Toxicon 78 (2014) 1–9

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Cyanotoxin occurrence and potentially toxin producing cyanobacteria in freshwaters of Greece: A multi-disciplinary approach

Spyros Gkelis*, Nikos Zaoutsos

Department of Botany, School of Biology, Aristotle University of Thessaloniki, GR-541 24 Thessaloniki, Greece

ARTICLE INFO

Article history:
Received 15 July 2013
Received in revised form 27 September 2013
Accepted 14 November 2013
Available online 23 November 2013

Keywords:
Cyanobacteria
Microcystin (MC)
Cylindrospermopsin (CYN)
Saxitoxin (STX)
Cylindrospermopsis raciborskii
Aphanizomenon flos-aquae

ABSTRACT

Cyanobacteria harmful algal blooms (or CyanoHABs) represent one of the most conspicuous waterborne microbial hazards in aquatic environments mostly due to the production of harmful secondary metabolites, known as cyanotoxins. In freshwaters of Greece only the presence of microcystins (MCs) has been reported despite the increasing occurrence of species able to produce other cyanotoxins too. In this paper, we studied the occurrence of potentially toxic cyanobacteria in water samples collected from six lakes and reservoirs in Greece. A multi-technique approach was applied by the use of microscopy, molecular, and immunological methods. Cyanobacteria were found in all the sites ranging from 4.7×10^3 to 5.3×10^8 individuals L^{-1}, representing >70% of the total phytoplankton abundance. Microcystins (MCs), cylindrospermopsins (CYNs), and saxitoxins (STXs) were detected using ELISA, in concentrations ranging from 3.9 to 108 μg L^{-1}, from 0.3 to 2.8 μg L^{-1} and from 0.4 to 1.2 μg L^{-1}, respectively. In half of the samples examined more than one cyanotoxins were detected. Our results document the first report on the occurrence of CYN and STX in freshwaters of Greece and show that potential STX producers are *Cylindrospermopsis raciborskii* and *Aphanizomenon flos-aquae*. Further studies are needed to assess potential CYN producers. This study provides further data on the distribution and toxicity of *C. raciborskii* and *Aph. flos-aquae* and documents a *C. raciborskii* dominated bloom producing STX in Europe.

1. Introduction

Cyanobacteria harmful algal blooms (or CyanoHABs) represent one of the most conspicuous waterborne microbial hazards to human and agricultural water supplies, fisheries production, and freshwater and marine ecosystems (Codd et al., 2005; Paerl et al., 2011). This hazard results from the production of cyanotoxins, harmful secondary metabolites, which can have deleterious effects within reservoirs and in downstream receiving water systems during releases. There are over 40 species representing 20 genera from three cyanobacterial orders known to produce cyanotoxins which include both cyclic peptides and alkaloids (Stewart and Falconer, 2008). However, differentiating toxin producers among cyanobacterial species/strains based on morphological features of the cells is beyond reach. Elucidation of the biosynthetic pathways of the toxins has paved the way for the development of molecular techniques for the detection and quantification of the producing cyanobacteria in different environments (Dittmann et al., 2013). After the elucidation of cyanotoxins genes clusters, several studies have applied molecular methods for monitoring the presence of harmful toxic cyanobacteria and the genes involved in the biosynthesis of

Abbreviations: MC, microcystin; CYN, cylindrospermopsin; STX, saxitoxin.

* Corresponding author. Tel.: +30 231 099 8083; fax: +30 231 099 8389.
E-mail address: sgkelis@bio.auth.gr (S. Gkelis).

0041-0101/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxicon.2013.11.010

cyanotoxins (e.g. Hisbergues et al., 2003; Ouahid et al., 2005; Vasconcelos et al., 2010; Mankiewicz-Boczek et al., 2012).

In Greece, the warm Mediterranean climate favors cyanobacteria blooms in eutrophic waters, which may start in spring and last until December (Cook et al., 2004) or in hypertrophic lakes throughout the year (Moustaka-Gouni et al., 2007). Several species of *Microcystis*, *Anabaena, Cylindrospermopsis* and *Aphanizomenon* are known to dominate blooms in Greece (Gkelis et al., 2005a; Vardaka et al., 2005) so the potential presence of several different toxins is probable. The presence of microcystins (MCs) has been already documented in *Microcystis* dominated blooms (Gkelis et al., 2005a; Papadimitriou et al., 2010). Until today there is no evidence of the occurrence of cylindrospermopsins (CYNs) or saxitoxins (SXTs) in Greek freshwaters despite the increasing occurrence of species such as *Cylindrospermopsis raciborskii* (Moustaka-Gouni et al., 2009), *Aphanizomenon ovalisporum* (Gkelis et al., 2005b), *Aphanizomenon flos-aquae* (Kormas et al., 2011).

In this work we present the first data on cyanotoxins occurrence from freshwaters of Greece using a multi-approach methodology. Microscopic (identification and counting of cyanobacteria), immunological (ELISA), and molecular techniques (PCR) were used to perform an analysis of water samples and blooms collected in several freshwater bodies in Greece.

2. Materials and methods

2.1. Sample collection and preparation

Samples were collected from six freshwaters in Greece (Table 1); for a detailed description of Kerkini Reservoir and Lakes Doirani, Volvi, Kastoria, Pamvotis, see Vardaka et al., (2005). Lake Karla is a newly reconstructed Lake, located in central Greece (39°29′02″N, 22°51′41″E). Its refilling started at 2009 and the suggested plan proposed the creation of a reservoir of about 38 km^2 (Papadimitriou et al., 2011). Water samples were collected from the surface layer (0–0.5 m). Sub-samples were preserved with both Lugol's solution (1% v/v) and formaldehyde (2% v/v). Water samples were stored in polyethylene bottles and transferred to the laboratory (<5 h) under cool and dark conditions. Immediately upon return to the laboratory, 100–1500 mL of lake water was filtered on a Whatman GF/C filter and the filter was stored at −20 °C.

2.2. Phytoplankton analysis

Fresh and preserved samples were examined using an inverted microscope (Olympus IX71) with phase-contrast. Species were identified using Komárek and Anagnostidis (1999, 2005), and the classification system described therein. The abundance of cyanobacterial filaments and cells was determined in accordance with Utermöhl (1958). Transepts were counted and the variation coefficient was always under 20%. Phytoplankton density is presented in number of individuals (filaments or cells mL^{-1}).

2.3. Molecular detection

In order to identify potentially toxic cyanobacteria, different primer pairs, previously described in the literature, were used to detect different gene targets known to be involved either in the biosynthesis of MC, CYN or STX. DNA was extracted using the protocol described in Atashpaz et al. (2010) for Gram negative bacteria, after slicing the filters with a sterile scalpel. PCR was carried out on the DNA extracts using the primer pairs presented in Table 2. For the successful detection of potential MC-producing cyanobacteria, a battery of primer pairs was used. The mcyA CD1F/mcyA CD1R primer pair (Hisbergues et al., 2003) was designed to amplify a 297-bp fragment of the mcyA gene from MC-synthesizing *Microcystis* and *Planktothrix* strains and was previously proved to be suitable to detect MC-producing cells from the genera *Anabaena*, *Microcystis* and *Planktothrix* (Hisbergues et al., 2003). Samples giving

Table 1
Sample number, sampling date and station, and cyanobacteria species abundance for samples collected from Greek freshwaters.

Freshwater	Sample number	Collection date	Sampling station*	Cyanobacteria (10^6 individuals L^{-1})	Cyanobacteria/Total phytoplankton (%)
Lake Doirani	1	29/9/2009	S1	0.05	88
	2	21/8/2010	S1	3.29	97
Kerkini Reservoir	3	21/8/2010	S1	246.25	100
	4	21/8/2010	S2	252.26	100
Lake Volvi	5	21/8/2010	S1	81.49	82
	6	16/5/2011	S1	0.004	71
Lake Kastoria	7	24/8/2010	S1	0.775	98
	8	24/8/2010	S2	1.011	99
Lake Pamvotis	9	7/10/2010	S1	0.572	100
	10	7/10/2010	S2	533.178	100
	11	7/10/2010	S3	0.230	100
Lake Karla	12	9/8/2010	S1	1.858	100
	13	9/8/2010	S2	0.874	100
	14	26/3/2010	S5	9.405	91
	15	20/4/2011	S1	0.022	92
	16	20/4/2011	S2	2.693	84
	17	20/4/2011	S3	0.408	53
	18	20/4/2011	S4	0.357	62

*For samples 1–11 and 12–18, sampling stations are those described in Gkelis et al., (2005a) and Papadimitriou et al., (2011), respectively.