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Co-occurrence of microcystin and anatoxin-a in the freshwater lake Aydat (France): Analytical and molecular approaches during a three-year survey

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ABSTRACT

Cyanobacterial mass occurrence is becoming a growing concern worldwide. They notably pose a threat to water users when cyanotoxins are produced. The aim of this study was to evaluate the occurrence and the dynamics of two cyanotoxins: microcystin (MC) and anatoxin-a (ANTX-a), and of two of the genes responsible for their production (respectively *mcyA* and *anaC*) during three consecutive bloom periods (2011, 2012 and 2013) in Lake Aydat (Auvergne, France). MC was detected at all sampling dates, but its concentration showed strong inter- and intra-annual variations. MC content did not correlate with cyanobacterial abundance, nor with any genera taken individually, but it significantly correlated with *mcyA* gene abundance ($R^2 = 0.51$; $p = 0.042$). MC content and *mcyA* gene abundance were maximal when cyanobacterial abundance was low, either at the onset of the bloom or during a trough of biomass. The LC-MS/MS analysis showed the presence of ANTX-a in the 2011 samples. To our knowledge, this is the first report of the presence of this neurotoxin in a French lake. The presence of ANTX-a corresponded to the only year for which *Anabaena* did not dominate the cyanobacterial community alone, and several cyanobacterial genera were present, including notably *Aphanizomenon*. *anaC* gene detection by PCR was not coherent with ANTX-a presence, both gene and toxin were never found for a same sample. This implies that molecular tools to study genes responsible for the production of anatoxin-a are still imperfect and the development of new primers is needed. This study also highlights the need for better monitoring practices that would not necessarily focus only on the peak of cyanobacterial abundance and that would take cyanotoxins other than MC into account.

1. Introduction

During the last few decades, cyanobacterial bloom occurrence has been constantly increasing worldwide (Paerl et al., 2011). The consequences of these blooms are diverse: aesthetics and odors, fish kills due to O_2 depletion, trophic dead-end, ecosystem imbalance, etc. But the most hazardous effect is probably the production of cyanotoxins that can be classified into 3 groups according to their target organ: hepatotoxins, neurotoxins and dermatotoxins. Their presence in drinking or recreational waters poses serious threats to human health and for the ecosystem functioning. Therefore, the assessment of the toxic potential associated with cyanobacterial blooms has become a growing concern.

The most ubiquitous cyanotoxins are the hepatotoxic microcystins (MC), a group of approximately 90 variants, which are found worldwide, and produced by several cyanobacterial genera, including *Microcystis*, *Planktothrix* and *Anabaena* (Welker and Von Döhren, 2006). MC are potent inhibitors of protein phosphatase

Abbreviations: ANTX-a, anatoxin-a; LC, Liquid Chromatography; LC-MS/MS, Liquid chromatography coupled to tandem mass spectrometry; MC, Microcystin; PCR, Polymerase Chain Reaction; qPCR, Quantitative Polymerase Chain Reaction.

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activity and they have been implicated in many cases of animals and humans poisonings (Jochimsen et al., 1998). They are also thought to be a cause of high levels of primary liver cancer in China (Duy et al., 2000). Consequently, MC are now actively monitored by health agencies in surface waters when potential producers' abundance is high. In the field of research, their occurrence and dynamics have also been thoroughly studied over the last few decades. The gene cluster *mcy*, responsible for MC production is now entirely sequenced for several genera (Dittmann et al., 1997; Tillett et al., 2000; Christiansen et al., 2003; Rouhiainen et al., 2004), allowing the development of genus-specific and universal PCR and qPCR primers for the detection and quantification of potentially toxic strains in the environment (Kaebernick et al., 2000; Nonneman and Zimba, 2002; Hisbergues et al., 2003; Kurmayer and Kutzenberger, 2003; Vaitomaa et al., 2003).

MC alone represents approximately half of the researches published on cyanobacterial toxins and a quarter of the literature available emphases on saxitoxins (Merel et al., 2013). In contrast, other cyanotoxins, such as nodularins, cylindrospermopsin or anatoxins are less studied and are usually not monitored by health agencies in freshwater ecosystems in France. Nevertheless, they have to be regarded as potential health hazard.

Among these toxins, anatoxin-a (ANTX-a) is a potent neurotoxin occurring both in lakes and rivers and it has been implicated in several animals casualties (Edwards et al., 1992; Wood et al., 2007). As most cases of reported animal deaths were due to benthic Oscillatoriales (Edwards et al., 1992; Gugger et al., 2005; Cadel-Six et al., 2007), fewer studies focus on planktonic producers, such as *Anabaena* or *Aphanizomenon* (Pawlik-Skowrońska et al., 2004; Ruiz et al., 2013). To our knowledge, ANTX-a production in pelagic compartment of lakes has never been reported in France. The gene cluster has been identified (Cadel-Six et al., 2009; Méjean et al., 2010; Rantala-Ylinen et al., 2011), allowing the development of PCR primers for the detection of potential ANTX-a producers.

Some cyanobacteria, such as *Anabaena*, may produce several toxins (Rapala and Sivonen, 1998) and several cyanobacteria producing different cyanotoxins often co-occur in lakes (Graham et al., 2010); yet, most environmental monitoring studies still focus on a single toxin. Nevertheless, a growing number of studies report the co-occurrence of several toxins in cyanobacterial blooms over the world (Park et al., 1998; Pawlik-Skowrońska et al., 2004; Ballot et al., 2005; Hedman et al., 2008; Graham et al., 2010; Li et al., 2010), emphasizing the need to monitor several toxins in cyanobacterial blooms.

Spatial and temporal variations of cyanotoxin concentrations are often observed, independently from cyanobacterial biomass fluctuations (Briand et al., 2008, 2009; Sabart et al., 2010). These changes can be partly explained by the variations in the proportion of cells possessing, or not, the genes responsible for toxin production. The development of qPCR primers targeting genes of the *mcy* cluster allowed the study of the dynamics of potential MC producers in lakes, and showed that the spatial and temporal variations can be quite important (Kurmayer and Kutzenberger, 2003; Yoshida et al., 2007; Briand et al., 2008, 2009; Hotto et al., 2008; Sabart et al., 2010). However, most of these studies focus on *Microcystis* or *Planktothrix* dominated blooms, using genus specific primers and little is known about *mcy*+ strains dynamics in other blooms.

In this context, the first objective of our study was to evidence the presence of MC and ANTX-a in the freshwater lake Aydat (France) which is subjected to annual *Anabaena*-dominated blooms. We also aimed to describe the inter- and intra-annual dynamics of these cyanotoxins during the bloom periods of three successive years by targeting both the toxins and the genes responsible for their production.

2. Material and methods

2.1. Site description and sampling

Lake Aydat (45°39′48″N, 02°59′04″E), is located in the Massif Central, France, at an altitude of 838 m above sea level. It originates from the damming of the river Veyre by a basaltic flow which occurred 7500 years ago. This river is now the only tributary of the lake. The surface of the lake is 60 ha, and its maximum depth is 15 m. Lake Aydat is typically eutrophic, and blooms of *Anabaena* develop every year, in late summer/early autumn (Lafforgue et al., 1995; Gerphagnon et al., 2013). The study was conducted in September and October (corresponding to the seasonal bloom of cyanobacteria in Lake Aydat) for three consecutive years: 2011, 2012 and 2013. The sampling station was located in the center of the lake, at the point of maximum depth (Fig. 1). Samplings were performed weekly in 2011 and 2012 and twice a week in 2013.

At each sampling date, oxygen and temperature profiles of the lake were performed using a multiparameter probe ProODO™ (Ysi, Germany). A chlorophyll fluorescence profile was obtained using a BBE fluoroprobe® (Moldaenke, Germany).

Twenty liter of water were taken at 0.5 m depth, using a 8-L Van Dorn Bottle, and phytoplankton was harvested with a plankton net (25-μm mesh size) in the whole euphotic zone.

Samples were kept in the dark and transferred to the lab within 1 h. Two hundred microliter of water sample were fixed with Lugol's iodine solution for upcoming counting. Known volumes of water sample were filtered through 8 μm polycarbonate filters (TETP filters, Merck Millipore) using a vacuum pump. Filters were stored in microtubes at −20 °C until MC and DNA extraction were performed. Phytoplankton concentrates harvested with the plankton net were freeze dried using an Alpha 1-2 LD Freeze Dryer (Christ) and stored at −20 °C until ANTX-a extraction.

2.2. Phytoplankton counting

For phytoplankton counting, 10–20 mL subsamples from the Lugol's iodine fixed samples were settled 24 h in counting chambers, and the phytoplanktonic cells were identified and counted under an inverted microscope (Zeiss Axiovert 200 M; 200× magnification) according to the Uthermöhl method (Utermöhl, 1958). At least 400 cells were counted on at least 30 randomly selected optical fields. Phytoplanktonic organisms were

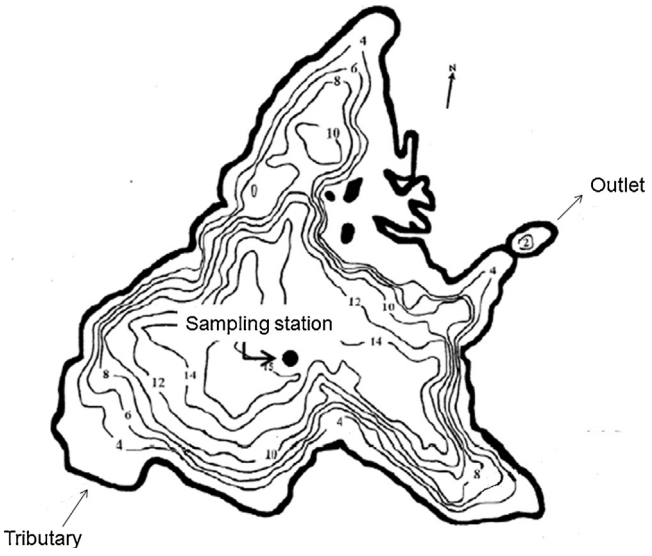

Fig. 1. Bathymetric map of Lake Aydat and location of the sampling station.