komárek & Komárková (2003) point out that *C. raciborskii* is a very plastic species and the entire genus of *Cylindrospermopsis* is apparently diversifying. Several new species have been described recently (e.g. Watanabe 1995; Komárková-Legnerová & Tavera 1996; Couté & Bouvy 2004; Couté *et al.* 2004) but the variability and stability of the characters are not well known yet and require further investigation (Komárek & Komárková 2003). Thus it is likely that both phys-

iological plasticity and generic diversification play important roles in the northwards expansion of *C. raciborskii* and that both mechanisms interact.

All three species, *C. raciborskii, Anabaena bergii* and *Aphanizomenon aphanizomenoides,* are able to develop akinetes to survive unfavourable conditions. It might be possible that these resting cells play a key role in the invasion of tropical cyanobacteria into temperate habitats and are the reason why tropical species lacking resting stages have not been detected in temperate habitats.

This study documented that cyanobacteria that were formerly considered tropical in origin are much more widely distributed in temperate habitats than previously known. This highlights the importance of investigating the mechanisms that enable these organisms to expand into temperate habitats. Is it due to their great physiological tolerance, to their diversification capacities, to their ability to form akinetes, to a changing environment, or to a combination of any of these factors? Did the process start only within the last decades, and if so, why not earlier? Moreover, information on the ecology of the northern populations is crucial to evaluate their further development and any potential impact on local ecosystems.

ACKNOWLEDGEMENTS

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1   Phylogenetic characterisation of the three cyanobacteria species *Anabaena bergii*, *Aphanizomenon*

2   *ovalisporum* and *Aphanizomenon aphanizomenoides* (order Nostocales)

3

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18   <u>Running title</u>: Phylogeny of *Anabaena bergii*, *Aphanizomenon ovalisporum* and *Aphanizomenon*

19   *aphanizomenoides*

20   <u>Subject category</u>: Evolution, Phylogeny and Biodiversity

21   The GenBank accession numbers are:

Strain	16S rRNA gene	cpcBA-IGS
LIE01AB	EF529483	EF529471
LIE02AB	EF529484	EF529474
PMC215.03	EF529485	EF529472
ZIE26AB	EF529487	EF529473
22C4-9	EF529479	EF534274
KA1.1	EF529482	EF529470
UAM290	EF529489	EF529468
UAM291	EF529488	EF529469
10E6	EF529475	EF529463
10E9	EF529476	EF529464
14E6	EF529477	EF534275
21C5	EF529478	EF529467
22D11	EF529480	EF529465
30D11	EF529481	EF529462
22 ZIE25AFA	EF529486	EF529466

23 SUMMARY

24 The aim of this study was to clarify the phylogenetic position of the three heterocystous cyanobacteria

25 species *Anabaena bergii*, *Aphanizomenon ovalisporum* and *Aphanizomenon aphanizomenoides* within the

26 order Nostocales. We determined and phylogentically analysed 16S rRNA gene and *cpcBA*-IGS

27 sequences of four *A. bergii*, three *A. ovalisporum*, one *A. aphanizomenoides* and seven *Aphanizomenon*

28 sp. strains isolated from Spain, Germany, Israel and Senegal and complemented the analyses with

29 morphometric descriptions of these strains. The phylogenetic clustering did not follow the current

30 botanical classification. All three species clustered separately from the majority of *Anabaena* and

31 *Aphanizomenon* strains. *A. bergii* and *A. ovalisporum* clustered close to *Nodularia*, whereas the position

32 of the cluster containing the *A. aphanizomenoides* strain varied between the trees and the different tree

33 constructing methods used. In addition to *A. aphanizomenoides,* this cluster contained the two *Anabaena*

34 species *A. kisseleviana* and *A. oumina*. All three species had highly similar DNA sequences at the two

35 fragments analysed and thus, based on evolutionary distances, might be assigned to a single species.

36 Further, our results contradict the previously formulated suggestion that *A. bergii* and *A. ovalisporum* are

37 morphotypes of a single species. Instead, *A. bergii* and *A. ovalisporum* consistently formed separate

38 clusters, which were less than 96.6 % similar to each other based on 16S rRNA gene sequence analysis.

39 Our results support the idea that the taxonomy of heterocystous cyanobacteria should be revised, but also

40 emphasize the importance of detailed morphological information when molecular data of new strains is

41 used for taxonomy.

42

43 INTRODUCTION

44 The three heterocystous cyanobacteria species, *Anabaena bergii* Ostenfeld, *Aphanizomenon ovalisporum*

45 Forti and *Aphanizomenon aphanizomenoides* (Forti) Horecká & Komárek (order Nostocales), have

46 recently been reported for the first time from various Mediterranean countries and Germany: *A. bergii*

47 from Turkey (Cirk-Altindag *et al.* 1992), Italy (Bazzichelli & Abdelahad, 1994), Slovakia (Hindák &

48 Hindáková, 2003) and Germany (Stüken *et al.* 2006); *A. ovalisporum* from Italy (Bazzichelli &

49 Abdelahad, 1994), Israel (Banker *et al.* 1997, Pollingher *et al.* 1998), Greece (Gkelis *et al.* 2005) and

50 Spain (Quesada *et al.* 2006); and *A. aphanizomenoides* from Turkey (Cirk-Altindag *et al.* 1992), Morocco

51 (Sabour *et al.* 2005) and Germany (Stüken *et al.* 2006).

52   All three species are toxic: Australian and Israeli strains of *A. bergii* and *A. ovalisporum* produce the

53   hepatoxin Cylindrospermopsin (CYN; Banker *et al.* 1997, Shaw *et al.* 1999, Fergusson & Saint, 2003),

54   and strains of *A. aphanizomenoides* synthesise different variants of the toxin microcystin (Sabour *et al.*

55   2005) as well as other, so far unidentified, toxins (Hiripi *et al.* 1998). Further, blooms of the

56   *Aphanizomenon* species have been reported to frequently occur: *A. ovalisporum* in Israel (Pollingher *et al.*

57   1998), Australia (Shaw *et al.* 1999) and Spain (Quesada *et al.* 2006), and *A. aphanizomenoides* in

58   Morocco (Sabour *et al.* 2005). No mass developments of *A. bergii* have been reported yet. However, it

59   has been suggested that *A. ovalisporum* and *A. bergii* are morphological variants of the same species,

60   even though they have originally been assigned to two different genera: *Anabaena* and *Aphanizomenon*.

61   The suggestion has been based on morphological (Komárek & Ettl, 1958) as well as molecular analyses

62   (Fergusson & Saint, 2000), but has not satisfactorily been resolved yet. For water management purposes

63   as well as to be able to answer ecological and evolutionary questions, it is important to know how closely

64   the two potentially CYN-producing species *A. bergii* and *A. ovalisporum* are related genetically.

65   Heterocystous cyanobacteria form a monophyletic group according to 16S rRNA gene sequence data (for

66   a review see Hoffmann *et al.* 2005). Within the heterocystous cyanobacteria, the phylogenetic

67   relationships are frequently inconsistent with the current classification. The divison of heterocystous

68   cyanobacteria into the orders Stigonematales and Nostocales has not been supported by genetic analyses;

69   these orders have been shown to be intermixed (Gugger & Hoffmann, 2004). The same has been

70   demonstrated for the genera *Aphanizomenon* and *Anabaena* (Lyra *et al.* 2001, Gugger *et al.* 2002, Iteman

71   *et al.* 2002, Rajaniemi *et al.* 2005). Further, several phylogenetic studies have shown that strains of *A.*

72   *bergii* and *A. ovalisporum* cluster separately from the majority of *Anabaena* and *Aphanizomenon* strains

73   (Fergusson & Saint, 2000; Wilson *et al.* 2000; Kellmann *et al.* 2006). All of these studies rely on the

74   sequences of only one *A. bergii* (283A), and two *A. ovalisporum* (ILC-146 & APH028A) strains. No

75   information on the morphology of the *A. bergii* strain is given, and the 16S rRNA gene sequence of the *A.*

76   *ovalisporum* strain APH028A is only 477bp long. This lack of data precludes a rigorous phylogenetic

77   analysis of *A. bergii* and *A. ovalisporum*. In the case of *A. aphanizomenoides*, the third species recently

78   reported for the first time from European freshwaters, the former name *Anabaena aphanizomenoides*

79   Forti is still widely used in the literature (Hiripi *et al.* 1998; Moisander *et al.* 2002; Sabour *et al.* 2005)

80   but no molecular data exists to verify its phylogenetic standing.

81

82 The aim of this study was to clarify the phylogenetic postion of the three heterocystous cyanobacteria

83 species *Anabaena bergii*, *Aphanizomenon ovalisporum* and *Aphanizomenon aphanizomenoides* within the

84 order Nostocales. For this purpose 16S rRNA gene and *cpcBA*-IGS sequences of four *A. bergii*, three *A.*

85 *ovalisporum*, one *A. aphanizomenoides* and seven *Aphanizomenon* sp. strains isolated from Spain,

86 Germany, Israel and Senegal were determined and phylogenetically analysed and the analyses

87 complemented with a documentation of the characteristic strain morphologies.

88

89 METHODS

90 **Strain isolation, cultivation and identification.** Single filaments were isolated from phytoplankton net

91 samples (mesh size 25µm) using ultra-thin Pasteur pipettes and transferred to 96-well plates, filled with

92 300µl BG11 culture medium (Rippka *et al.* 1979) per well. Plates were incubated at 25°C and 65 $\mu Em^{-2}s^{-1}$

93 or 20 °C and 80 $\mu Em^{-2}s^{-1}$ and 12/12 h dark/light cycle. Successfully isolated strains were transferred into

94 5 ml-reaction tubes and in 50 ml Erlenmeyer flasks for cultivation under the same light and temperature

95 conditions. The final culture medium was either BG11 or O2 (Van Liere & Mur, 1978) and cultures were

96 maintained under continuous shake. Cyanobacteria identification was carried out according to: Geitler,

97 1932; Huber-Pestalozzi, 1938; Komárek & Ettl 1958; Horecká & Komárek 1979; Hindák, 1992, 2000.

98 Photographs were taken with an Olympus BX51 system microscope at 400 and 1000x magnification with

99 the Cell D software from Olympus Soft Imaging Solutions GmbH, Münster, Germany.

100

101 **DNA isolation, PCR amplification, and sequencing.** 16S rRNA gene and *cpcBA*-IGS sequences were

102 determined for the 15 strains listed in table 1. DNA was extracted from culture material using the Qiagen

103 Dynabeads kit. A 1226 bp long fragment of the 16S rRNA gene was amplified using the primers 16SF2

104 and R4, the *cpcBA*-IGS was amplified using primers PcβF and PcαR (Table 2). All PCR-reactions were

105 performed using a PTC-200 Peletier thermal cycler and carried out in final volumes of 50 µl reaction

106 mixture, containing 10 - 100 ng of template DNA, 1 x PCR-buffer (Qiagen, Hilden/Germany), 2.5 mM

107 $MgCl_2$, 20 pmol of each, 200 µM of each dNTP, and 1.5 U Qiataq DNA Polymerase (Qiagen, Hilden/

108 Germany). Thermal cycling conditions for the 16S rRNA gene were: 94°C/ 5 min, 30 x [94°C/30s, 55°C/

109 30s, 72°C/60s], 72°C/ 3 min, 4°C hold. Thermal cycling conditions for the *cpcBA*-IGS were: 96°C/ 5 min,

110   30 x [96°C/ 30s, 55°C/20s, 72°C/ 60s], 72°C/3 min, 4°C hold. The PCR-products were purified using the

111   QIAquick PCR purification kit (Qiagen), and sequenced with Big Dye Terminator Cycle Sequencing

112   Ready Reaction Kit 3.1 (Applied Biosystems, Darmstadt) according to the manufacturers' protocols.

113   Sequencing was carried out using an ABI Prism 3100-Avant Genetic Analyzer. Both DNA strands were

114   sequenced, and several internal primers were used to sequence the 16S rRNA gene (Table 2). Accession

115   numbers see table 1.

116

117   **Sequence alignment and phylogenetic analyses.** The sequences were aligned with ClustalX 1.83

118   (Thompson *et al.* 1997) and edited manually using Align (Hepperle, 2000). Hyper variable and

119   ambiguous sites as well as poorly aligned columns (i.e., most of the IGS between *cpcB* and *cpcA*) were

120   removed. The alignments used in phylogenetic analyses consisted of 959 (16S rRNA gene sequences) and

121   388 (*cpcBA*-IGS sequences) nucleotide positions.

122   Trees based on either alignment were constructed by neighbour-joining (NJ), maximum-parsimony (MP)

123   and maximum likelihood (ML) algorithms in the program PAUP 4 beta 10 Win (Swafford, 1998). For

124   ML, the evolutionary model of substitution was evaluated by MODELTEST v3.7 (Posada & Crandall,

125   1998). The models used were Jukes and Cantor pair wise distances for NJ and the TrN+I+G model of

126   evolution for the ML. For NJ and MP analyses, 1000 bootstrap and for ML, 100 bootstrap values were

127   performed. The bootstrap values are given at the nodes of the trees (Fig. 1 & Fig. 2).

128   To obtain comparable trees, we only included those strains, for which sequences of both gene fragments

129   (*cpcBA*-IGS, 16S rRNA gene) were available from Genbank. The only exceptions were the *A. bergii*

130   strain 283A and the *A. ovalisporum* strain ILC-146, for which only 16S rRNA gene sequences were

131   available. If multiple sequences of the same strain and fragment were present in Genebank, the sequences

132   were compared. If the sequences were identical, then one was chosen at random to be included in the

133   analysis; otherwise, the strain was excluded. Likewise, if species designation was ambiguous, the strain

134   was excluded from the analysis. Sequences of both gene fragments were only available for one

135   *Aphanizomenon* sp. strain (strain TR 183). To adequately represent the genus *Aphanizomenon* in the

136   analyses, the 16S rRNA gene and *cpcBA*-IGS sequences of seven *Aphanizomenon* sp. strains isolated

137   from various German lakes were determined and included in the phylogentic analyses. The

138   *Synechococcus sp.* MW28B3 was used as outgroup.

139

140   RESULTS AND DISCUSSION

141   **Phylogenetic trees.** Four main and five sub-clusters were consistently formed in the analysis of 16S

142   rRNA gene and *cpcBA* (Fig. 1 & 2). Cluster 1 contained all *Cylindrospermopsis* strains, cluster 2 the

143   German *Aphanizomenon aphanizomenoides*, one *Anabaena oumiana* and two *Anabaena kisseleviana*

144   strains, cluster 3 all *Nodularia*, *Anabaena bergii* and *Aphanizomenon ovalisporum* strains and cluster 4

145   the remaining *Anabaena* and *Aphanizomenon* strains. Cluster 3 was subdivided into three and cluster 4

146   into two sub-clusters. All clusters and sub-clusters were stable in both trees and under the different tree-

147   constructing methods applied. In contrast, the tree topologies of both trees and between the different tree-

148   constructing methods were inconsistent.

149   According to Wilmotte and Herdman (2001), inconsistencies in branching order occur quite often when

150   the same sequences are analysed by different tree-constructing methods. These inconsistencies make it

151   difficult to interpret the hierarchical arrangement of taxa. Stable groups of sequences, however, which

152   consistently occur at the tips of branching regardless of the tree-constructing method used, indicate

153   relatedness and true phylogenetic significance (Wilmotte & Herdman, 2001). Thus, we are confident that

154   the clusters obtained in our analysis represent closely related taxa.

155

156   ***Aphanizomenon aphanizomenoides.*** The *Cylindrospermopsis* (cluster 1) and the *A. aphanizomenoides/*

157   *A. oumiana/ A. kisseleviana* cluster (cluster 2) were the main causes for the variable branching pattern,

158   they "jumped" within the trees; i.e. their positions within the trees were not fixed. For cluster 1, these

159   results are in agreement with those obtained by Iteman *et al.* (2002) who found that the

160   *Cylindrospermopsis* cluster was variable within the 16S rRNA gene phylogenetic tree, but never grouped

161   with other organisms, and concluded that *Cylindrospermopsis* is genetically distant from other planktonic

162   heterocystous cyanobacteria. For cluster 2, these results are novel. Cluster 2 is made up of four strains

163   that have been attributed to three different species and two different genera based upon morphological

164   criteria. In contrast, the molecular data shows that all four strains have virtually identical DNA sequences

165   at the two fragments analysed (table 4). If the boundary for species circumscription of > 97 % 16S rRNA

166   gene similarity (Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001) is applied, then all strains

167   of cluster 2 might be considered to be the same species. This view is supported by the observation that *A.*

168   *kisseleviana* and *A. oumiana* have very similar DNA base compositions (Li & Watanabe, 2002). Further,

169   all three species, *A. aphanizomenoides*, *A. kisseleviana* and *A. oumiana*, share one distinct morphological

170   characteristic: they all have spherical akinetes adjacent to their heterocysts, either on one or on both sides

171   (Horécka & Komárek, 1979; Watanabe, 1996; Li *et al.* 2000). Akinete parameters have previously been

172   shown to be useful for the classification of selected *Anabaena* and *Aphanizomenon* strains (Rajaniemi *et*

173   *al.* 2005). The main morphological difference between the three species is that *A. oumiana* has coiled and

174   *A. aphanizomenoides* and *A. kisseleviana* have straight trichomes. Similar differences in filament form are

175   also known from other species of Nostocales. Strains of *C. raciborskii* have been shown to be almost

176   identical at their 16S rRNA gene sequences, even though their filament morphologies differed

177   significantly; filaments were either straight or coiled (Saker *et al.* 1999).

178   Pictures of *A. aphanizomenoides* with its characteristic akinetes next to the heterocyst are given in Fig. 3a

179   and in Stüken *et al.* (2006). These pictures are taken from field samples, not from strain 22C4-9. The

180   morphologies of 22C4-9 and the field populations photographed are alike, only that strain 22C4-9 rarely

181   develops more than one akinete under culture conditions. The main morphological characteristics of *A.*

182   *aphanizomenoides* are given in table 3. Pictures and descriptions of *A. oumiana* can be found in Watanabe

183   (1996) and of *A. kisseleviana* in Li *et al.* (2000).

184   *Aphanizomenon aphanizomenoides* clusters separately from *Anabaena* and *Aphanizomenon*, the two

185   genera to which it has originally been assigned; and with *A. aphanizomenoides* the remaining strains of

186   cluster 2. Further, based on sequence similarity, cluster 2 is only as similar to the *Anabaena*/

187   *Aphanizomenon* cluster (cluster 4) as it is to the *Cylindrospermopsis* (cluster 1) or the *Nodularia*/ *A.*

188   *bergii*/ *A. ovalisporum* cluster (cluster 3; table 4).

189

190   **Anabaena bergii and Aphanizomenon ovalisporum.** All *Anabaena bergii* and *Aphanizomenon*

191   *ovalisporum* strains clustered separately from *Anabaena* and *Aphanizomenon*. Instead, they clustered

192   close to *Nodularia*. This supports the previous suggestion that *A. bergii* and *A. ovalisporum* may have

193   been taxonomically misclassified (Kellmann *et al.* 2006).

194   Our results, however, challenge the suggestion based on *rpoC1* (Fergusson & Saint, 2003) and 16S rRNA

195   gene sequence analyses (Schembri *et al.* 2001) that *A. bergii* and *A. ovalisporum* are morphotypes of the

196   same cyanobacterium. The *A. ovalisporum* and *A. bergii* strains analysed in this study formed distinct

197   sub-clusters, which were less than 96.6 % similar based on 16S rRNA gene sequence comparisons (table

198   3). Sequence similarity within the two sub-clusters was notably higher. The only exception was the *A.*

199   *bergii* strain 283A, which clustered with the *A. ovalisporum* strains. The three *A. ovalisporum* strains

200   sequenced had identical 16S rRNA gene, *cpcBA* and intergenic spacer (IGS) sequences. Their 16S rRNA

201   gene sequence was identical to that of *A. bergii* 283A, differed only slightly from that of the Israeli *A.*

202   *ovalisporum* strain ILC-146 (Fig. 1) and from the Australian *A. ovalisporum* strain APH028A

203   (AF044270, 16S rRNA gene sequence was only 477 bp long). Likewise, three of the four sequenced *A.*

204   *bergii* strains had identical 16S rRNA gene sequences, which differed only slightly from the fourth strain,

205   LIE01AB. The *cpcBA* region of the *A. bergii* strains was more variable than of the *A. ovalisporum* strains.

206   The IGS sequences of all *A. bergii* strains were identical and 22bp longer compared to those of *A.*

207   *ovalisporum*. The two different IGS sequences of *A. bergii* and *A. ovalisporum* were not alignable. These

208   differences in sequence length and order support the notion that *A. bergii* and *A. ovalisporum* should be

209   considered as separate species. The length of the IGS has been shown to adequately reflect phylogenetic

210   groups of picocyanobacteria (Crosbie *et al.* 2003), and comparable sequences were useful in studying

211   relationships within genera of Oscillatoriales (Manen & Falquet, 2002) and Nostocales (Dyble *et al.*

212   2002). It is noteworthy that the IGS sequences, which are thought to be highly variable due to their non-

213   coding character, are identical between strains isolated from as far apart as Senegal and Germany or as

214   Israel and Spain, but are significantly different between strains isolated from within Europe, that have

215   been proposed to be morphotypes of a single species.

216   Our genetic results are supported by the distinctive morphology of the *A. bergii* and *A. ovalisporum*

217   strains analysed. The main morphological features of each are given in table 3. All strains ascribed to *A.*

218   *bergii* were morphologically indistinguishable and likewise, all *A. ovalisporum* strains looked alike under

219   culture conditions. Thus, only pictures of one representative strain of each species are given (*A. bergii*:

220   Fig. 3b, e, f, g; *A. ovalisporum*: Fig. 3c, h, i, j). The vegetative cells in *A. bergii* were shorter than wide,

221   whereas in *A. ovalisporum*, the vegetative cells were mostly as long as or longer than wide. The most

222   striking difference between *A. bergii* and *A. ovalisporum* were the terminal cells. *A. bergii* had clearly

223   arrow-shaped pointed terminal cells, whereas those of *A. ovalisporum* were rounded at the tips. This is in

224   agreement with field observations from Shaw *et al.* (1999), who described *A. ovalisporum* from Lake

225   Kinneret with rounded terminal cells.

226   The 16S rRNA gene sequence of *A. bergii* strain 283A is genetically identical to the 16S rRNA gene

227   sequence of the three *A. ovalisporum* strains analysed in this study and clearly separate from the other *A.*

228   *bergii* strains (Fig. 1). No morphological description of this strain is available from the studies it has been

229   used in (Schembri *et al.* 2001; Fergusson & Saint, 2003). Based on the sequence data, we suggest that the

230   *A. bergii* strain 283A might have been misidentified. Its morphology should be checked and, if our

231   suspicion proves correct, should be re-named *A. ovalisporum*. A misidentification of strain 283A as *A.*

232   *bergii* would explain why other authors suggested that *A. bergii* and *A. ovalisporum* are morphotypes of

233   the same species (Schembri *et al.* 2001; Fergusson & Saint, 2003), a suggestion that is clearly rebutted by

234   our analyses.

235

236   ***Aphanizomenon* sp. strains.** Cluster 4 contained two sub-clusters, one containing all remaining

237   *Anabaena* and the other all remaining *Aphanizomenon* strains (Fig.1 & 2). However, the sequence-

238   similarity between the sub-clusters was high (> 97.5 %). Thus, the observed sub-clustering may be due to

239   artefacts of sequence choice, specifically to the close relatedness of the *Aphanizomenon* sp. strains.

240   Therefore, our results are in agreement with earlier conclusions that the genera *Anabaena* and

241   *Aphanizomenon* are intermixed (Lyra *et al.* 2001, Gugger *et al.* 2002, Iteman *et al.* 2002, Rajaniemi *et al.*

242   2005). All strains were originally either isolated as *Aphanizomenon gracile* (strain 21C5, ZIE25AFA) or

243   as *Aphanizomenon flos-aquae* (strains 22D11, 10E6, 10E9, 14E6, 30D11). However, under culture

244   conditions all strains looked morphologically alike and a distinction between *A. flos-aquae* and *A. gracile*

245   was not possible. Thus, we choose to classify them as *Aphanizomenon* sp. Figure 3d and Fig. 3k depict

246   strain 30D11. The cell morphologies of the vegetative and of the end cells are clearly different from those

247   of *A. aphanizomenoides, A. bergii* and *A. ovalisporum*.

248

249   **Conclusions.** Our results show that the phylogenetic relationships of the strains analysed are in conflict

250   with their traditional classification. The genera *Anabaena* and *Aphanizomenon* were paraphyletically

251   distributed. Strains of both genera isolated from as far apart as Japan and Germany and morphologically

252   corresponding to three different species, were virtually identical in their *cpcBA*-IGS and 16S rRNA gene

253   sequences. The cluster that contained these strains was only as closely related to the main

254   *Aphanizomenon*/*Anabaena* cluster, as it was to the other two clusters, which contained strains classified

9

255 as belonging to the genera *Nodularia* and *Cylindrospermopsis* based on morphology. Further, strains

256 morphologically corresponding to the species *Anabaena bergii* and *Aphanizomenon ovalisporum* shared

257 much higher DNA sequence similarities with strains corresponding to the genus *Nodularia*, than with

258 strains within the main *Aphanizomenon*/*Anabaena* cluster. Thus, our results strengthen the proposition

259 that the taxonomy of the heterocystous cyanobacteria, and especially of the genera *Anabaena* and

260 *Aphanizomenon*, need to be revised. In addition, our results stress the importance of including a minimum

261 of both, genetic and morphological information when data of novel cyanobacteria strains are published.

262 This will aid to avoid misidentification and confusion, when data of different origins are compared.

263

272

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391   Table 1: List of strains sequenced during this study. Accession numbers of the 16S rRNA gene and

392   *cpcBA*-IGS sequences, the country of origin and the cluster number of each strain are given. * obtained

393   from the Algothèque du Laboratoire de Cryptogamie, Muséum National d'Histoire Naturelle, Paris,

394   France ; † strain provided by Aaron Kaplan, The Hebrew University of Jerusalem, Jerusalem, Israel;

395

396   Table 1:

Species	Strain	Country	Location	16S rRNA gene	cpcBA-IGS	Cluster
Anabaena bergii						
	LIE01AB	Germany	Lake Lieps	EF529483	EF529471	3.3
	LIE02AB	Germany	Lake Lieps	EF529484	EF529474	3.3
	*PMC215.03	Senegal	Lake Guiers	EF529485	EF529472	3.3
	ZIE26AB	Germany	Lake Zierker See	EF529487	EF529473	3.3
Aphanizomenon aphanizomenoides						
	22C4-9	Germany	Lake Heiliger See	EF529479	EF534274	2
Aphanizomenon ovalisporum						
	†KA1.1	Israel	Lake Kinneret	EF529482	EF529470	3.2
	UAM290	Spain	Juan Carlos First Park	EF529489	EF529468	3.2
	UAM291	Spain	Juan Carlos First Park	EF529488	EF529469	3.2
Aphanizomenon sp.						
	10E6	Germany	Lake Melangsee	EF529475	EF529463	4.2
	10E9	Germany	Lake Melangsee	EF529476	EF529464	4.2
	14E6	Germany	Lake Melangsee	EF529477	EF534275	4.2
	21C5	Germany	Lake Zermützelsee	EF529478	EF529467	4.2
	22D11	Germany	Lake Heiliger See	EF529480	EF529465	4.2
	30D11	Germany	Lake Petersdofer See	EF529481	EF529462	4.2
	ZIE25AFA	Germany	Lake Zierker See	EF529486	EF529466	4.2

397

398

399   Table 2: Primer employed during this study.

400   Table 2:

Primers	Sequence (5' - 3')	Reference
16S rRNA gene		
16SF2	GAAGAGCTTGCGTCTGATTA	This study
530f	GTGCCAGCAGCCGCGG	Neilan et al. 1997
601f = F3	GTGTAGCGGTGAAATGCGTA	Li et al. 2001
942f	GGGCCCGCACAAGCGG	Neilan et al. 1997
R4	TACGGCTACCTTGTTACGAC	Li et al. 2001
683R = F3reverse	TCTACGCATTTCACCGCTACAC	Li et al. 2001
S10r	CCGTCAATTCCTTTGAGTT	Iteman et al. 2002
Phycocyanin Locus (cpcBA-IGS)		
pcbβF	GGCTGCTTGTTTACGCGACA	Neilan et al. 1995

401

402

403   Table 3:

404   Table 3: Main morphological characteristics of the *Anabaena bergii* and *Aphanizomenon*

405   *aphanizomenoides* populations from Germany and the *Aphanizomenon ovalisporum* populations from

406   Israel and Spain.

Species	Filament	Vegetative cells	Heterocysts	Akinets	References
Aphanizomenon ovalisporum from Spain	Free floating; solitary; unbranched; straight or slightly curved, often narrowing towards the end	With gas vesicles; barrel shaped, longer than wide; 4.9 - 9.5 µm long, 3.9 - 4.9 µm wide; end cells hyaline (1 or 2 cells), 8.6 µm long, 2.45 µm wide	Solitary; intercalary; 5 - 8 cells distant from the akinets; spherical; 4.6 µm diameter	Solitary; intercalary; oval; 11.2 µm long, 9.7 µm wide	This study
Aphanizomenon ovalisporum from Israel	Free floating; solitary; unbranched; constricted at the cross walls	With gas vesicles; 5 - 13.75 µm long, 5 - 6.25 µm wide; apical cells rounded; apical cells in some trichomes hyaline	Solitary; intercalary; 5 -11.5 µm long, 5 - 7.5 µm wide	Solitary; intercalary; oval; distant from heterocysts; 10 -12.5 µm in diameter	Banker *et al.* 1997; Shaw *et al.* 1999
Anabaena bergii from Germany	Free floating; solitary; unbranched; straight or slightly curved; usually slightly narrowing towards the ends	With gas vesicles; short barrel shaped; shorter than wide; 2.8 - 5.2 µm long, 3.6 - 6.6 µm wide; one or both apical cells distinctly attenuated; some of the apical cells hyaline	Solitary; intercalary; 1 - 3 per filament; generally distant from the akinets (10 - 22 cells); roundish; 4.0 - 8.7 µm diameter	Solitary; intercalary; oval - cylindrical; 11-20 µm long, 10-16 µm wide	This study
Aphanizomenon aphanizomenoides from Germany	Free floating; solitary; unbranched; straight or slightly curved; slightly narrowing towards the ends	With gas vesicles; very variable in form and size; generally roundish barrel shaped, shorter than wide; 2.5 - 7.8 µm long, 2.7 - 7.2 µm wide; apical cells rounded	Solitary; intercalary; 0 - 3 per filament; round-oval; 4.2 - 8.6 µm long, 3.9 - 8.3 µm wide	Solitary or several in a row; intercalary; directly next to the heterocysts, either on one or both sides; spherical; 9.2 -13.5 µm diameter	This study

407

408   Table 4: 16S rRNA gene similarities in %, based on 959bp alignment.

409   Table 4:

Cluster	1	2	3.1	3.2	3.3	4.1	4.2
1	99.58 - 99.90						
2	96.25 - 96.66	99.9 - 100					
3.1	93.12 - 93.95	94.26 - 94.58	98.75 - 100				
3.2	94.37 - 94.79	95.10 - 95.41	96.66 - 97.18	99.58 - 100			
3.3	94.47 - 94.89	93.64 - 93.85	96.87 - 97.29	96.14 - 96.56	99.79 - 100		
4.1	93.85 - 94.79	93.95 - 94.16	94.79 - 95.72	94.37 - 95.72	94.47 - 95.31	98.44 - 99.79	
4.2	93.95 - 94.79	94.06 - 94.37	95.10 - 95.62	95.10 - 95.52	94.58 - 94.89	97.60 - 98.23	99.17 - 100

410

411   Figure 1: Neighbour-joining Jukes- Cantor phylogenetic tree based on the 959bp long alignment of partial

412   16S rRNA gene sequences. Bootstrap values of NJ, MP and ML analyses are given at the nodes

413   respectively. Strain names in **bold**: sequenced during this study. * Strain NIES-73 as *Anabaena flos-*

414   *aquae* in Genebank, as *Anabaena oumiana* in Watanabe (1996). † KA1.1 is a re-isolate of ILC-146,

415   originally isolated by Banker *et al.* 1997. ILC-146 was not a single genetic strain. It was made unialgal

416   and axenic by the laboratory of A. Kaplan, The Hebrew University of Jerusalem, Jerusalem, Israel. The

417   observed difference between KA1.1 and ILC-146 is probably due to this singling process.

418   Figure 1:
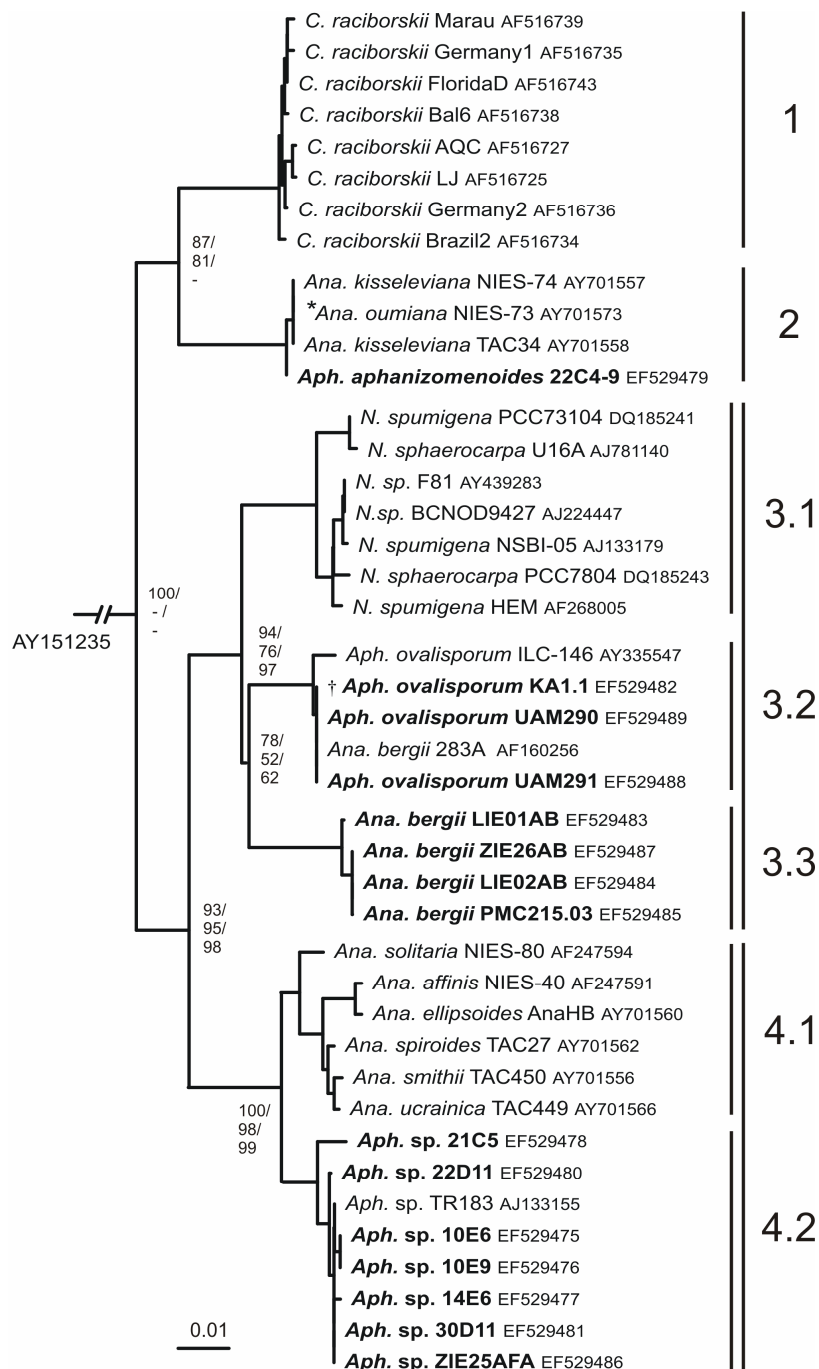
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420   Figure 2: Neighbour-joining Jukes- Cantor phylogenetic tree based on the 388bp long alignment of partial

421   *cpcBA*-IGS sequences. Bootstrap values of NJ, MP and ML analyses are given at the nodes respectively.

422   Strain names in **bold**: sequenced during this study. * Strain NIES-73 as *Anabaena flos-aquae* in

423   Genebank, as *Anabaena oumiana* in Watanabe (1996).

424   Figure 2:
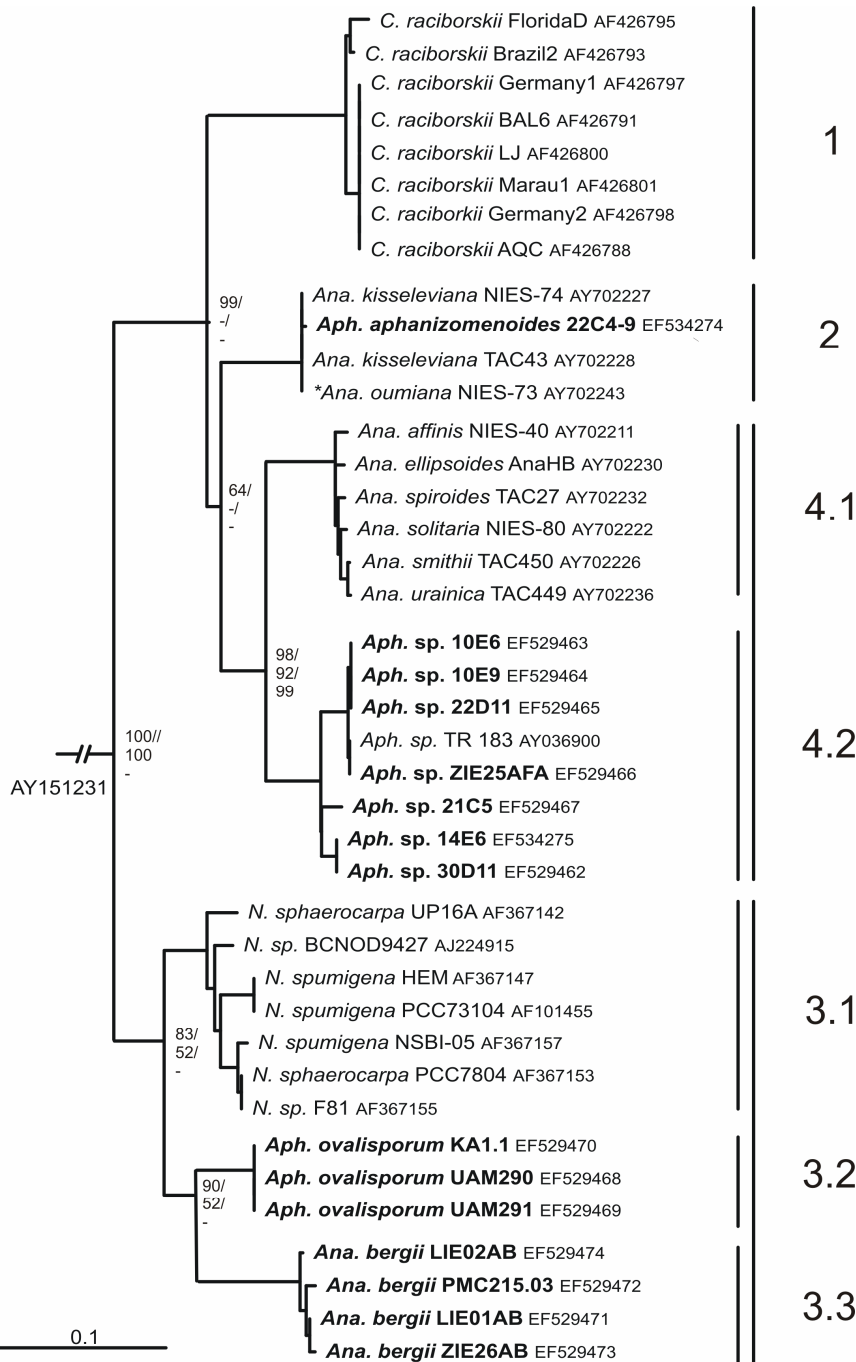
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426 Figure 3: Photographs of *Aphanizomenon aphanizomenoides*, *Anabaena bergii* and *Aphanizomenon*

427 *ovalisporum*; each bar represents 10 µm: a) *A. aphanizomenoides* with two akinets on either side of the

428 heterocyst, taken from an environmental sample of Lake Müggelsee, Berlin, Germany; b, e) filaments of

429 *A. bergii* strain ZIE26AB, in Fig. b each with one heterocyst; c, j) filaments of *A. ovalisporum* strain

430 KA1.1 with heterocyst; d, k) filaments of *Aphanizomenon* sp. strain 30D11 with heterocysts; f, g) akinets

431 of *A. bergii* strain ZIE26AB; h, i) akinets *A. ovalisporum* filaments from field samples from Spain.

432 Figure 3:
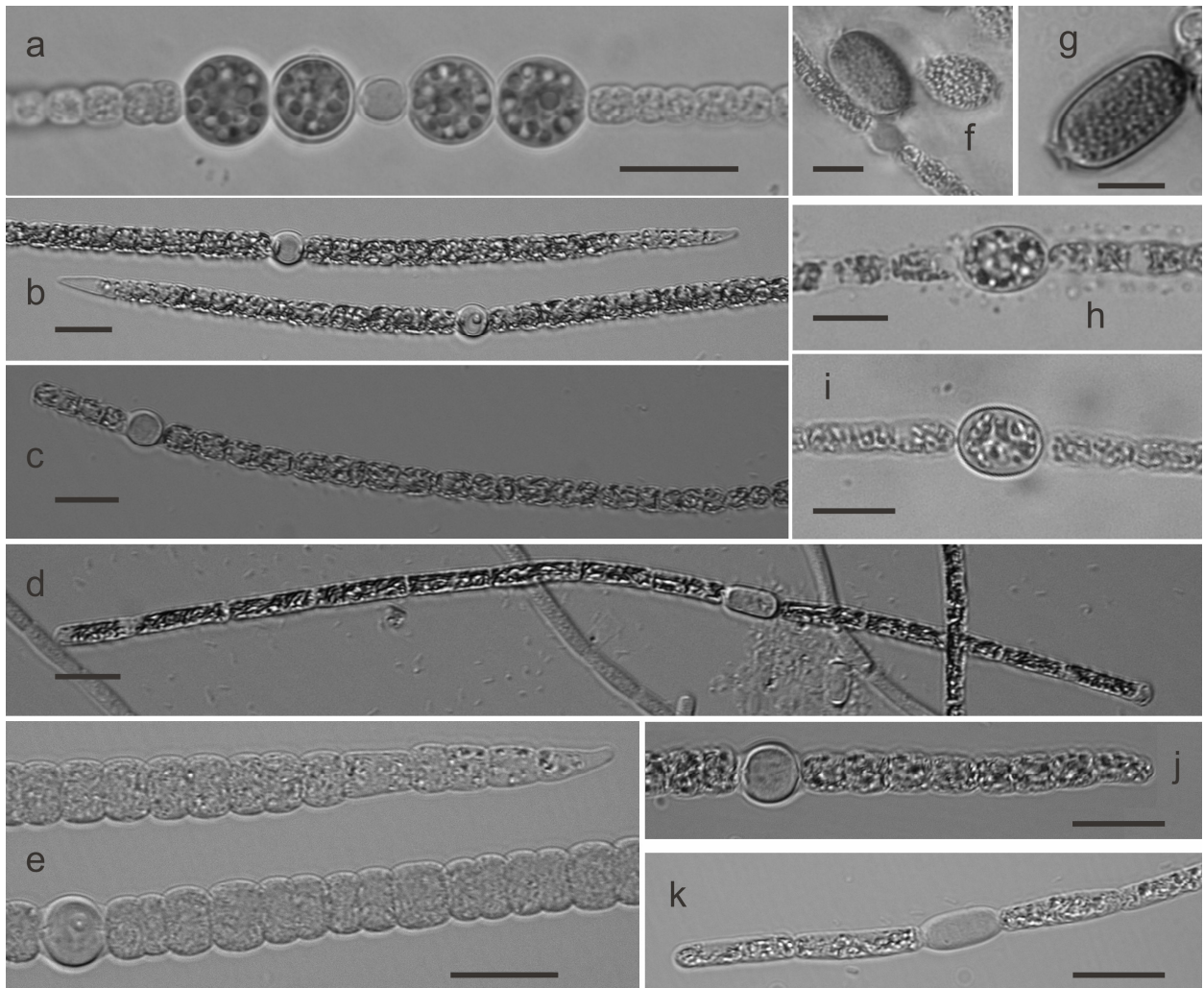
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434

Germination of *Cylindrospermopsis raciborskii* and *Aphanizomenon* species under natural and experimental conditions

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Introduction

Cylindrospermopsis raciborskii (Woloszynska) Seenayya et Subba Raju and *Aphanizomenon* spp. are both freshwater cyanobacteria of the order Nostocales. *C. raciborskii* was thought confined to tropical and sub-tropical environments but spread to temperate climatic regions on all continents except Antarctica (Padisák, 1997). It is known to be widely distributed in Northern Germany reaching the northernmost margin of its distribution at latitudes of 53 - 54°N (Krienitz & Hegewald, 1996; Stüken et al., 2006). Strains of this species are detected to produce the hepatotoxin cylindrospermopsin, from which human injury has been clearly identified (Falconer & Humpage, 2006).

Nostocales are characterized by trichomal structures such as vegetative cells, heterocysts and akinetes. The heterocysts can fix atmospheric nitrogen (N_2) when aquatic nitrogen is depleted (Kim et al., 2005). Akinetes are non-motile, resistant cells that accumulate proteinaceous reserves in the form of cyanophycin granules (Wetzel, 2001). These akinetes can survive low temperatures, desiccation and other adverse environmental conditions. When favourable conditions return, they germinate to produce trichomes. The ability of Nostocales to form akinetes confers a distinct advantage in environmental adaptation and subsequent bloom formation (Kim et al., 2005; Wetzel, 2001).

C. raciborskii akinetes are more commonly observed in subtropical and temperate populations late in the population cycle as a component of seasonal population dynamics (Kravchuk et al., 2006). Particular set of physico-chemical conditions stimulate akinetes to germinate (Moore et al., 2005). Light has been implicated as well as temperature and nutrients as triggering factors (Huber, 1985). According to Moore (2004) germination of akinetes occurs at temperatures between 15 °C and 30 °C in tropical Australian strains of *C. raciborskii*. Mischke (2003) found filaments of this species at temperatures of about 17 °C in temperate lakes with those in the Scharmützelsee region in Germany.

We therefore, tried to find out at what temperature the akinetes germinate in temperate region hypothesizing it should be below 15 °C. Since *Aphanizomenon* species like *A. gracile* and *A. flos-aquae* are the most abundant natural Nostocales in that habitat, we compare it to the invaded *C. raciborskii*. To figure out the germination temperature for both species, field observations are complemented by experiments at different temperatures.

Study site

The study site is Lake Melangsee (52.17 lat; 13.98 long.) in Brandenburg, Germany. This lake with its catchment area of 5 km^2 belongs to the catchment area of the River Dahme. It receives inflow from groundwater and from mesotrophic Lake Tiefer See (Schmitt & Nixdorf, 1999). The residence time of the water is 22 days. For more details see Nixdorf & Deneke (1997), Rücker (2004).

Table 1: Some morphometrical and trophic parameters (annual means, 2004-2005 of Lake Melangsee (TP=total phosphorus; SRP=Soluble reactive inorganic phosphorus)

Maximum depth	3.3 m	TP	42.33 μg l^{-1}
Mean depth	1.6 m	SRP	5.32 μg l^{-1}
Area	0.11 km^2	Secchi depth	0.92 m
Volume	0.17x10^6 m^3	Chl a	55.68 μg l^{-1}

Materials and methods

Sediment cores were taken monthly to bi-weekly in vegetation period. Vertical profiles of water temperature were measured in 0.5 m intervals using a Hydolab© H20 probe. Underwater light was measured by two Licor quantum sensors in a distinct distance of 39 cm. Mean PAR in the mixed layer (Imix) was calculated after Nixdorf & Rücker (2006).

The uppermost 2 cm of the sediment were sliced off from the sediment cores. For enumeration of seasonal course of akinete number the material was fixed with formaldehyde (final concentration 4 %). For germination experiments fresh sediment was suspended in N-free culture medium (modified after Nicklisch, 1992) or in filtered (pore size 0.45 µm) lake water (experiment at 19 ^{o}C). The flasks were placed in a culture chamber and cultivated at temperatures of 13, 15, 17 and 19 °C, respectively. All the flasks were placed in a light intensity of 130 µE m^{-2}s^{-1} which corresponds to the mean light in the mixed layer in Lake Melangsee in May in a light:dark cycle of 12:12 h. Subsamples of 20 ml were taken from the flask daily over two weeks, fixed with Lugols' solution and stored in the refrigerator until counting.

Enumeration and estimation of biovolume employed the Utermöhl technique (Utermöhl, 1958) using a Limnos© sedimentation chamber and an inverse Nikon© Diaphot microscope. To each fixed sample two different sedimentation chambers were prepared to do a parallel counting. Akinetes were counted at 400x magnification. Since filaments and germlings of different *Aphanizomenon* species hardly can be distinguished, *Aphanizomenon gracile* and *A. flos-aquae* which are the most important species of the genus Aphanizomenon in Lake Melangsee are summed up together. Whereas the samples of germination experiments were counted in the original concentration, formaldehyde fixed sediment samples for annual course were diluted 1:100 with thinned Lugol's solution. Following phases of development were differentiated: i) akinete, ii) growing akinete (elongating envelope and formation of holes at the poles of the akinete) iii) germling without envelope or in germination in the size of the akinete (up to 30 µm), iiii) growing filament.

Results

Following the seasonal dynamics of these two species (fig. 1), *Aphanizomenon* spp. appeared earlier in the water column than *C. raciborskii*. In both years, *Aphanizomenon* spp. were found as early as April corresponding to the beginning of spring with increasing water temperatures. The appearance

of *C. raciborskii* in 2004 came in later towards the beginning of June 2004 and towards the end of June in 2005 when the water temperature was about 18 °C. The biovolume of *Aphanizomenon* spp. in 2004 was more than that of *C. raciborskii*. Same trend was noted in 2005, but unlike 2004 the biovolume of both species decreased. The population peaks too were different for the two species. Whereas that of *Aphanizomenon* spp. came up as early as June that of *C. raciborskii* was around August corresponding to the peak of the summer period.
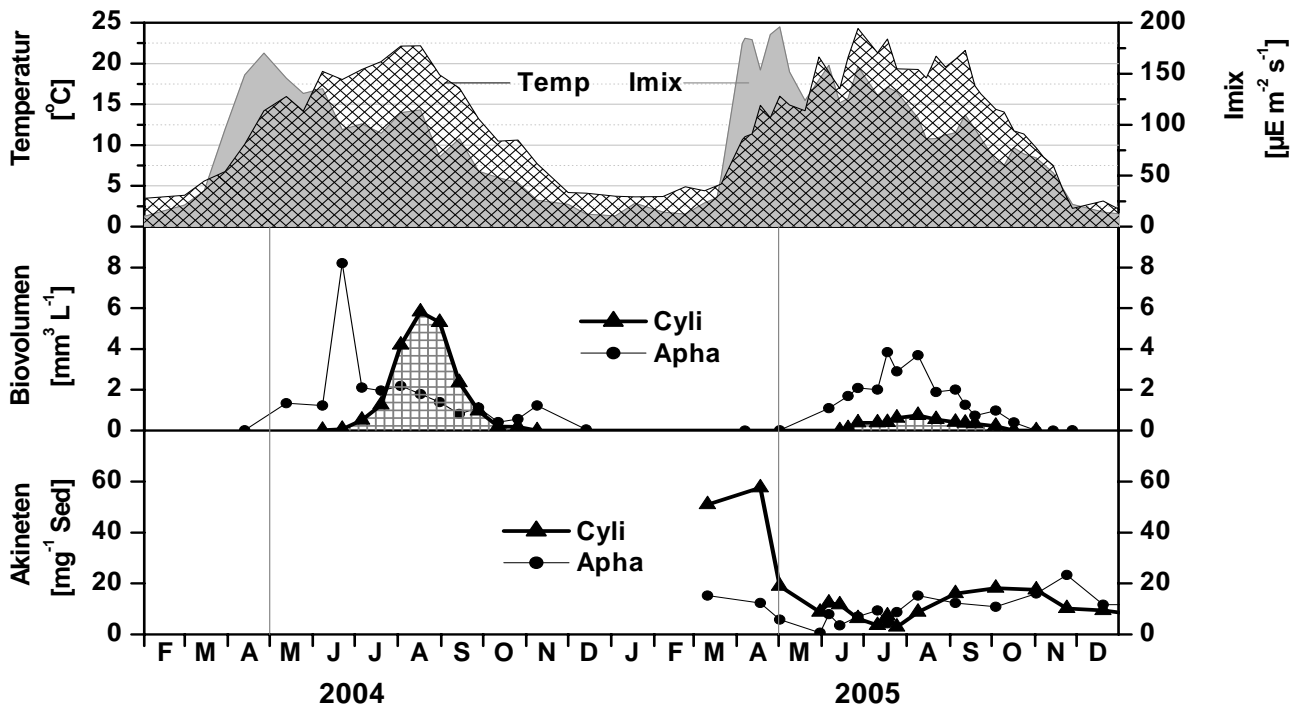

Figure 1: Seasonal development of water temperature (Temp), underwater light (Imix; upper panel), biovolume (middle) and number of akinetes per mg fresh weight of the uppermost 2 cm of sediment for *Cylindrospermopsis raciborskii* (Cyli) and *Aphanizomenon gracile* plus *A. flos-aquae* (Apha; lower panel) in Lake Melangsee 2004 and 2005.

The number of akinetes per mg fresh weight of sediment was monitored starting in 2005. At the beginning of May, it was noticed that at a water temperature of between 13 °C and 15 °C, there was a sharp decrease in the number of akinetes counted from the uppermost 2 cm of sediment.

This too was observed in the samples germinated in the laboratory (fig. 2). *Cylindrospermopsis* and *Aphanizomenon* species germinated at temperatures as low as 13 °C as can be seen from the increasing biovolume of growing akinetes and germlings in the sample cultured at this temperature. Most of the akinetes at all the cultured temperatures started germinating within the first 3 days of incubation. One important aspect of the germination observed was that germlings of *Cylindrospermopsis* hatched out of the envelope of the akinetes but do not show any significant further growth at low temperatures like 13 °C. But for *Aphanizomenon* spp., it exhibited growth and heterocyte differentiation already at 13 °C within the first three days and increasingly with growing temperatures. Considerable growth and heterocyte formation was observed for both species at 17 °C and above.

Discussion

In the field, the akinete number in the sediment of both genera declined synchronously at the beginning of May 2005 when the temperature at the sediment surface reached 13 – 14 °C. We suppose

that this decrease was as a result of the akinetes germination. After a lag phase of 5 weeks for *Aphanizomenon* spp. and 7 weeks for *C. raciborskii* filaments of the investigated species were found in the water column. The same could be observed in the culture experiments. *C. raciborskii* hatched out of the akinete envelope at 13 °C so does those of *Aphanizomenon*. Whereas young filaments of *Aphanizomenon* with first heterocysts were detected after one week in the culture at 13 °C, *Cylindrospermopsis* germlings exhibited much slower growth with growing filaments appearing in the 13 °C sample only on the 9th day of incubation. They also stayed in the size of akinetes without any further cell differentiation at this temperature and the total number of individuals decreased.
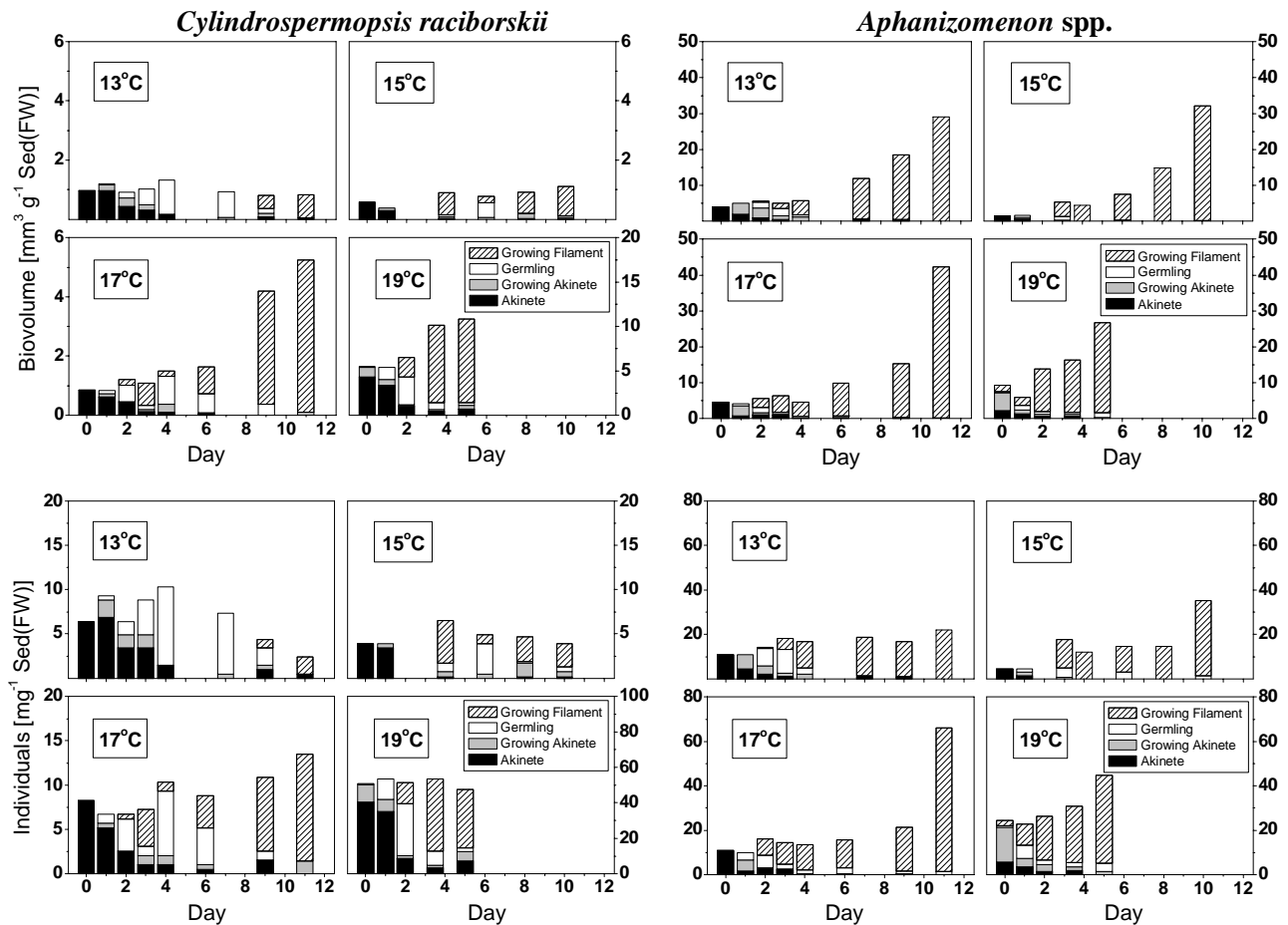
decreased.

Figure 2: Biovolume (above) and abundance (below) of different phases of development of Cylindrospermosis raciborskii (left) and Aphanizomenon species (right) in the course of germination experiments at different temperatures with sediment of Lake Melangsee.

At this point we can say if *C. raciborskii* germinates already at low temperatures, further population development is dependent on increasing temperatures in the course of the seasons. In other words, should the temperature at which the akinete started germinating remains constant the naked germlings which are supposed to be more sensitive than complete akinete would be destroyed. We assume that fluctuating temperatures observed in 2005 resulted in the death of some naked germlings. This caused the observed decrease in *C. raciborskii* population of 2005 as compared to 2004.

Aphanizomenon species exhibited higher growth rates at lower temperatures. From this we can deduce that they are more adapted to the temperate habitat, thus are more successful under low temperatures in northern habitats. Contrary to previous studies the akinete germination of the tem-

perate population of *C. raciborskii* is initiated at temperatures as low as 13 °C but requiring an increase in temperature for further growth. Unfavourable conditions in the phase after germination delayed the development of the *C. raciborskii* population in the year 2005. An open window which is still left for investigation is to find whether this germination can be initiated below 12 °C and its dependence on light. Should there be higher spring temperatures in the consequence of global warming it would promote the flourishing of this species and why not its colonisation of other freshwater bodies.

Acknowledgement

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POPULATION ECOLOGY

Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions

Claudia Wiedner · Jacqueline Rücker ·
Rainer Brüggemann · Brigitte Nixdorf

Abstract *Cylindrospermopsis raciborskii*, an invasive freshwater cyanobacterium, originated from the tropics but has spread to temperate zones over the last few decades. Its northernmost populations in Europe occur in North German lakes. How such dramatic changes in its biogeography are possible and how its population dynamics in the newly invaded habitats are regulated are still unexplained. We therefore conducted a long-term (1993–2005) study of two German lakes to elucidate the mechanisms behind *C. raciborskii* population dynamics and to identify the abiotic constraints on its development. Our data revealed that pelagic populations of *C. raciborskii* thrived for three months during the summer, contributing up to 23% of the total cyanobacteria biovolume. Population sizes varied greatly between years without exhibiting any distinct long-term trends. In the annual lifecycle, *C. raciborskii* filaments emerged in the pelagic habitat when the temperature rose above 15–17 °C. At that time, mean photosynthetically active radiation in the mixed water column (I_{mix}) oversteped its maximum. Rates of population net increase were highest at the beginning of the season (0.15–0.28 day^{-1}), declined continuously over time, and were significantly positively correlated with I_{mix}. This indicates that the onset of the pelagic population is temperature-mediated and that I_{mix} controls its growth. Since I_{mix} peaks before the population onset, the time of germination is of crucial importance for successful development. To test this hypothesis, we designed a model to simulate pelagic population size, starting at different dates in the annual cycle. Moving the population onset forward by 30 days resulted in a doubling of the population size. We therefore conclude that an earlier rise in water temperature associated with climate change has promoted the spread of *C. raciborskii* to the temperate zone. Earlier warming permits earlier germination, thereby shifting the pelagic populations to a phase with higher I_{mix}, which advances growth and the population establishment.

Keywords Nostocales · *Cylindrospermopsis raciborskii* · Population dynamics · Lifecycle · Biogeography

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Introduction

Cylindrospermopsis raciborskii is a filamentous freshwater cyanobacterium of the order Nostocales. It originated in tropical and subtropical regions and was first discovered in a lake in Java (Woloszynska 1912). Like other Nostocales, it can produce two types of specialized cells: (1) hetero-cysts, which can fix molecular nitrogen and thereby offset nitrogen demand under conditions of nitrogen deficiency, and (2) akinetes, which are resting stages that allow the species to survive unfavorable growth conditions in the sediment. In tropical and subtropical regions, vegetative

pelagic populations of this species can survive all year round; they can dominate the phytoplankton community and even form mass developments (e.g., Fabbro and Duivenvoorden 1996; Bouvy et al. 1999). *C. raciborskii* can produce toxins such as paralytic shellfish poisons (Lagos et al. 1999) or cylindrospermopsin (Hawkins et al. 1997), which are harmful to humans, as was observed on Palm Island, Australia, in 1979 (Bourke et al. 1983; Hawkins et al. 1985; Ohtani et al. 1992).

During the last few decades, *C. raciborskii* has spread from the tropics to northern temperate zones, as was first reported by Padisák (1997). In Europe, the cyanobacterium was first recorded in Greece in 1938 (Skuja 1938); it appeared in Hungary in the 1970s (Padisák 1997) and in Germany (Krienitz and Hegewald 1996; Rücker et al. 1997), Austria (Dokulil and Mayer 1996), and France (Couté et al. 1997) during the 1990s. Several reports of the species followed thereafter from other European countries (e.g., Portugal; Saker et al. 2003) as well as from Canada (Hamilton et al. 2005) and New Zealand (Wood and Stirling 2003). The species has already formed mass developments in some northern habitats, e.g., Hungary, Austria, and Canada.

No other freshwater cyanobacterium or phytoplankton species has achieved such an incredibly rapid and successful spread from tropical to temperate regions all over the world. Two hypotheses have been put forward to explain the changes in the biogeography of *C. raciborskii*: (1) its spread to temperate regions could be due to increasing water temperatures associated with climate change; (2) selected ecotypes with lower temperature and light requirements have spread northwards. The second hypothesis was tested by comparing the effects of temperature and light on the growth of strains from different geographical regions. Briand et al. (2004) found neither temperature- nor light-dependent differences in the growth of strains from different regions and concluded that the spread of the species results from climate change rather than from ecotype selection. In contrast, Chonudomkul et al. (2004) found differences between strains from Thailand and Japan in terms of their ability to acclimatize and grow at low temperatures, and presumed that ecotype selection plays an important role in the process of species dispersion. However, it is still not clear which out of climate change or ecotype selection or both has triggered the spread of this species.

Three types of differences between populations from different geographical regions have been found so far: phylogenetic, morphological, and chemical. DNA sequence analyses have identified three distinct clusters—American, European, and Australian or African (Gugger et al. 2005). Three morphotypes of *C. raciborskii* have been identified in the tropics—straight, coiled and

sigmoid-shaped (Fabbro and Duivenvoorden 1996), whereas only straight filaments occur in temperate regions. Finally, strains differ in their ability to produce certain secondary metabolites; for example, strains from tropical regions produce cylindrospermopsin (Saker and Griffiths 2000; Li et al. 2001), while only non-cylindrospermopsin-producing strains have been isolated in Europe (Saker et al. 2003; Fastner et al. 2003). However, it is not currently known whether these phylotypes, morphotypes, and chemotypes match with certain ecotypes. Therefore, the question of why this tropical species has spread to temperate regions has still not been answered satisfactorily.

Another important question is how *C. raciborskii* populations are regulated in the newly invaded regions, which differ significantly in abiotic conditions, especially in terms of temperature and light. Tropical field studies have shown that *C. raciborskii* akinetes germinate at temperatures above 23 °C, and that maximum population densities occur between 27 and 30 °C. Population growth was considered to be enhanced by a high pH, a stratified water column, a long water residence time, and high levels of incident radiation (e.g., Branco and Senna 1994; Fabbro and Duivenvoorden 1996; Souza et al. 1998; Bouvy et al. 1999; McGregor and Fabbro 2000). In temperate regions, *C. raciborskii* populations are restricted to the summer months and occur mainly in eutrophic, polymictic shallow lakes (Stüken et al. 2006). Temperature has been considered to be the main limiting factor for population growth. At Lake Balaton, Hungary, the estimated temperature required for the germination of akinetes was >22 °C (Górzo 1987; Padisák 1997). However, population growth has been observed at temperatures of 15 °C in Austrian lakes and 17 °C in German lakes (Dokulil and Mayer 1996; Mischke 2003). Population peaks mainly coincide with the annual temperature peak. Light intensity was considered to be the second most important factor regulating population dynamics. Population growth was found to correlate positively with the mixing depth:Secchi depth ratio (z_{mix}:z_{SD}), which was used as a proxy for light intensity in the water column, and it was concluded that the population size of the species increases with decreasing light intensity (Dokulil and Mayer 1996). High pH (Hamilton et al. 2005) and low dissolved inorganic nitrogen and phosphorus concentrations (Mischke 2003; Briand et al. 2002) are also thought to promote *C. raciborskii* growth.

Most studies are limited to a description of the circumstances under which the species occurs. Many factors are considered to be important in regulating *C. raciborskii* population dynamics, and different studies have produced conflicting results. The underlying regulatory mechanisms have not been adequately explained. Here, we present the

first long-term data series covering a 13-year period of *C. raciborskii* population dynamics in two polymictic shallow lakes in Northern Germany, the species' northernmost European habitat. Data were analyzed to elucidate the regulatory mechanisms and abiotic constraints on population dynamics, and a simple mathematical model was designed to test whether the time of population onset affects population size. Results are discussed in the context of climate change as a promoter of changes in species biogeography.

Materials and methods

Study sites

The two lakes studied—Langer See and Melangsee—are eutrophic and polymictic shallow lakes located in northeast Germany (57°20′N; 54°35′E). The main characteristics of the lakes are given in Table 1; more detailed information on the lakes can be found in Nixdorf and Deneke (1997). The phytoplankton communities of both lakes were recurrently dominated by cyanobacteria from early spring until late autumn. Taxa of the order Oscillatoriales—*Planktothrix agardhii* in Lake Langer See and *Limnothrix* spp. and *Pseudanabaena* spp. in Lake Melangsee—form mass developments in late summer (Rücker et al. 1997). Before Oscillatoriales become dominant, taxa of the order Nostocales, including *C. raciborskii*, and several taxa of the genus *Aphanizomenon*, *Anabaena*, and *Anabaenopsis* are more abundant (Wiedner et al. 2002). More detailed information on phytoplankton can be found in Mischke (2003) and Nixdorf et al. (2003).

Sampling and analyses

The two lakes have been sampled monthly to biweekly at their deepest point from July 1993. Mixed samples from the whole water column were prepared by taking samples at half-meter intervals with a 2.3 l Limnos (Turku, Finland) sampler. Aliquots of the mixed samples were analyzed to determine the concentrations of ammonia (NH_4–N), nitrate plus nitrite (NO_{total}–N), dissolved inorganic phosphorus

(DIP), total phosphorus (TP), and total nitrogen (TN), according to standard methods (DEV 1976–1998). Detection limits of the dissolved nutrients were 1.7 µg l^{-1} for DIP, 64 µg l^{-1} for NH_4–N and 3.2 µg l^{-1} for NO_{total}–N. Dissolved inorganic nitrogen (DIN) represents the sum of NH_4–N plus NO_{total}–N. Phytoplankton composition and biovolume were estimated using an aliquot fixed with Lugol's solution studied under an inverse microscope according to Utermöhl (1958) and Rott (1981). On each sampling date, we determined the Secchi depth (z_{SD}) as well as depth profiles of water temperature, pH and oxygen saturation at 0.5-m intervals using a multiparameter probe (H20, Hydrolab, Austin, TX, USA). Photosynthetically active radiation (PAR) was measured at half-meter intervals along the water column using two spherical quantum sensors (SA 193, Li-Cor , Lincoln, NE, USA).

Calculation of mean PAR in the mixed water column (I_{mix})

The vertical attenuation coefficient K_d was calculated according to Kirk (1994) as follows:

$$K_d = \frac{\ln I_1 - \ln I_2}{z_1 - z_2} \tag{1}$$

whereby I_1 is the PAR at depth z_1 and I_2 is the PAR at depth z_2. Missing K_d values, e.g., for the years 1993–1995, were computed from the Secchi depth using Eq. 2 (previously unpublished) with pooled long-term data derived from regional plankton turbid lakes in different trophic states:

$$K_d = 1.5276 \cdot z_{SD}^{-0.7253} (r^2 = 0.84; N = 345) \tag{2}$$

Mean PAR in the mixed water column (I_{mix}) was calculated according to Riley (1957):

$$I_{mix} = 0.45 \cdot I_o \left(\frac{1 - e^{-K_d \cdot z_{mix}}}{K_d \cdot z_{mix}} \right) \tag{3}$$

where z_{mix} is the depth of the mixed water column, which was assumed to be equal to the mean depth of the lake (see Table 1). I_{mix} was calculated as the fortnightly mean prior

Table 1 Morphometric and trophic characteristics of Melangsee (MEL) and Langer See (LAN)

Lake	A (km^2)	V (10^6 m^3)	z_{mean} (m)	z_{max} (m)	TP (µg l^{-1})	TN (mg l^{-1})
LAN	1.55	3.27	2.1	3.5	95.75	1.262
MEL	0.11	0.17	1.6	2.8	59.58	1.042

Total phosphorus *TP* is given as the long-term annual mean for the years 1994–2005, total nitrogen *TN* for 1998–2005

A surface area, *V* volume, z_{mean} mean depth, z_{max} maximum depth of the water column

to the sampling date based on the daily total global radiation (I_0). Daily K_d values were interpolated linearly between sampling dates. The fraction of the total global radiation comprising the PAR and the reflection at the surface of the water were calculated using a correction factor of 0.45, as proposed by Behrendt and Nixdorf (1993).

Specific net growth rate (μ)

The specific net growth rate (μ) between sampling dates t_1 and t_2 ($t_2 > t_1$) is defined by Eq. 4:

$$\mu = \frac{\ln \text{Cbio}v_2 - \ln \text{Cbio}v_1}{t_2 - t_1} \tag{4}$$

where $\text{Cbio}v_1$ is the biovolume at time t_1 and $\text{Cbio}v_2$ is the biovolume at time t_2. In the data matrix for statistical analyses, μ was assigned to t_2. Since this study is based on field data, the net growth rate encompasses all loss processes, e.g., grazing and dilution.

Mean annual courses of I_{mix}, temperature, DIN, DIP, biovolume and specific growth rate of *C. raciborskii* (denoted as z_j $j = 1,..., 6$) were derived from pooled data for each lake over the whole study period (1993–2005) using the polynomial fit tool of Origin Pro 6.1 (OriginLab Corporation, Northampton, MA, USA). μ_{trend} is an exception (see below). To fit the data of the abiotic parameters properly at the end of the Julian day axis, the data set was extrapolated by appending the data from the first and last 50 days of each year, respectively. The order of polynomial fit (n_j), which was chosen to give optimal statistical results and curve fitting, depends on the different quantities of DIP, DIN, etc. Therefore, the summation (Eq. 5) runs between 0 and 9. The polynomials can generally be expressed by the equation:

$$z_j = \sum_{i=0}^{n_j} a_{j,i} \cdot t^i \quad j = 1, \dots, 6 \tag{5}$$

Mean annual courses

The mean annual course of the specific growth rate, $\mu_{\text{trend}}(t)$, was derived from pooled data, $[t, \mu(t)]$, by linear regression (Eq. 6):

$$\mu_{\text{trend}} = a \cdot t + b \tag{6}$$

Here, a is the slope of the estimated linear $\mu_{\text{trend}}(t)$ function, and b is the intercept with the μ-axis. Nonlinear curve fittings (exponential and quadratic forms) were additionally tested by means of SPSS, but this did not improve the statistical results.

Correlation (Pearson) analysis

In order to evaluate the main factors controlling population growth, a correlation analysis was performed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA).

Model design

A simple model (Eq. 7) was used to simulate time-dependent changes in *C. raciborskii* population size during the annual cycle (where d*Cbiov* is the change in the biovolume of *C. raciborskii* and dt the change in time):

$$\text{d Cbiov}/\text{d}t \sim \mu(t) \tag{7}$$

The model is based on the specific growth rate (μ) as a function of time (t), which is $\mu_{\text{trend}}(t)$ for Lake Melangsee (Eq. 6). The coefficients a and b are specified in Table 2. Together with the proportionality constant *skal* (=0.15), we arrive at the following model:

$$\frac{\text{d }Cbiov}{\text{d}t} = \begin{cases} \text{skal} \cdot \mu_{\text{trend}}(t) & \text{if } t \geq t_{\text{begin}} \\ 0 & \text{else} \end{cases} \tag{8}$$

Simulations were performed using ModelMaker 3.0 (FamilyGenetix, Oxford, UK). Population size was modeled for three starting times t_{begin} during the annual cycle (t_{begin} = Julian days 90, 120 and 150, respectively) to simulate the effects of earlier spring activities. Two scenarios were simulated for each starting time. In the first scenario, the same inoculum size (1 mm³ l⁻¹) was used for all three start times. In the second scenario, different inoculum sizes were used to reflect the growing number of akinetes in the sediment over the course of *C. raciborskii* invasion: 1.5 mm³ l⁻¹ at t_{begin} = day 90; 1.0 mm³ l⁻¹ at t_{begin} = day 120; 0.5 mm³ l⁻¹ at t_{begin} = day 150.

Table 2 Statistical results from the linear regression analysis for growth rate μ_{trend} over time at Melangsee (MEL, $N = 52$) and Langer See (LAN, $N = 57$) using pooled six- and nine-year sampling data, respectively

Lake	r^2_{DF}	F	a	$t(a)$	SD(a)	b	$t(b)$	SD(b)
MEL	0.353	28.8	-1.177×10^{-3}	−5.365	0.000	0.280	5.281	0.053
LAN	0.299	24.8	-1.304×10^{-3}	−4.986	0.000	0.323	4.999	0.065

a slope of regression curve, b intercept with the y-axis (all a and b values were significant at the level of <0.001)

Results

The long-term development of *C. raciborskii*, total cyanobacteria and total phytoplankton biovolume in the two lakes is shown in Fig. 1. *C. raciborskii* was found in both lakes over a three-month summer period during all years studied. Population peaks occurred between mid-July and late August, with maximum values of 9.2 mm^3 l^{-1} in Melangsee and 5.3 mm^3 l^{-1} in Langer See, corresponding

to 19.3 and 13.6% of the total phytoplankton and 22.6 and 16.8% of the total cyanobacteria biovolume, respectively. The average conditions under which the population peaks occurred were 86 and 116 µg l^{-1} TP, 1.5 mg l^{-1} TN, 21.2 and 20.6°C, 106 and 117% O$_2$ saturation, and pH 8.2 and 8.7 at Melangsee and Langer See, respectively (means for six years in Melangsee and nine years for Langer See).

The seasonal dynamics of *C. raciborskii* biovolume and of relevant growth factors (I_{mix}, temperature, DIN and DIP) showed a distinct pattern of recurrence in all years studied. Mean annual courses of each parameter were therefore extrapolated by polynomial fitting (Fig. 2; Table 3) and by linear regression in the case of the growth rate [$\mu_{trend}(t)$] (Figs. 2, 3; Table 2). In both lakes, I_{mix} rose steeply in spring and peaked on average 45 days before the maximum temperature had been reached; by then, I_{mix} had dropped to one-third its peak value. DIN and DIP concentrations were around the detection limit during the *C. raciborskii* population growth phase. The annual course of temperature development was similar in both lakes; the first *C. raciborskii* filaments appeared in the pelagial habitat around Julian day 140, when the water temperature was above 17 °C. The initial pelagic population grew, reaching estimated specific growth rates of between 0.15 and 0.28 day^{-1}, and then declined continuously over the season, dropping to values of around 0.035 day^{-1}, when the population peaks coincided with or occurred shortly after the temperature peaks. The growth of *C. raciborskii* (μ) showed a significantly positive correlation with I_{mix} in the first place and
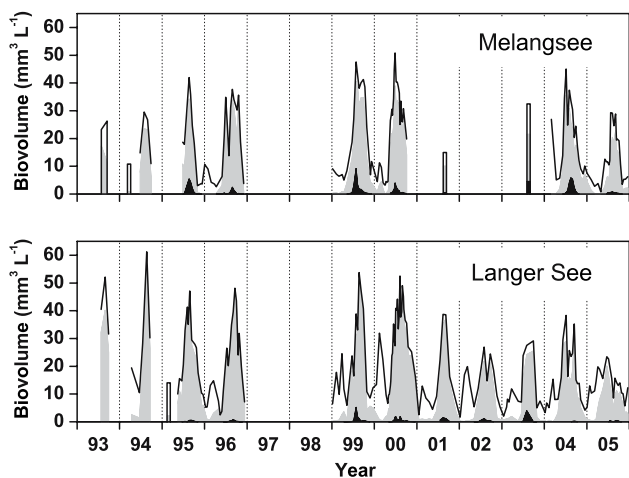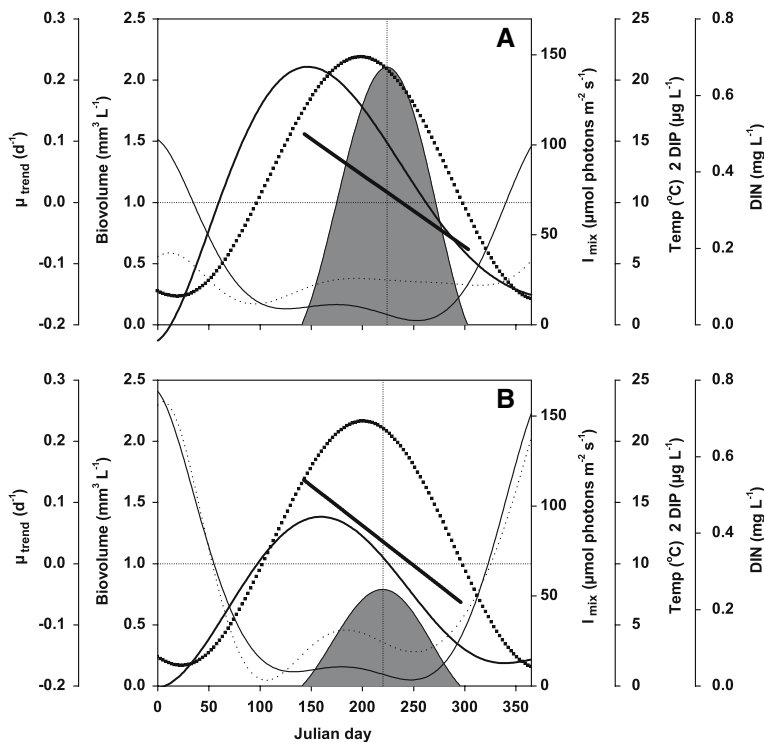

Fig. 1 Long-term development of the biovolumes of total phytoplankton (*black line*), total cyanobacteria (*gray area*), and *C. raciborskii* (*black area*) at Melangsee and Langer See from July 1993 to August 2005

Fig. 2A–B Mean annual course of *C. raciborskii* biovolume (*gray area*), mean PAR in the mixed water layer (I_{mix} *bold line*), temperature (Temp *thick dotted line*), dissolved inorganic nitrogen (DIN *thin line*), and dissolved inorganic phosphorus (DIP *thin dotted line*), derived from polynomial fits of pooled data from 1993 to 2005 at Melangsee **A** and Langer See **B** (statistics are given in Table 3) and specific growth rate of *C. raciborskii* (μ_{trend}: *thickest solid line*) calculated using linear fit (see Fig. 3; Table 2). *Straight dotted lines* mark zero growth rate and maximum biovolume

Table 3 Order (n_j, Eq. 5) and statistical results from polynomial fits of pooled data for mean PAR in the mixed water layer (I_{mix}), temperature (Temp), dissolved inorganic nitrogen (DIN) and phosphorus (DIP), and biovolume of *C. raciborskii* at Melangsee (MEL) and Langer See (LAN) as functions of Julian day, using pooled data from 1993 to 2005

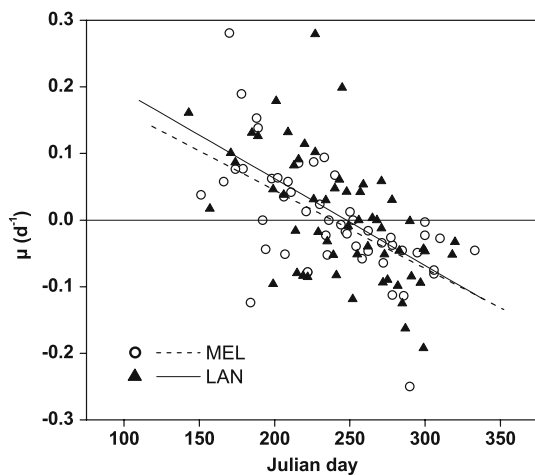| Lake	Parameter	N	n_j	r^2_{DF}	F
MEL	I_{mix}	349	6	0.80	228.7
	Temp	345	6	0.94	906.9
	DIN	255	6	0.54	49.9
	DIP	264	9	0.11	4.6
	Biovolume	131	9	0.30	7.2
LAN	I_{mix}	376	6	0.80	261.9
	Temp	375	6	0.94	937.3
	DIN	281	6	0.65	86.5
	DIP	289	9	0.51	33.9
	Biovolume	194	9	0.23	7.5

Fig. 3 Pooled data for the specific growth rate (μ) of *C. raciborskii* from 1993 to 2005 for Melangsee and Langer See, and mean annual course (μ_{trend}) of the growth rate calculated using linear regression analysis (statistics are given in Table 2)

temperature in the second place, but it was not significantly correlated with DIN and DIP (Table 4; Fig. 4).

Population peaks varied greatly between the years at both lakes without any distinct long-term increasing or decreasing trends (Fig. 5). In our simulations of growth rate as a function of time, the population size nearly doubled when the time of *C. raciborskii* emergence was set forwards by 60 days in the annual cycle with a constant inoculum size (Fig. 6A). When a proportional increase in inoculum size (number of akinetes germinating in spring) was also considered, a 1.9-fold increase in population size was achieved by shifting the onset 30 days forward, and a 2.9-fold increase occurred when the onset was shifted 60 days forward (Fig. 6b).

Discussion

Regulation of population dynamics

Photosynthetically active radiation

Our results clearly demonstrate that I_{mix} is of major importance for regulating the dynamics of the pelagic population of *C. raciborskii*. After germination, the pelagic population started to grow at high rates that were limited by I_{mix}, and then decreased over time as I_{mix} decreased. These findings conflict with most of the conclusions drawn from other field studies. Some authors have assumed that *C. raciborskii* is shade-tolerant and benefits from low light conditions (Padisák and Reynolds 1998) because high biovolumes of the species were found under conditions of high light attenuation and low euphotic depth (Bouvy et al. 1999), or that the biovolume, its changing rates or population growth correlates positively with the mixing depth:Secchi depth (z_{mix}:z_{SD}) ratio (Dokulil and Mayer 1996; Mischke 2003). However, genuine PAR in the water column results from global radiation, light attenuation, and mixing depth (Kirk 1994). Thus, frequently used proxies for PAR, e.g., z_{mix}:z_{SD}, Secchi depth, euphotic depth (z_{eu}), or z_{mix}:z_{eu}, do not account for global radiation and are therefore inadequate for describing the mean PAR in the water column. Likewise, global radiation parameters such as hours of sunshine do not account for light attenuation. The use of inadequate proxies for PAR might even lead to conflicting findings in the same study; Briand et al. (2002), for example, found that *C. raciborskii* populations benefit from small Secchi depths (low light conditions) as well as from more hours of sunshine (high light conditions). The role of PAR in regulating the population dynamics of *C. raciborskii* was not recognized before because either it was not analyzed or it was not interpreted properly due to the use of inadequate proxies. The only observation on *C. raciborskii* dynamics that agrees with our findings is that of Fabbro and Duivenvoorden (1996), who found that the population of this species increased in Fitzroy River in tropical Queensland (Australia) under conditions of decreasing turbidity and increasing global radiation, which automatically leads to an increase in PAR in the water column. *C. raciborskii* is also thought to benefit from low light intensities because culture experiments on its light-dependent growth yielded low I_k values of 15–26 μmol photons m^{-2} s^{-1} (Shafik et al. 2001; Briand et al. 2004); these findings were taken as an indication of low light adaptation. However, the culture experiments also showed that *C. raciborskii* requires high light intensities of 80–120 μmol photons m^{-2} s^{-1} for maximal growth, and that it is able to grow at almost maximum rates at very high light intensities of up to 500 μmol photons m^{-2} s^{-1}, which

Table 4 Correlation matrix (Pearson) for the specific growth rate of *C. raciborskii* (μ) and the influencing variables, I_{mix}, Temp, DIN, and DIP (for abbreviations, see Table 2) at Melangsee (MEL, $N = 52$) and Langer See (LAN, $N = 57$)

Melangsee		μ	I_{mix}	Temp	DIN	DIP
Pearson correlation	$\mu_{measured}$	1.000	0.673	0.561	–0.165	–0.185
	I_{mix}	0.673	1.000	0.786	–0.275	0.023
	Temp	0.561	0.786	1.000	–0.546	0.087
	DIN	–0.165	–0.275	–0.546	1.000	–0.125
	DIP	–0.185	0.023	0.087	–0.125	1.000
Significance (one-tailed)	$\mu_{measured}$	–	0.000	0.000	0.121	0.095
	I_{mix}	0.000	–	0.000	0.024	0.436
	Temp	0.000	0.000	–	0.000	0.269
	DIN	0.121	0.024	0.000	–	0.189
	DIP	0.095	0.436	0.269	0.189	–
Langer See		μ	I_{mix}	Temp	DIN	DIP
Pearson correlation	$\mu_{measured}$	1.000	0.537	0.494	–0.068	–0.197
	I_{mix}	0.537	1.000	0.723	–0.140	–0.258
	Temp	0.494	0.723	1.000	–0.301	–0.230
	DIN	–0.068	–0.140	–0.301	1.000	–0.008
	DIP	–0.197	–0.258	–0.230	–0.008	1.000
Significance (one-tailed)	$\mu_{measured}$	–	0.000	0.000	0.307	0.071
	I_{mix}	0.000	–	0.000	0.150	0.026
	Temp	0.000	0.000	–	0.012	0.042
	DIN	0.307	0.150	0.012	–	0.478
	DIP	0.071	0.026	0.042	0.478	–

indicates high light tolerance. In our study, populations started to grow at average rates of 0.17 day^{-1} at I_{mix} values of around 90 and 130 μmol photons m^{-2} s^{-1} in Langer See and Melangsee, respectively; growth rates dropped to zero at I_{mix} values of 60 μmol photons m^{-2} s^{-1}. It is important to note that the PAR requirement of *C. raciborskii* in our lakes is most likely enhanced due to the fact that the species complements its nitrogen demand by N_2 fixation (see below). N_2 fixation is generally an energy-consuming process, and Nostocales are known to require higher intensities of photon irradiance to maintain the same growth rate under conditions of N_2 fixation (Ward and Wetzel 1980; De Nobel et al. 1997). In the case of *C. raciborskii*, the maximum growth rate with N_2 as a nitrogen source was 69 and 87% of that of NH_4–N and NO_3–N, respectively (Shafik et al. 2001). Although I_{mix} in the lakes cannot be compared one-to-one with controlled stable PAR in the laboratory, our data are similar to the laboratory findings and may reflect the enhanced light requirement of N_2-fixing *C. raciborskii* populations.

Temperature

In the present study, the population peaks coincided with the temperature peaks, which is in agreement with most other field observations (e.g., Hamilton et al. 2005). Like I_{mix}, temperature displayed a significantly positive (albeit lesser) correlation with growth rate, indicating that temperature is not the primary growth-limiting factor. Temperature can definitely be excluded as the growth-limiting factor up to the point where the population peak occurs since the growth rate declined continuously over the season, whereas the temperature increased until the growth rate dropped to nearly zero (Fig. 2). *C. raciborskii* population growth was also found to be unaffected by temperature in Alte Donau, Austria (Dokulil and Mayer 1996). This is surprising, since 17–22 °C is far below the optimal growth temperature, which was determined to be 30 °C in culture experiments with strains from tropical and temperate zones (Saker and Griffiths 2000; Shafik et al. 2001; Briand et al. 2004). However, it is not totally clear whether *C. raciborskii* populations from different climatic zones differ in their ecophysiology. The results of Chonudomkul et al. (2004), for example, suggest that clones in the temperate zone may be able to adapt to low temperatures.

Nevertheless, temperature is a crucial factor in the regulation of the onset of the pelagic population. Since filaments of the species are frequently detected at temperatures of 15–17 °C, the akinetes must have germinated at even lower temperatures. This is significantly less than the

Fig. 4A–D Scatter plots showing the specific growth rate (μ) of *C. raciborskii* at Melangsee (MEL) and Langer See (LAN) versus I_{mix} (**A**), temperature (**B**), DIN (**C**), and DIP (**D**). For abbreviations see Fig. 2
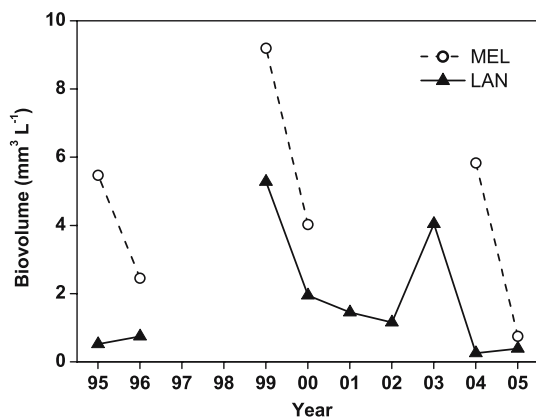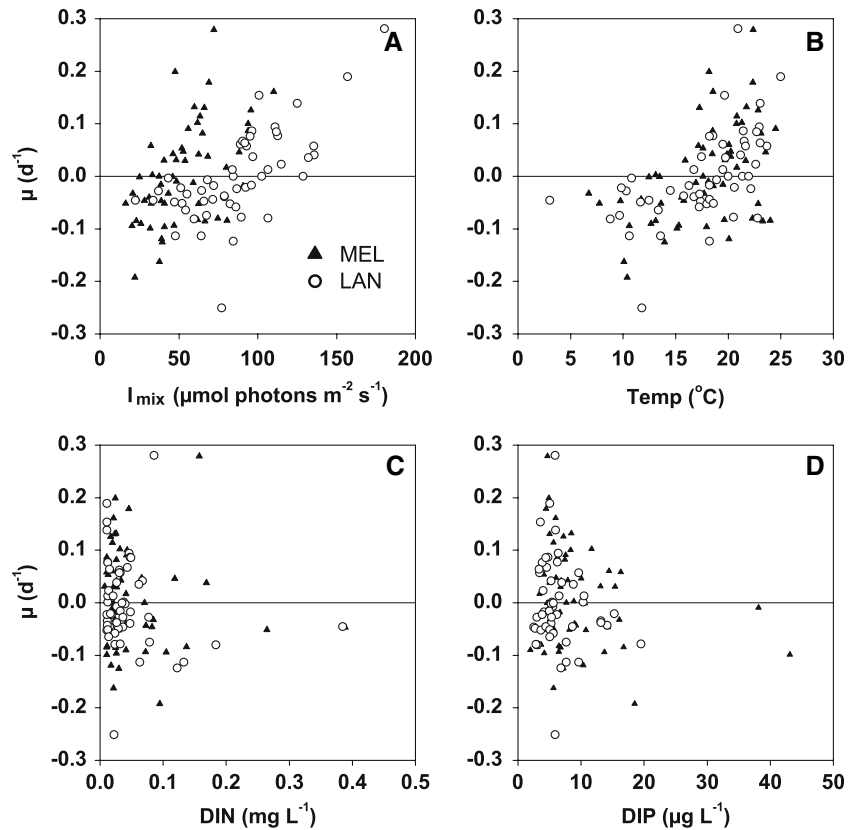

Fig. 5 Biovolume of *C. raciborskii* at their annual population maximums in Melangsee (MEL) and Langer See (LAN) from 1995 to 2005

temperatures observed in Lake Balaton, where germination occurred at 22 °C (Górzo 1987; Padisák 1997). However, the time of the onset of the population is important for the population size (as discussed below); thus, temperature indirectly has a major impact on the population dynamics.

Other factors

The low DIN and DIP concentrations observed indicate a shortage of nutrients. Hence, the different species most

likely competed for available nutrients, which deplete rapidly under such conditions. Consequently, significant effects on *C. raciborskii* growth may have gone undetected. In the case of nitrogen deficiency, *C. raciborskii* and other Nostocales have a competitive advantage, namely N_2 fixation, which supplies up to 49% of the nitrogen demand of *C. raciborskii* in Lake Balaton (Présing et al. 1996). Since N_2 fixation results in an increased light demand or a reduced growth rate (Shafik et al. 2001), it is more beneficial at the beginning of the season when I_{mix} is high. *C. raciborskii* is also a strong competitor for phosphorus. Strains isolated from Lake Balaton had a high uptake affinity and a high storage capacity for phosphorus (Istánovics et al. 2000). *C. raciborskii* was also found to exploit phosphorus very efficiently in Florida lakes, where increases in phytoplankton biomass yield per unit phosphorus occurred when the species was dominant (Dobberfuhl 2003). Nevertheless, since it needs high phosphorus concentrations for akinete formation (Moore et al. 2003, 2005), low DIP concentrations in the studied lakes might limit the size of the inoculum in the following season. However, novel experimental approaches are necessary to gain further insights into the role of nutrients and the competitive potential of *C. raciborskii* during nutrient shortages.

From many field studies, it was concluded that *C. raciborskii* benefits from high pH values (e.g., Hamilton et al.

2005). The pH reflects the relative fraction of inorganic carbon species. Since the CO_2 fraction decreases as pH increases, this might be a limiting factor for photosynthesis. Cyanobacteria generally tend to be strong competitors under conditions of high pH and low CO_2, but it is difficult to distinguish the effects of these parameters individually (Shapiro 1990). Moreover, CO_2 concentrations were not measured in any of the field studies, and carbon uptake and storage by *C. raciborskii* have not been examined until now. Thus, the effects of pH and CO_2 remain unclear.

Finally, top–down control of *C. raciborskii* by zooplankton grazing must also be considered. Grazing pressure on cyanobacteria is generally low because most zooplankton species are too small to ingest the organisms. Instead, zooplankton eliminates competing phytoplankton, allowing the cyanobacteria to thrive with lessened competition (e.g., Haney 1987). Large species, such as *Daphnia pulex* or *D. magna*, which would be able to ingest *C. raciborskii* filaments, did not occur in the two lakes studied. We therefore conclude that grazing does not play a major role in controlling *C. raciborskii* populations.

Ecological niche

To outline the main dimensions of the abiotic niche of *C. raciborskii* in its northernmost European habitats, we will now briefly summarize the data on regulation of its growth. Temperatures of 15–17 °C are necessary for the onset of the pelagic population. PAR limits the growth of the vegetative pelagic population from its inception. Low nitrogen concentrations enhance the organism's competitiveness. Over the course of the season, *C. raciborskii* must alternate between taking advantage of either N_2 fixation or light-limited growth.

Little is known about the position of *C. raciborskii* within the community structure or about how it interacts with the biota of the habitats it invades. Since the species contributes up to 23% of the total cyanobacteria biovolume, it can be postulated that it has forced out or suppressed one or more species by approximately 23%. We cannot specify which species were suppressed, since our study does not date back to the time prior to *C. raciborskii* invasion. However, the species that largely overlap with *C. raciborskii* in terms of ecological niche and the timing of their occurrence are most likely to be affected; these include co-occurring Nostocales, such as *Anabaena* spp. and *Aphanizomenon* spp., and Oscillatoriales, such as *Planktothrix agardhii* or *Limnothrix* spp. Unfortunately, most reports on the invasion of *C. raciborskii* into new habitats lack data on the prior phytoplankton composition. The only report of this kind was published by Dobberfuhl (2003), who observed a dramatic decrease in phytoplankton diversity in Florida lakes after the introduction of *C. raciborskii*; however, the author did not specify which species were involved. At a higher level, changes in phytoplankton composition might cause changes in the whole community. Moreover, effects of toxic secondary metabolites of *C. raciborskii* might affect other species.

Long-term trends

When studying a neocyanobacterium such as *C. raciborskii*, it is of great interest to determine whether its population size is still increasing and whether it might become a dominant or even bloom-forming species in the future, as has already been observed in some new habitats currently invaded (e.g., Tóth and Padisák 1986). Unfortunately, a period of 13 years is too short to determine any long-term trends due to the great interannual variability in the population size of *C. raciborskii* (Fig. 5). Furthermore, the exact date at which *C. raciborskii* first invaded the studied lakes is not known. Although the lakes have been studied since 1993, the invasion probably occurred even earlier and remained undetected until a certain population size had been reached. In all probability, its occurrence does not date back to the 1930s, since early limnological studies (Czensny 1938; Wundsch 1940; Schäperclaus 1941) prove that the lakes were dominated by cyanobacteria, mainly Oscillatoriales; however, *C. raciborskii* was not reported. The definitive causes of interannual variability in *C. raciborskii* population size cannot be determined at this time. Further studies are needed to clarify whether this is caused by interannual variability in climatic conditions or by lake specific variability in certain controlling parameters. However, as discussed below, one can assume that any further seasonal advance in temperature increase will accelerate the increase in species population size.

Biogeographic changes

It is generally assumed that changes in the biogeography of *C. raciborskii* have occurred due to rising water temperatures associated with climate change and/or to northward migration of ecotypes with lower temperature and light requirements. Ecological responses to recent climate changes, especially rising air and water temperatures, have been observed worldwide. In general, spring activities have begun to occur progressively earlier since the 1960s (Walther et al. 2002). In aquatic ecosystems of the northern temperate zone, changes in the seasonal phenology of plankton, which are connected to enhanced winter–spring water temperatures, were widely reported: *Daphnia* populations, for example, developed two weeks earlier in the German lakes Constance and Müggelsee (Straile 2000; Gerten and Adrian 2000). Spring peaks in phytoplankton occurred 20 days earlier in Lake Washington, USA

Fig. 6A–B *C. raciborskii* population size simulated using three starting points at Julian days 90, 120 and 150 in the given annual cycle with a constant inoculum size of 1 mm³ l⁻¹ (**A**) and variable inoculum sizes of 0.5, 1 and 1.5 mm³ l⁻¹ (**B**), respectively
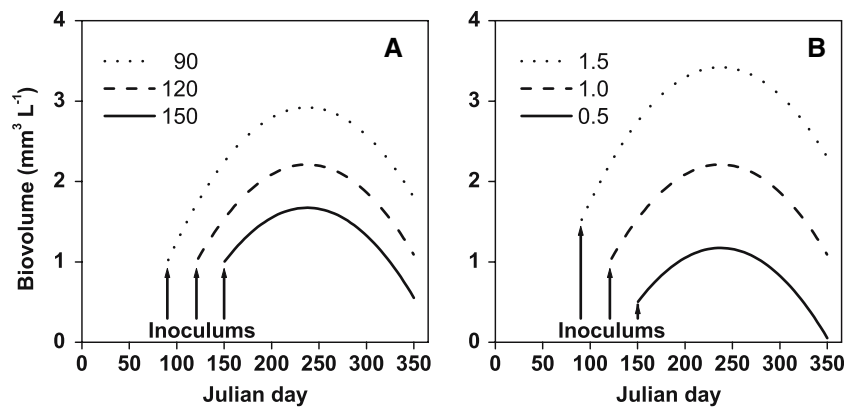
(Winder and Schindler 2004), 30 days earlier in Lake Erken, Sweden (Weyhenmeyer et al. 1999), and 30 days earlier in Lake Müggelsee (Gerten and Adrian 2000), which is located in our study area. In the North Sea, seasonal onsets of light-mediated plankton species were found to remain fixed in time, while those of temperature-mediated populations moved forward in time (Edwards and Richardson 2004). In the present study, we found that the population onset of *C. raciborskii* is temperature-mediated; our simulations predicted that a 30-day forward shift in emergence will double the population size (Fig. 6). We therefore conclude that the earlier increase in water temperature due to climate change has promoted the spread of *C. raciborskii* to the temperate zone. This change results in earlier germination and shifts the pelagic populations into a phase with higher light intensity, thereby promoting *C. raciborskii* growth and recurrence. The same mechanism could also explain the northward shift of the biogeographic boundaries of two other cyanobacteria, *Anabaena bergii* and *Aphanizomenon ovalisporum*, which were first detected in several German lakes in 2004 (Stüken et al. 2006).

Ecotype selection may be co-responsible for the observed changes in the biogeography of *C. raciborskii*. Interactions between ecology and evolution on the level of genetic changes or strong selection of ecotypes influence contemporary invasion dynamics in meaningful ways, as outlined by Lambrinos (2004). Phylogenetic studies have revealed genetic differences in *C. raciborskii* strains from different climatic regions (Gugger et al. 2005). Morphological differences (Fabbro and Duivenvoorden 1996) and differences in secondary metabolites (Saker and Griffiths 2000) have also been identified. Strains do not differ in their light-dependent growth (Briand et al. 2004), but they do differ in their ability to adapt to low temperatures (Chonudomkul et al. 2004). Our data showed that temperature does not limit the growth of the northern population until their maximum is reached during the annual cycle.

Of greater importance are our findings that *C. raciborskii* populations emerged at temperatures of 15–17 °C compared to 22 °C at Lake Balaton in Hungary (Górzo 1987; Padisák 1997). This provides further evidence in support of the selection of ecotypes with lower critical temperatures for akinete germination in the northern habitats. However, further experiments are needed to study whether strains from various climate regions differ in their temperature requirements for germination.

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Seasonal dynamics of cylindrospermopsin and cyanobacteria in two German lakes

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ABSTRACT

Cylindrospermopsin (CYN) is a potent hepatotoxin produced by different cyanobacteria of the order Nostocales, which is widely distributed in freshwaters and considered a hazard to wildlife and humans. In Europe, only *Aphanizomenon flos-aquae* is identified as CYN producer so far and the seasonal variability of CYN was not studied yet.

Therefore, we studied the seasonal dynamics of the particulate and dissolved CYN concentrations in relation to the cyanobacterial occurrence and environmental factors in two German lakes over two years.

Total CYN reached maximum concentrations of 0.34 and 1.80 µg L^{-1} in Melangsee and Langer See, respectively. In both lakes, the dissolved CYN fraction peaked after the particulate fraction, and reached higher values indicating that CYN is poorly decomposed and accumulates in the water. The cyanobacterial community was very diverse in both lakes, including potentially CYN producing species such as *Cylindrospermopsis raciborskii*, *Anabaena bergii* and *Aphanizomenon flos-aquae*. However, these species could be excluded as the major CYN producers. The strongest significant correlation was found for *Aphanizomenon gracile* and CYN, strongly indicating that the species is the main CYN producer in Langer See. CYN was also correlated with *Planktothrix agardhii* in Langer See and *Pseudanabaena limnetica* in Melangsee, but species of the order Oscillatoriales are not known to produce CYN. In Melangsee the CYN producer could not be identified. Different correlations of CYN with abiotic factors in the two lakes indicate the presences of further undetected CYN producers as well as different regulation mechanisms of their dynamic and the variability of CYN.

INTRODUCTION

Worldwide, many freshwaters are dominated by cyanobacteria. This is of major concern

for drinking water supply or for recreational water use, because cyanobacteria can

produce a wide range of bioactive and toxic substances, which can affect both wildlife

and humans (e.g. Carmichael and Falconer, 1993). One of these substances is

cylindrospermopsin (CYN), a potent hepatotoxin (Falconer et al., 1998). In 1979 at Palm

Island (Australia) humans suffered from hepatoenteritis after being supplied with

drinking-water from a reservoir (Bourke et al., 1983). Subsequent investigations revealed

that this was caused by CYN produced by the cyanobacterium *Cylindrospermopsis*

raciborskii blooming in the reservoir (Hawkins et al., 1985). Thereafter CYN was

detected in several other Australian waters (Hawkins et al., 1997; Saker and Griffiths,

2000) as well as in other countries in tropical regions e.g. Brazil (Carmichael et al.,

2001). Besides *C. raciborskii* other CYN-producing cyanobacteria were detected:

Umezakia natans in Japan (Harada et al., 1994), *Aphanizomenon ovalisporum* in

Australia and Israel (Shaw et al., 1999; Banker et al., 1997), *Anabaena bergii* in Australia

(Schembri et al., 2001), *Raphidiopsis curvata* in China (Li et al., 2001), and very

recently, *Aphanizomenon flos-aquae* in Germany (Preußel *et al.* 2006).

Up to this identification of *Aphanizomenon flos-aquae* as CYN-producing species,

C. raciborskii was the only known candidate for CYN-production in North European

waters. Its spread from tropical to temperate regions gave cause to study the occurrence

of CYN in German lakes, currently the northernmost distribution of this species (Krienitz

and Hegewald, 1996; Rücker et al., 1997). CYN was first detected in Melangsee

(Germany) in 2000 with maximum quantities of 17.3 µg CYN/g dry weight. However,

none of the strains of *C. raciborskii* isolated from the same lake produced CYN (Fastner et al., 2003). This is in line with other observations, as none of the *C. raciborskii* strains isolated from various European waters so far produces the toxin (Bernard et al., 2003; Saker et al., 2003). This raised the key question, who the CYN-producing species are in northern Europe. Also, little was known about the frequency of CYN occurrence and concentrations, as well as about the conditions under which this toxin occurs.

In 2004, a qualitative survey on the occurrence of CYN and cyanobacteria in 127 German lakes revealed that CYN occurs in 50 % of the lakes with quantities up to 73.2 µg CYN/g DW (Fastner et al., submitted). Due to the great diversity of cyanobacteria in these field samples CYN producers could not be identified. However, during the survey one potentially CYN producing species, *Anabaena bergii*, was found for the first time in Germany (Stüken et al., in press). Furthermore, CYN-producing strains of *Aphanizomenon flos-aquae* were isolated from some of these lakes (Preußel et al., 2006), and this finding is the first unambiguous detection of CYN producing cyanobacteria in Northern Europe. A subsequent study selected 20 lakes on the basis of the results of the earlier qualitative survey (Fastner et al., submitted) for more detailed quantitative assessment of CYN-occurrence, including not only the intra– but also the extracellular concentrations in relation to biovolume of cyanobacterial taxa. This showed that total CYN concentrations attained 12.1 µg L^{-1}, with the concentrations of the dissolved extra cellular CYN contributing the major portion to total CYN – on average 80 % (Rücker et al., submitted). Similar findings on total CYN concentrations in the range of 1.2-120 µg L^{-1} as well as high portions of the extra cellular CYN concentrations between 19-98 % were reported for Australian waters (Chiswell et al., 1999; Shaw et al., 1999; McGreggor

and Fabbro, 2000; Hoeger et al., 2004). In most Australian reservoirs the concentrations of CYN closely track the biovolume of *C. raciborskii* (McGreggor and Fabbro, 2000), and CYN-producing *C. raciborskii* strains have been isolated from Australian waters (e.g. Saker and Griffiths, 2000). However, the correlation between CYN concentrations and *C. raciborskii* abundance was weak (Saker and Griffiths, 2000). Other CYN incidences in Australia and Israel have been attributed to *Aphanizomenon ovalisporum* (Banker *et al*. 1997; Shaw *et al*. 1999).

In German lakes, the occurrence and quantities of CYN could not be explained by the abundance of the three so far known potentially CYN producing species (*C. raciborskii*, *A. bergii*, and *A. flos-aquae*), indicating the presence of so far undetected CYN producers (Rücker et al., submitted). Information on the seasonal variability of CYN exists only for two Australian lakes with year round occurrence of *C. raciborskii* (McGreggor and Fabbro, 2000). The regulation mechanisms of the occurrence and variability of CYN are poorly understood. Since CYN concentrations exceed the guideline value of 1 µg L^{-1} recommended by Humpage and Falconer (2003) in various German waters (Rücker *et al*., submitted), further identification of the CYN producers as well as information on the regulation of this hepatotoxin is of major importance for water quality assessment.

We studied for the first time the seasonal dynamics of cell bound and extracellular CYN in two German lakes over a two years period in relation to the occurrence of cyanobacterial species and in relation to environmental factors.

MATERIALS AND METHODS

Study sites. The two lakes studied — Langer See and Melangsee — are located in northeast Germany (57°20' N; 54° 35'E). Langer See and Melangsee are eutrophic and polymictic shallow lakes with mean depths of 2.1 m and 1.6 m, respectively. More detailed information on the lakes can be found in Nixdorf et al. (2003).

Sampling and analyses. The two lakes have been sampled monthly to bi-weekly at the deepest point during 2004 and 2005. On each sampling date, the secchi depth (z_{SD}) and depth profiles of water temperature, pH and oxygen saturation at 0.5 m intervals were measured using a multiparameter probe (Hydrolab H2O). Photosynthetically active radiation (PAR) was measured at half-meter intervals through the water column using two spherical quantum sensors (Li Cor SA 193). Attenuation of PAR in the water column was calculated according to Sommer (1994). Mean photosynthetically active radiation in the mixed water column (I_{mix}) was calculated from data of global radiation and PAR attenuation as described in Wiedner et al. (2002). Mixed samples from the whole water column were prepared by taking samples at half-meter intervals with a 2.3 L LIMNOS sampler. Aliquots of the mixed samples were analyzed to determine the concentrations of ammonia (NH_4-N), nitrate plus nitrite (NO_{total}-N), dissolved inorganic phosphorus (DIP), total phosphorus (TP) and total nitrogen (TN) according to standard methods (DEV, 1976 - 1998). Detection limits of the dissolved nutrients were 1.7 µg L^{-1} for DIP, 64 µg L^{-1} for NH_4-N and 3.2 µg L^{-1} for NO_{total}-N. Dissolved inorganic nitrogen (DIN) represents the sum of NH_4-N plus NO_{total}-N. Phytoplankton composition and biovolume were estimated using an aliquot fixed with Lugol's solution studied under an inverse microscope

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according to Utermöhl (1958) and Rott (1981). For the toxin analyses, water from the

mixed samples was filtered through glass microfibre filters (GF/C, 47mm

diameter,Whatman). Two filters and 200ml filtrate (2x 100ml) were frozen at –20°C

until analysis.

Cylindrospermopsin analysis.

Filters were placed in Eppendorf tubes and CYN extracted by adding 1.5 ml of water

(Welker et al. 2002). The samples were sonicated for 10 min, shaken for 1 hour and then

centrifuged. The extraction was repeated once and the pooled supernatants were dried by

vacuum centrifugation. For analysis the dried extracts were dissolved in 1 ml of water

and filtered (0.45 µm). The filtrate was filtered (0.45 µm) prior to analysis. CYN was

determined in the aqueous extract by LC-MS/MS using an Agilent 1100 series HPLC

system (Agilent Technologies, Waldbronn, Germany) coupled to an API 4000 triple

quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Framingham, MA)

equipped with a turbo-ionspray interface. The extract was separated using a Nova-Pak

C_{18} column (150 X 4.6 mm, 5 µm particle size; Waters, USA) at 30 °C and a flow rate of

0.8 ml min^{-1} with the following gradient programme: 100% A for 1 min, ramped to 100%

B in 5 minutes, held for 3 minutes and then to 100% A in 1 minute and equilibrated for 7

minutes (solvent A: 1% methanol/deionised water, solvent B 60% methanol/ deionised

water, both solvents contained 5 mM ammonium acetate (Eaglesham et al., 1999). The

mass spectrometer was operated in the multiple reaction-monitoring mode (MRM) with a

collision energy of 48eV. For the determination of CYN the transitions *m/z* 416.1

$(M+H^+)$ to 194 and 416.1/176 were monitored with a dwell time of 0.2 seconds.

Quantitation of CYN (purchased from Dr. A. Humpage, Australian Water Quality Centre,

Salisbury, Australia) was achieved using the 416.1/194 transition with the 416.1/176

transition monitored as confirmation ion. The detection limit was 10 pg on column.

Statistical analysis. Correlations between the CYN concentrations and the biovolume of

cyanobacteria as well as CYN concentrations and environmental factors were analyzed

by Spearman Rank correlation analysis using SPSS 12.0 for Windows (SPSS Inc.).

RESULTS

CYN concentration. The maximum concentration of total CYN in Langer See was 1.80

$\mu g\ L^{-1}$ in 2004, which is 3.9 fold higher than the maximum of 0.46 $\mu g\ L^{-1}$ in Melangsee

(Fig. 1A and 2A). Similar maximum concentrations of 0.49 and 0.32 $\mu g\ L^{-1}$ were

measured during 2005 in Langer See and Melangsee, respectively. In both lakes the

dissolved CYN fraction reached higher values than the particulate fraction, and peaked

after the particulate fraction except for Langer See in 2005, where both fractions reached

equivalent concentrations and displayed a similar dynamic.

Cyanobacteria diversity and dynamics. The phytoplankton was dominated by

cyanobacteria from early summer until late autumn. Both lakes are characterized by a

great diversity of cyanobacterial species. Over the investigation period 48 different

species were determined in Langer See and 46 in Melangsee, which are all listed here

(Tab.1), because at the present state of knowledge it is unclear which species potentially

produce CYN. Seasonal dynamics are presented for the predominating species and for

those that were already reported as potential CYN producers (Fig. 1 and 2). In both lakes,

the cyanobacteria community was dominated by species of the order Oscillatoriales -

Pseudanabeana limnetica in Melangsee and *Planktothrix agardhii* in Langer See - as well as by *Aphanizomenon gracile* of the order Nostocales. Subdominating species of the order Nostocales, of which the seasonal dynamics are presented here were: 1. *Cylindrospermopsis raciborskii*, which occurred in both lakes and years, but reached significant higher biovolume in Melangsee compared to Langer See; 2. Species of the genus *Anabaena* and *Anabaenopsis*, which were grouped due to their great species diversity; 3. *Aphanizomenon issatschenkoi* that occurred in both lakes and years. Other potentially CYN producing cyanobacteria of the genus *Aphanizomenon* were: 1. *A. flos-aquae* that was present only in Langer See, and co-occurred with CYN three times; 2. *A. aphanizomenoides*, that was present only in Melangsee, and co-occurred with CYN twice; 3. One *Aphanizomenon* species that could not unambiguously be identified and that was only present in Langer See, and co-occurred with CYN five times. These species are not presented individually and were not included in the correlation analysis.

Environmental parameters. Temperature and I_{mix} displayed a similar time course in both lakes (Fig. 3A and 4A), although I_{mix} reached clearly lower values in Langer See with a maximum of 100 µmol photons m^{-2} s^{-1} compared to Melangsee with a maximum of 200 µmol photons m^{-2} s^{-1}. Concentrations of TP, TN and DIP were higher in Langer See than in Melangsee (Fig. 3B and 4B), while similar concentrations of NH_4-N were found in both lakes. NH_4-N was the predominant fraction of the total dissolved nitrogen concentration, therefore, data on NO_{total}-N are not shown here. In both lakes the fraction of the dissolved phosphorous and nitrogen were around the detection limit during the summer months.

Correlations between CYN concentration and cyanobacteria or environmental factors. The analysis of correlations between the biovolume of cyanobacteria and CYN concentrations revealed different results for the two studied lakes (Tab. 2). In Langer See the highest significant correlation coefficient was found between *A. gracile* and the particulate CYN concentration. Significantly positive correlations were also found between *A. gracile* and the total and dissolved CYN concentration as well as between *P. agardhii* and all CYN concentrations. Additionally, the biovolumes of *A. gracile* and *P. agardhii* were significantly correlated (coefficient = 0.484, p = 0.002, N = 33). In Melangsee *P. limnetica* was significantly positive correlated with the total CYN concentration as well as with the particulate and the dissolved CYN fraction. Correlation analysis of abiotic environmental factors and CYN concentrations also revealed different results for the two studied lakes. In Langer See, the total and the dissolved CYN concentrations were significantly positive correlated with the NH_4-N and TN concentrations. In Melangsee, the total and the dissolved CYN concentrations were significantly positive correlated with I_{mix} and the particulate CYN concentration was significantly positive correlated with TN. Analysis of a pooled data set from both lakes revealed lower correlation coefficients in all cases (data not shown).

DISCUSSION

Dynamics of the cylindrospermopsin concentration. The maximum concentrations of total CYN determined for Langer See (1.80 and 0.49 µg L^{-1}) and Melangsee (0.34 and 0.32 µg L^{-1}) are in the lower range of the values reported for 20 German lakes in summer 2005, where the average total CYN concentration was 1.03 (SD 2.00) µg L^{-1} and maxima

reached 12.10 µg L^{-1} (Rücker et al. submitted). They were also low in comparison to values reported for Australian waters in the range of 1.2-120 µg L^{-1} (Chiswell et al., 1999; Shaw et al., 1999; McGreggor and Fabbro, 2000; Hoeger et al., 2004). A proposed guideline value of 1 µg L^{-1} for CYN in drinking water (Humpage and Falconer, 2003) was exceeded only in Langer See during 2004.

Seasonal variability of the particulate and dissolved CYN concentrations was not reported for any other lake before, and our data provide for the first time information on their seasonal pattern. Except for Langer See during 2005, the particulate CYN fraction increased first and then – with a delay of several weeks – the dissolved fraction increased. Thus, CYN remains cell bound and is not released for extended periods, which are most probably periods of exponential growth of the producer population. The inverse time course of the particulate and dissolved fraction points towards CYN release as a consequence of cell lysis during periods when the producer population collapses and decays. Interestingly, the concentrations of the dissolved fractions exceed by far the values of the particulate fraction. Several explanations are possible: 1. Enhancement of CYN production under unfavorable growth conditions; 2. Release of CYN from intact cells under certain environmental conditions; 3. A decay of producer cells before the maximum of the whole producer population is reached combined with an accumulation of the released CYN in the water.

High percentages of dissolved CYN were also reported from other field studies (Chiswell et al., 1999: Shaw et al., 1999; Rücker et al., submitted). Additionally our data show that CYN remains firstly cell bound and that the peak of dissolved CYN exceeds by far that of the particulate concentrations, indicating CYN accumulation in the water. Our

scenario that CYN remains cell bound during the exponential growth phase of the producer population agrees with laboratory findings of high percentages of intra cellular CYN during exponential growth of *C. raciborskii* strains and a shift to higher extra cellular CYN portions when the cultures move towards the stationary phase (Saker and Griffiths, 2000: Hawkins et al., 2001). Our theory on the accumulation of CYN in the water is supported by results from laboratory studies which show that low decomposition rates of CYN are found under several abiotic conditions in laboratory experiments. Only when samples were exposed to sunlight in the presence of plant pigments, rapid degradation took place (Chiswell et al., 1999). Long periods of CYN persistence for up to 6 weeks after cells of *C. raciborskii* were below the detectable level are also reported from field studies (McGregor and Fabbro, 2000).

Unlike to the pattern discussed above, both CYN fractions reached equal quantities and displayed a similar time course in Langer See during 2005. Whether this was caused by the loss of the producer cells by sedimentation or grazing before they released CYN, or resulted from optimal conditions for CYN decomposition, can not be answered here.

CYN producers. A question of major concern still is, who is/are the CYN producer(s) in German lakes? Several potential CYN producers occurred in the two lakes studied. Populations of *C. raciborskii* occurred in both lakes and years studied. Their biovolume was six-fold higher in Melangsee compared to Langer See, whereas CYN concentrations in Melangsee were only one third of those in Langer See in 2004. The biovolume of *C. raciborskii* was not significantly correlated with the CYN concentrations. Although many strains of *C. raciborskii* isolated from tropical regions do produce CYN (e.g. Saker and

Griffiths, 2000), strains isolated from Melangsee in 2000 did not produce CYN (Fastner et al., 2003), and this applies also for other European strains (Saker et al., 2003; Bernard et al., 2003). Thus, it is very unlikely that *C. raciborskii* is a main CYN producer, if it is a CYN producer in our lakes at all. *Anabaena bergii*, which was first detected in German waters in 2004 (Stüken *et al*. in press), is included here under the genus *Anabaena* and occurred only during 2005 in low quantities in Langer See at one and in Melangsee at five sampling dates. Strains of the species isolated from Australian waters were found to produce CYN (Schembri et al., 2001), and it is likely that it is the same species as *Aphanizomenon ovalisporum* (Komárek and Ettl, 1958; Hindák, 2000; Fergusson and Saint, 2003), of which CYN producing strains are reported from Australia and Israel (Shaw et al., 1999; Banker et al., 1997). Information on isolates from German waters does not exist yet. Thus the species has to be considered as one potential CYN producer but not the main one.

Species of the order Oscillatoriales – *Planktothrix agardhii* in Langer See and *Pseudanabaena limnetica* in Melangsee – were positively correlated with the CYN concentration. Reports on CYN producing Oscillatoriales do not exist so far. The correlations might be by coincidence, in case the species co-occur with the CYN producers; for example the biovolume of *P. agardhii* was strongly correlated with that of *A. gracile*. However, one species that does not belong to the order Nostocales – *Umezakia natans* of the order Stigonematales (Harada et al., 1994) – is already known to produce CYN. Thus, further studies on individual strains of Oscillatoriales from the two lakes are necessary to prove whether or not they do produce CYN. Surprisingly, *A. flos-aquae* the only species in Europe for which CYN production has unambiguously been

proven (Preußel et al., 2006), can be excluded as a major CYN producer in the two lakes studied here, because it did not occur in Melangsee at all, and was present in Langer See only at 3 and 5 sampling occasions in 2004 and 2005, respectively. However, an important result is that the strongest correlation was found between a closely related species, *A. gracile,* and the particulate CYN fraction in Langer See (Tab. 2 and Fig. 3). Together with the findings that other species of the genus *Aphanizomenon* (*A. flos-aquae* and *A. ovalisporum*) do produce CYN confine *A. gracile* as the main CYN producer in Langer See. Interestingly, *A. gracile* was not significantly correlated with CYN in Melangsee, where the CYN producing species cannot be inferred from the data at this point.

A difficulty in identifying the CYN producers is the fact that not all clones of a population necessarily produce CYN. For example not all strains of *C. raciborskii* or *A. flos-aquae* isolated so far produce CYN and populations of other cyanobacterial species have been shown to be composed of toxin-producing and non-producing genotypes (e.g. Kurmayer et al., 2001; Mbedi et al., 2005). The relationship between the abundance of CYN producing clones and CYN might remain undiscovered when analyzing the correlation between the total biovolume of individual species and CYN in case the relative abundance of CYN-producing clones shifts during the season or in case the cellular CYN content of the producer clones changes. The latter mechanism was not observed for strains of *C. raciborskii*, whose relative CYN content was observed to vary only within a narrow range between 0.14 - 0.20 % of cell dry weight (Saker et al., 1999), but such information is lacking for other CYN-producing species.

Impact of environmental factors on CYN variability. It is remarkable that CYN was correlated with different environmental parameters in the two lakes. This indicates different growth limitations of the CYN producers or different limitations of their CYN production in the two lakes. High abundances of cyanobacteria generally occurred under conditions of high TP concentrations that were correlated with CYN in Langer See as well as under conditions of high TN concentrations that were correlated with CYN in both lakes. Low NH_4-N concentrations are generally known to favor the development of N-fixing species (e.g. Blomqvist et al., 1994), which can explain the negative correlation between CYN and NH_4-N in Langer See. Nostocales were also often found to be limited by I_{mix} in polymictic shallow lakes (e.g. Wiedner et al., 2002), which can be the reason for the negative correlation between CYN and I_{mix} in Melangsee.

Environmental parameters can also affect the decomposition of dissolved CYN in the water. For example CYN was rapidly decomposed in samples exposed to sunlight (Chiswell et al., 1999). The strong negative correlation between I_{mix} and dissolved CYN in Melangsee support the findings on decomposition of CYN by light. However, the portion of dissolved CYN to total CYN in Melangsee is not lower compared to Langer See where I_{mix} is significantly lower, and thus, lower CYN decomposition by light could be expected. Therefore, there must be other mechanisms regulating the CYN decomposition.

In summary, the analyses of the relationships between CYN and cyanobacteria as well as CYN and environmental factors strongly indicate different CYN producers or at least different regulations of CYN producing clones in both lakes. To gain further insights into

the regulation of CYN and to create a basis to predict CYN in natural waters, further

studies are needed to identify all potentially CYN producing species, to estimate whether

changes in the relative abundance of CYN producing clones occur within populations,

and to clarify the regulation of their dynamics and CYN production.

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	Melangsee				Langer See			
	2004		2005		2004		2005	
	Max.	N	Max.	N	Max.	N	Max.	N
Anabaena sp.	0.07	5	0.05	4	0.28	8	0.09	4
Anabaena affinis	0.11	1	< 0.01	1	0.28	6	0.37	3
Anabaena bergii			0.04	5			0.00	1
Anabaena circinalis					0.08	3	0.01	3
Anabaena compacta					0.01	1	0.03	2
Anabaena crassa	0.35	1			0.08	1		
Anabaena flos-aquae	0.45	2	0.39	4	0.63	6	0.43	8
Anabaena macrospora			0.02	1				
Anabaena planctonica			0.18	2	1.03	3		
Anabaena viguieri			0.04	3			0.14	8
Anabaenopsis sp.					3.64	3	0.01	1
Anabaenopsis cunningtonii							0.12	5
Anabaenopsis elenkinii					0.35	2	0.14	6
Aphanizomenon sp.							0.19	10
Aphanizomenon aphanizomenoides			0.04	2				
Aphanizomenon flos-aquae					0.07	3	0.14	5
Aphanizomenon gracile	8.22	15	3.84	18	11.25	16	7.35	22
Aphanizomenon issatschenkoi	0.08	4	0.07	3	1.84	13	1.11	12
Aphanocapsa sp.			0.07	4	< 0.01	1		
Aphanocapsa elachista			0.03	3			0.05	5
Aphanocapsa holsatica	0.04	6			0.12	10	0.03	3
Aphanothece clathrata	0.37	12	0.15	15	0.08	12	0.02	10
Chroococcus sp.	0.00	3			0.02	4		
Chroococcus minutus			0.03	7			0.01	3
Coelosphaerium sp.			0.11	1				
Cyanodictyon sp.			0.34	8				
Cylindrospermopsis raciborskii	5.83	11	0.74	13	0.26	5	0.39	9
Gomphosphaeria sp.			0.08	3			< 0.01	1
Limnothrix sp.	5.25	4	2.57	11			3.93	9
Limnothrix planctonica	0.58	5			0.07	2		
Limnothrix redekei			0.74	4	0.01	2	3.85	13
Merismopedia tenuissima	0.01	7	< 0.01	10	0.01	6	0.04	6
Microcystis sp.			0.01	1			0.14	4
Microcystis aeruginosa	0.19	7	0.45	15	0.60	10	0.14	9
Microcystis flos-aquae			0.03	5	0.34	2	0.10	4
Microcystis viridis			0.16	6			0.16	1
Microcystis wesenbergii	0.03	1	0.14	10			0.05	1
Phormidium sp.	0.20	10	0.65	6	0.01	1	0.06	5
Planktolyngbya sp.	0.06	10	0.25	8	0.03	13		
Planktolyngbya limnetica			0.15	3			0.19	15
Planktothrix agardhii	2.54	17	1.55	20	15.03	19	5.36	22
Pseudanabaena limnetica	32.21	17	18.90	21	16.38	20	6.94	23
Raphidiopsis mediterranea			0.22	5			0.06	4
Snowella sp.	0.08	6	0.02	4	0.10	8	0.17	6
Spirulina sp.							0.02	1
Synechocystis sp.	0.01	3	0.02	7	0.00	5	0.01	5
Woronichinia compacta	0.01	1						
Woronichinia naegeliana			0.03	1			0.03	3

Table 1. List of all cyanobacterial species detected in the two studied lakes in 2004 and 2005. For each species the maximum biovolume (Max.) and the number of times it occurred is presented for each lake and year. Not included in the list are 9 species of the order Chroococcales, which either occurred less than twice with a biovolume less than 0.01 mm^3 L^{-1} or which could not be determined on the level of genus or species.

	Langer See			Melangsee		
	CYN part.	CYN dis.	CYN tot.	CYN part.	CYN dis.	CYN tot.
Anabaena spp.	0.262 (22)	-0.074 (22)	-0.094 (22)	-0.192 (22)	-0.385 (22)	-0.386 (22)
Anabaenopsis spp.	-0.065 (22)	-0.349 (22)	-0.333 (22)	-	-	-
A. gracile	0.803** (22)	0.512** (22)	0.608** (22)	0.259 (22)	-0.319 (22)	-0.067 (22)
A. isatschenkoi	0.352 (18)	0.485 (18)	0.455 (18)	0.714 (6)	0.068 (6)	0.486 (6)
C. raciborskii	0.000 (13)	-0.179 (13)	0.014 (13)	0.270 (19)	0.378 (19)	0.388 (19)
Limnothrix spp.	-0.294 (22)	-0.465 (22)	-0.429 (22)	0.069 (22)	0.069 (22)	0.069 (22)
P. agardhii	0.721** (22)	0.574** (22)	0.654** (22)	0.019 (22)	0.476 (22)	0.346 (22)
P. limnetica	0.321 (22)	0.357 (22)	0.361 (22)	0.691** (21)	0.382 (21)	0.653** (21)
Chroococcales	0.039 (22)	-0.292 (22)	-0.302 (22)	0.154 (22)	-0.007 (22)	0.108 (22)
Temperature	0.250 (23)	0.121 (23)	0.150 (23)	0.269 (24)	-0.026 (25)	0.173 (25)
I_{mix}	-0.132 (23)	-0.112 (23)	-0.121 (23)	-0.647** (24)	-0.632** (25)	-0.717** (25)
NO_{total}-N	-0.038 (23)	-0.336 (23)	-0.271 (23)	-0.106 (24)	0.052 (25)	-0.045 (25)
NH_4-N	-0.362 (23)	-0.614 ** (23)	-0.564** (23)	-0.363 (24)	-0.104 (25)	0.287 (25)
DIP	0.153 (23)	-0.290 (23)	-0.208 (23)	-0.049 (24)	0.174 (25)	0.075 (25)
TP	0.529** (23)	0.544** (23)	0.579** (23)	0.413 (24)	0.070 (25)	0.329 (25)
TN	0.486 (23)	0.850** (23)	0.839** (23)	0.820** (24)	0.274 (25)	0.649** (25)

Table 2. Spearman rank correlation coefficients; significance level of $p < 0.01$ is indicated by **, number of cases included in the analysis are given in parenthesis.
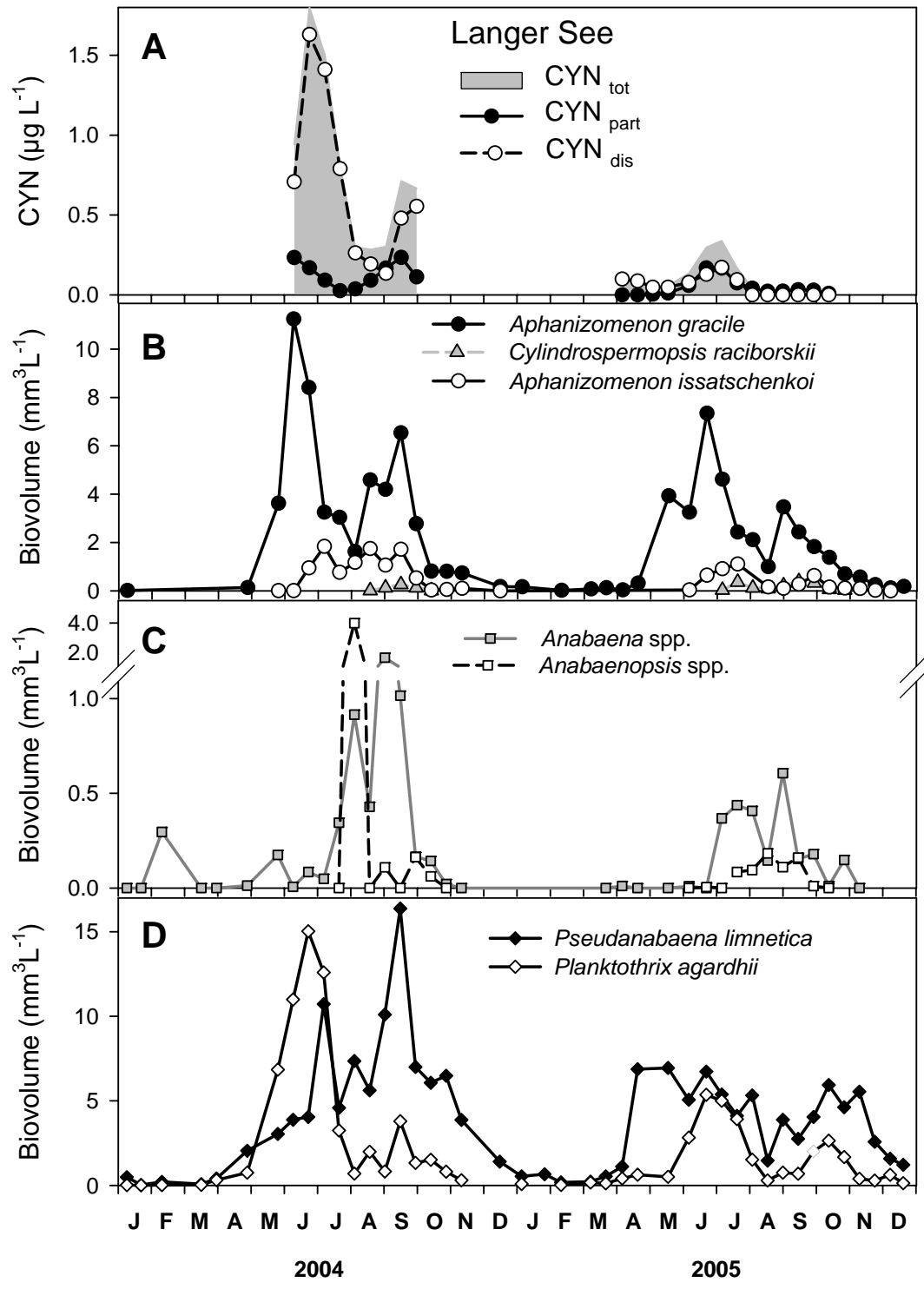
Figure 1. Seasonal dynamics of the total, particulate and dissolved CYN concentrations

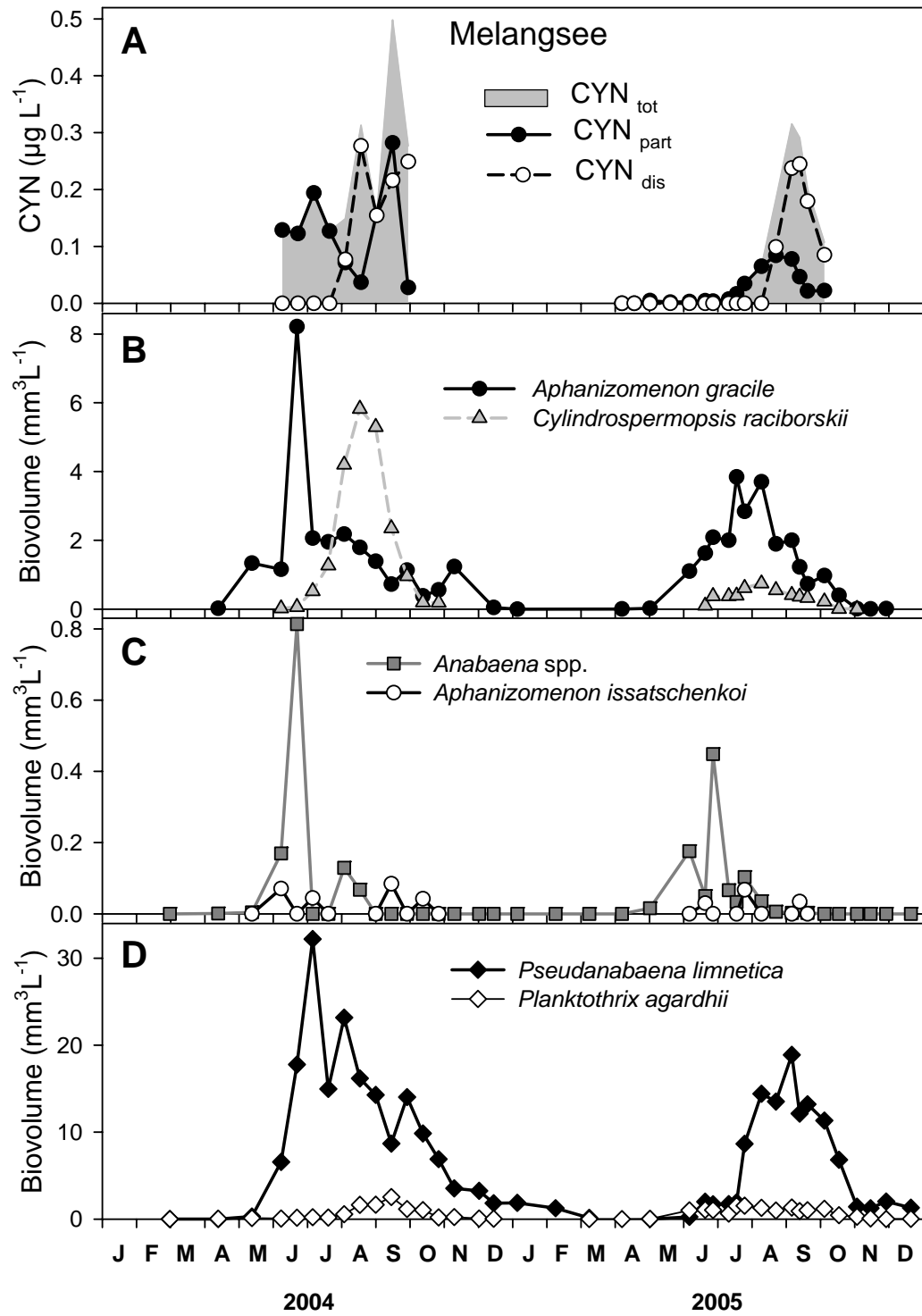(A), and of the cyanobacteria biovolumes (B-D) in Langer See during 2004/05.

Figure 2. Seasonal dynamics of the total, particulate and dissolved CYN concentrations

(A), and of the cyanobacteria biovolumes (B-D) in Melangsee during 2004/05.
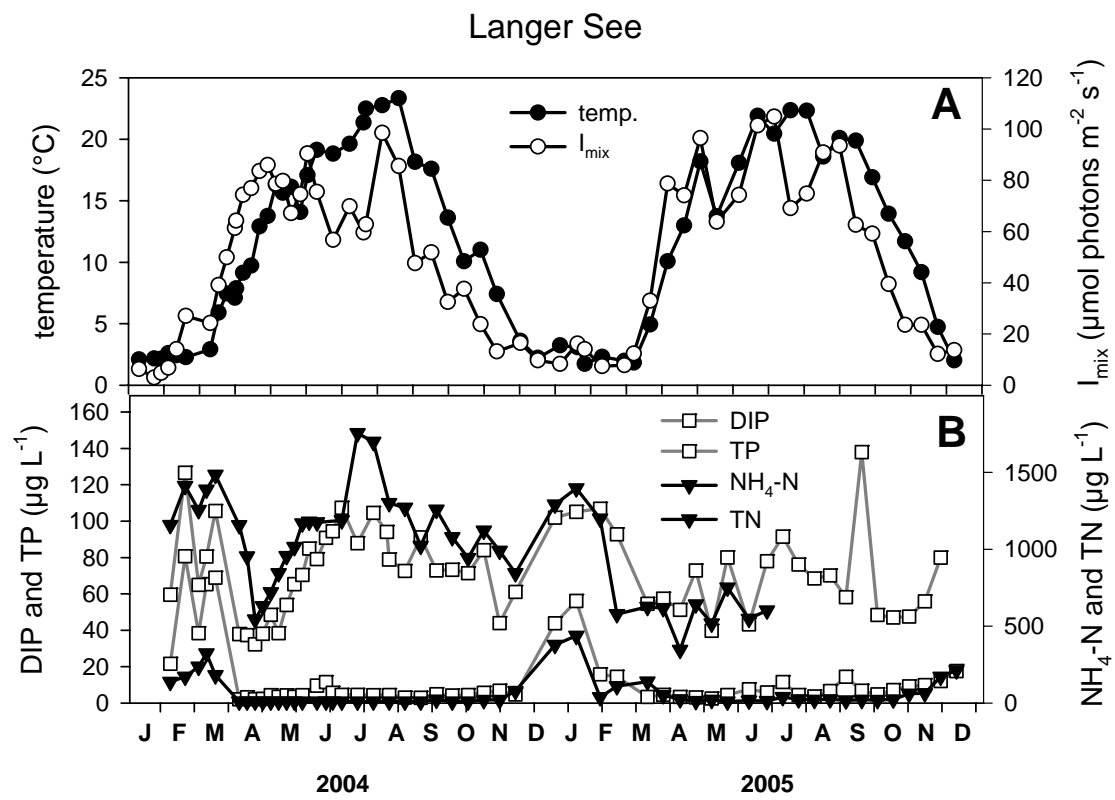
Figure 3. Seasonal dynamics of temperature and I_{mix} (A), phosphorous and nitrogen
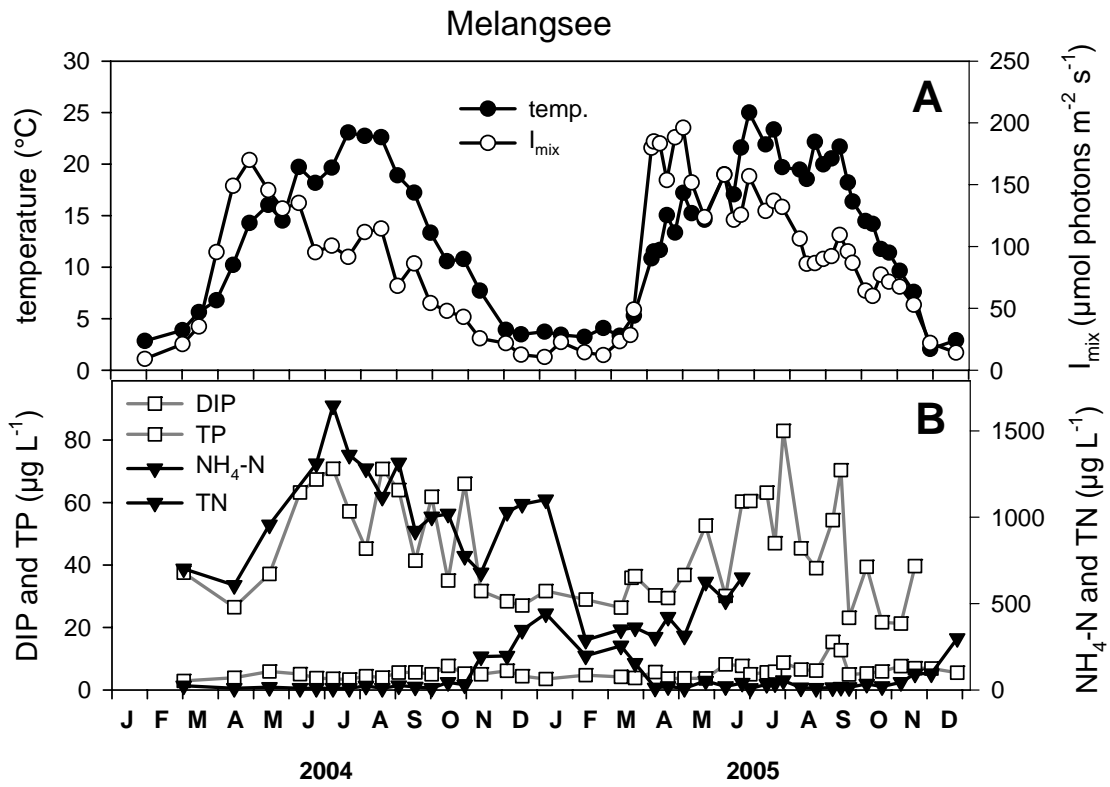
concentrations in Langer See during 2004/05.

Figure 4. Seasonal dynamics of temperature and I_{mix} (A), phosphorous and nitrogen concentrations in Melangsee during 2004/05.
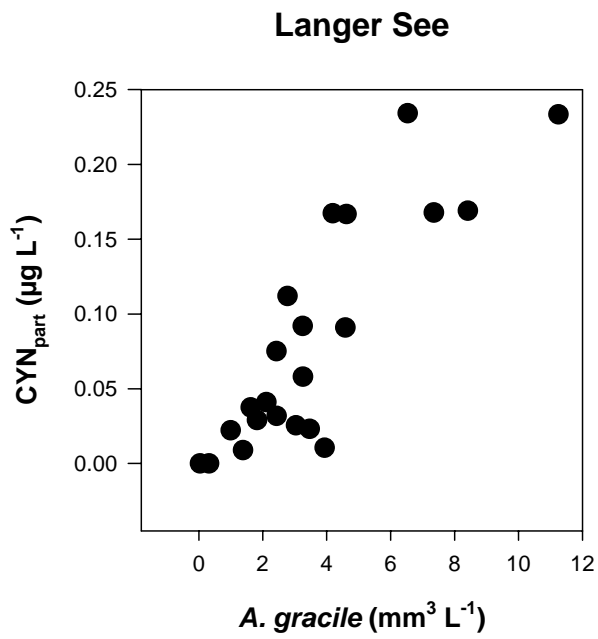
Langer See

Figure 5. Biovolume of *A. gracile* versus the concentration of particulate CYN in Langer See (n=22, r_S=0.803, p=0.000).