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Anatoxin-a producing *Tychonema* (Cyanobacteria) in European waterbodies

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ABSTRACT

In order to identify the cyanobacterial species responsible of anatoxin-a (ATX) production in Lake Garda (Northern Italy), an intensive isolation and culturing of filamentous cyanobacteria were established since 2014 from environmental samples. In this work, we report a detailed account of the strategy adopted, which led to the discovery of a new unexpected producer of ATX, *Tychonema bourrellyi*. So far, this species is the first documented example of cultured Oscillatoriales able to produce ATX isolated from pelagic freshwater ecosystems. The isolated filaments were identified adopting a polyphasic approach, which included microscopic species identification, genetic characterisation and phylogenetic analyses based on 16S rRNA genes. The taxonomic identification was further confirmed by the high (>99%) *rbcLX* sequence similarities of the *T. bourrellyi* strains of Lake Garda with those deposited in DNA sequence databases. More than half of the isolates were shown to produce a significant amount of ATX, with cell quota ranging between 0.1 and 2.6 μg mm^{-3}, and 0.01 and 0.35 pg $cell^{-1}$. The toxic isolates were tested positive for *anaC* of the anatoxin-a synthetase (*ana*) gene cluster. These findings were confirmed with the discovery of one ATX producing *T. bourrellyi* strain isolated in Norway. This strain and a further non-ATX producing Norwegian *Tychonema bornetii* strain tested positive for the presence of the *anaF* gene of the *ana* gene cluster. Conversely, none of the Italian and Norwegian *Tychonema* strains were positive for microcystins (MCs), which was also confirmed by the absence of *mcyE* PCR products in all the samples analysed. This work suggests that the only reliable strategy to identify cyanotoxins producers should be based on the isolation of strains and their identification with a polyphasic approach associated to a concurrent metabolomic profiling.

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1. Introduction

The long evolutionary history is the basis of the high competitive ability that characterizes cyanobacteria. They are distributed in most aquatic and terrestrial habitats, including extreme environments (Paerl et al., 2003; Boyer and Zimba, 2007; Kleinteich et al., 2012). In waterbodies characterized by high concentrations of nutrients, limited water exchange and high temperatures and thermal stability, cyanobacteria can develop with high biomasses, giving rise to the formation of blooms at the surface, euphotic zone or in the metalimnic layers, largely depending on the respective species (Paerl and Paul, 2012). Cyanobacteria represent one of the major causes of ecosystem degradation and impairment of the economical value of freshwater resources. Specific strains produce a wide range of powerful toxins, with important implications for health risks associated with the human exploitation of recreational and drinking waters (Meriluoto and Codd, 2005; Mankiewicz-Boczek et al., 2011; Zamyadi et al., 2012). The principal classes of cyanotoxins are microcystins, nodularins, anatoxin-a and homoanatoxin-a, anatoxin-a(S), saxitoxins and cylindrospermopsins (Metcalf and Codd, 2012; Méjean et al., 2014).

Compared with microcystin (MC) producers, only a few anatoxin-a (ATX) producing taxa have been distinctly isolated and characterized (Table 1). Other reports, based on analyses carried out on bulk environmental samples, suggest the existence of a wide spectrum of potential cyanobacterial taxa able to produce ATX (see, among the others, Carrasco et al., 2007; Van Apeldoorn et al., 2007; Aráoz et al., 2010; Metcalf and Codd, 2012; Quiblier et al., 2013). Many reports, however, were not confirmed by analyses made on isolated strains. Toxic species can be detected using direct analytical chemical approaches (Meriluoto and Codd, 2005; Humpage et al., 2012; Metcalf et al., 2012) as well as molecular methods able to detect the presence of toxin biosynthetic genes (Pearson and Neilan, 2008; Sivonen, 2008; Rantala-Ylinen et al., 2011a). Nevertheless, until a few years ago, a genetic molecular approach to identify ATX encoding genes was not feasible because of the unknown biosynthetic pathway leading to the production of anatoxin. Biosynthetic genes coding for ATX have been characterized only recently in a benthic *Oscillatoria* PCC 6506 (Méjean et al., 2009, 2010) and planktonic *Anabaena* sp. strain 37 (Rantala-Ylinen et al., 2011b), opening the way to the design and use of primers for the detection of genes coding ATX in *Oscillatoria*, *Phormidium*, *Aphanizomenon* and *Anabaena* strains (Cadel-Six et al., 2009; Ballot et al., 2010; Wood et al., 2010; Rantala-Ylinen et al., 2011b).

In a recent work, Cerasino and Salmaso (2012) documented a widespread presence of ATX in the lake district south of the Alps. Based on analyses carried out on environmental samples collected during the warmer months, detectable concentrations of ATX ranging between 0.1 and 0.6 μg L^{-1} were found in the lakes Garda, Iseo, Como and Maggiore, i.e. the largest lakes that experienced a recent colonization and summer surface blooms of *Dolichospermum lemmermannii* (Salmaso et al., 2012). However, a clear identification of producers in the different seasons was not possible because biological analyses on isolated strains were not available.

Based on the hypothesis that filamentous cyanobacteria could possibly be amongst the ATX producers, cultures of Oscillatoriales were established from environmental samples collected since 2014 in Lake Garda with the aim to isolate potential new producers. Owing to the very low abundance of cyanobacteria usually recorded in the winter months (Salmaso, 2011), samples were collected using plankton nets and initial cultures established. The isolated cyanobacteria were then examined and identified following a polyphasic approach (Vandamme et al., 1996; Lee et al., 2014), which included microscopic species identification, genetic and phylogenetic analyses. Culture strains were further screened for cyanotoxins, particularly ATX and MCs, and tested for the presence of ATX and MCs biosynthesis encoding genes. Above approach led to the discovery and characterization of a new unexpected filamentous cyanobacterial producer of ATX.

2. Methods

2.1. Study site

Lake Garda is located at the southern border of the north eastern Italian Alps, at 65 m a.s.l. With a volume of more than 49×10^9 m^3, a maximum depth of 350 m and a surface of

Table 1 – Cyanobacterial anatoxin-a producers. The list, at the genus level, includes only the results obtained from analyses carried out on isolated strains in culture conditions.

	Genus	Selected references
Heterocystous genera	*Dolichospermum/Anabaena*	Sivonen et al. (1989), Lakshmana Rao et al. (2002) and Rantala-Ylinen et al. (2011b)
	Aphanizomenon	Sivonen et al. (1989) and Osswald et al. (2009)
	Cuspidothrix (*Aphanizomenon*)	Wood et al. (2007a), Ballot et al. (2010) and Hodoki et al. (2013)
	Cylindrospermum	Sivonen et al. (1989)
Oscillatoriales	*Oscillatoria*[a]	Sivonen et al. (1989), Edwards et al. (1992), Aráoz et al. (2005) and Rantala-Ylinen et al. (2011b)
	Phormidium[b]	Wood et al. (2012) and Harland et al. (2013, 2014)
	Tychonema	This work

[a] Including *O. limnetica* (*Pseudanabaena limnetica*).
[b] Populations of *Phormidium* producing ATX were observed for the first time in benthic river mats (Wood et al., 2007b).