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First report of anatoxin-a-producing cyanobacterium *Aphanizomenon issatschenkoi* in northeastern Germany

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ABSTRACT

The neurotoxin anatoxin-a (ATX), has been detected in several northeast German lakes during the last two decades, but no ATX producers have been identified in German water bodies so far. In 2007 and 2008, we analyzed phytoplankton composition and ATX concentration in Lake Stolpsee (NE Germany) in order to identify ATX producers. Sixty-one *Aphanizomenon* spp. strains were isolated, morphologically and phylogenetically characterized, and tested for ATX production potential by liquid chromatography–tandem mass spectrometry (LC–MS/MS). New primers were specifically designed to identify a fragment of a polyketide synthase gene putatively involved in ATX synthesis and tested on all 61 *Aphanizomenon* spp. strains from L. Stolpsee and 92 non-ATX-producing *Aphanizomenon* spp., *Anabaena* spp. and *Anabaenopsis* spp. strains from German lakes Langersee, Melangsee and Scharmützelsee.

As demonstrated by LC–MS/MS, ATX concentrations in L. Stolpsee were undetectable in 2007 and ranged from 0.01 to 0.12 $\mu g\, l^{-1}$ in 2008. Fifty-nine of the 61 strains isolated were classified as *Aphanizomenon gracile* and two as *Aphanizomenon issatschenkoi*. One *A. issatschenkoi* strain was found to produce ATX at concentrations of $2354 \pm 273\ \mu g\, g^{-1}$ fresh weight, whereas the other *A. issatschenkoi* strain and *A. gracile* strains tested negative. The polyketide synthase gene putatively involved in ATX biosynthesis was found in the ATX-producing *A. issatschenkoi* strain from L. Stolpsee but not in the non-ATX-producing *Aphanizomenon* spp., *Anabaena* spp. and *Anabaenopsis* spp. strains from lakes Stolpsee, Langersee, Melangsee, and Scharmützelsee.

This study is the first confirming *A. issatschenkoi* as an ATX producer in German water bodies.

1. Introduction

Cyanobacterial blooms and mats are increasingly recognized as sources of potent hepatotoxic and neurotoxic cyanotoxins (Codd et al., 1999; Chorus and Bartram, 1999). The alkaloid anatoxin-a (ATX), is one of the neurotoxic compounds produced by some cyanobacterial species. In fact, it was the first cyanobacterial toxin to be chemically and functionally defined (Huber, 1972; Devlin et al., 1977). Anatoxin-a is a potent nicotinic acetylcholine receptor agonist causing toxic effects including muscle fasciculation, gasping, convulsions and death by respiratory arrest in vertebrates (Carmichael, 1992).

Anatoxin-a has been detected in freshwater bodies worldwide (Codd et al., 1999). However, only few cyanobacterial strains producing anatoxin-a have been identified so far. These include *Anabaena* spp. strains from Irish lakes (James et al., 1997), *Anabaena flos-aquae* Breb. ex Born. et Flah., *Aphanizomenon flos-aquae* Ralfs ex Born. et Flah. and *Cylindrospermum* sp. strains from Finnish lakes (Rapala et al., 1993; Sivonen et al., 1989), *Anabaena planctonica*

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Brunnth. and *Planktothrix rubescens* (D. C. ex Gomont) Anagn. et Komárek strains from Italian lakes (Bruno et al., 1994; Viaggiu et al., 2004), *Raphidiopsis mediterranea* Skuja strains from Japanese lakes (Namikoshi et al., 2003), *Phormidium favosum* (Bory) Gomont strains from French lakes (Gugger et al., 2005), and *Aphanizomenon issatschenkoi* (Usacev) Proshkina-Lavrenko and *Phormidium autumnale* (Agardh) Trevisan ex Gomont strains from New Zealand lakes (Wood et al., 2007a,b).

ATX was detected in 25% of eighty German lakes surveyed in 1995–1997, (Bumke-Vogt et al., 1999), but no anatoxin-a could be detected in 92 *Anabaena*, *Aphanizomenon* and *Anabaenopsis* strains recently isolated from five northeast German lakes (Ballot et al., 2010). In fact, no sources of ATX production have been identified in German water bodies to date.

Cadel-Six et al. (2009) recently developed a PCR-based method to detect the presence of anatoxin-a- and homoanatoxin-a-producing *Oscillatoria* spp. in field samples or cultures. The primers developed by these investigators amplify a part of a polyketide synthase encoding gene putatively involved in anatoxin-a and homoanatoxin-a production by *Oscillatoria* spp.

We used the following polyphasic approach to identify producers of ATX in L. Stolpsee in NE Germany. Water samples taken from L. Stolpsee in 2007 and 2008 were analyzed for cyanobacterial composition and for the presence of ATX using liquid chromatography with tandem mass spectrometry (LC–MS/MS). Sixty-one *Aphanizomenon* spp. strains were isolated from L. Stolpsee, classified morphologically and phylogenetically, screened for ATX with LC–MS/MS, and tested for the presence of putative ATX biosynthesis encoding genes. Additionally, 92 non-ATX-producing *Aphanizomenon* spp., *Anabaena* spp., and *Anabaenopsis* spp. strains isolated from lakes Langersee, Melangsee and Scharmützelsee (Ballot et al., 2010) were tested for the presence of putative ATX biosynthesis encoding genes.

Six non-ATX-producing *A. issatschenkoi* strains isolated from L. Langersee (Ballot et al., 2010) were included in the phylogenetic classification.

2. Material and methods

2.1. Sampling and analysis

Water samples were taken fortnightly from L. Stolpsee (NE Germany) between June 2007 and December 2008 at the deepest part of the lake. Mixed samples were prepared using samples collected at meter intervals from the epilimnion (0–10 m maximally) with a 2.3 l LIMNOS sampler.

For determination of cyanobacterial toxins 150–300 ml of the mixed sample were filtered through 0.45 μm RC 55 membrane filters (Whatman, Dassel, Germany). Filters and filtrates were stored at −80 °C until further analysis.

100 ml of the mixed sample were preserved with Lugol's solution for morphological determination and enumeration of phytoplankton taxa. Enumeration was conducted using Utermöhl sedimentation chambers (Utermöhl, 1958) and an inverse microscope (Wilovert S; Hund, Wetzlar, Germany). Measurements of length and width of 20 vegetative cells, heterocytes and akinetes were conducted for each cyanobacterial taxon to calculate mean cell biomass.

2.2. Isolation of strains and morphological characterization

Sixty-one *Aphanizomenon* spp. strains were isolated from L. Stolpsee. The isolated filaments were washed 5 times and placed on microtiter plates containing 300 μl Z8 medium (Kotai, 1972). After successful growth, the samples were placed in 50-ml flasks containing 20 ml Z8 medium and maintained at 20 °C and a photon flux density of 80 μmol of photons $m^{-2}\,s^{-1}$. From each strain between 10 and 20 ml of culture material was filtered in triplicates through 0.45 μm RC 55 membrane filters (Whatman, Dassel, Germany) for cyanotoxin analysis. Filters and filtrates were stored at −80 °C until further analysis. Ten ml of culture material was preserved with Lugol's solution for biomass determination.

Morphological studies were conducted using an Olympus BX51 light microscope and color view imaging system (Olympus, Germany). Fifty measurements of lengths and width of filaments were made to calculate mean biomass of filaments. Length and width of 50 vegetative cells (to calculate mean cell biomass) and of heterocytes and akinetes were measured. Enumeration of filaments was carried out using Utermöhl sedimentation chambers (Utermöhl 1958) and an inverse microscope Eclipse TS 100 (Nikon Corporation, Tokyo, Japan). Strains were classified based on morphological traits according to Komárek and Komárkova (2006).

2.3. Genomic DNA extraction and PCR amplification and sequencing

Fresh culture material of all 61 *Aphanizomenon* spp. strains from L. Stolpsee was frozen and thawed three times, then boiled for 5 min and subsequently centrifuged for 5 min (13,000 rpm or ~16,000 × *g*). The supernatants were discarded. Each pellet was resuspended in 100 μl distilled water and vortexed for 1 min. Genomic DNA was extracted using the MoleStrips DNA blood kit and the DNA-Cyano protocol on GeneMole (Mole Genetics, Lysaker, Norway) according to the manufacturer's instructions. All PCRs were performed on a Peltier thermal cycler PTC 200 (MJ Research, Inc., San Francisco, CA) using the Taq PCR core kit (Qiagen GmbH, Hilden, Germany). The reaction mixture contained 0.1 μl Taq DNA polymerase (5 U/μl), 0.5 μl deoxynucleoside triphosphate mix (10 mM), 2 μl Qiagen PCR buffer, 1 μl each forward and reverse primer (10 μM), and 1 μl genomic DNA, yielding a total volume of 20 μl. PCR of the 16S rRNA gene was conducted for both *A. issatschenkoi* strains from L. Stolpsee and for 6 *A issatschenkoi* strains from L. Langersee (Ballot et al., 2010) using primers pA and B23S (Edwards et al., 1989; Gkelis et al., 2005). The cycling protocol for the 16S rRNA gene fragment was one cycle for 5 min at 94 °C, then 30 cycles of 30 s at 94 °C, 30 s at 57 °C and 1 min at 70 °C; with a final elongation step of 72 °C for 3 min. To amplify the gene encoding the polyketide synthase (PKS) fragment of the putative anatoxin (ATX)