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# 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*.


*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/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. 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 ×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