

## Can cyanobacteria infect underground water sources? Indications from small scale monitoring of a natural drinking water source

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### ABSTRACT

The expansion of harmful cyanobacterial blooms is of worldwide concern as they have increased globally in frequency and intensity in recent decades. A cyanobacterial colony was found in a bottle of natural mineral water of a small water company in July 2012, which led to a further examination for a period of five months (July-November 2012) of both the bottled filtered water and the originating groundwater source (N. Greece) for the occurrence of Cyanobacteria. Cyanobacteria occurrence was monitored by microscopy and cyanospecific 16S rDNA amplification; potentially toxic species occurrence was screened by *mcyA* gene (known to take part in the MC-biosynthetic gene cluster) amplification. The highest abundance of cyanobacterial cells without the simultaneous presence of the *mcyA* gene, was measured in July, in contrast to October when the presence of cyanobacteria was only identified by tracing cyanospecific 16S rDNA and the *mcyA* gene region in the underground water source. The results of this small scale monitoring program indicate the potential existence of an emerging danger for human health in a relatively manageable product such as the bottled natural mineral water.

*Key words:* Microcystis; microcystin; bottled water; natural mineral water.

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### INTRODUCTION

Cyanobacterial harmful algal blooms represent one of the most conspicuous waterborne microbial hazards to 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 (Paerl and Otten, 2013). Harmful cyanobacterial blooms have increased globally in frequency and intensity in recent decades. Eutrophication and warmer temperatures are often cited as key factors which promote these events (Hudnell and Dortch, 2008; Paerl and Huisman, 2008; Gkelis *et al.*, 2014). In Greece, the warm Mediterranean climate favors cyanobacterial blooms in eutrophic waters, which may start in spring and last until December; increased temperatures due to global warming may further enhance cyanobacteria dominance and promote toxic over non-toxic strains (Gkelis *et al.*, 2014).

After the elucidation of cyanotoxins genes clusters, several studies have applied molecular methods for monitoring the presence of toxic cyanobacteria and the genes involved in the biosynthesis of cyanotoxins (Hisbergues *et al.*, 2003; Vasconcelos *et al.*, 2010; Gkelis and Zaoutos, 2014). Furthermore, molecular methods based on 16S rRNA gene amplification are widely employed for the

analysis of natural samples and/or monitoring freshwaters (Kormas *et al.*, 2011; Loza *et al.*, 2013).

Bottled water can come from a variety of sources including natural aquifers, springs, glacier run-off, and municipal water supplies, and these sources could potentially be contaminated with microcystins (CFIA, 2011). To our knowledge, only two studies on levels of microcystins in bottled water have been published. Those studies, performed in Italy (Ferretti *et al.*, 2007) and Canada (CFIA, 2011; CFIA, 2012), analysed domestic bottled water samples for the presence of microcystins and nodularin, and did not detect cyanotoxins. This work presents the results of a small scale monitoring program for drinking natural mineral water and highlights the necessity to initiate research and possibly establish official monitoring programs in cases where similar results are obtained in order to cope with the new demands for drinking water.

### METHODS

#### Sample collection and preparation

The bottled natural mineral water originated from a water source (250 m depth), which is located in the Prefecture of Central Macedonia, Northern Greece (N: 40°15'4.88" and E: 22°32'12.71") with a water temperature ranging between 16°C in winter and spring and 18°C in autumn and summer. Water samples were collected

monthly between July and December 2012 from two drilling points (SUP1 and SUP2) used for the production of bottled natural mineral water. The final product which had undergone filtration was also sampled (bottled filtered water-BF). Aliquots of these 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 reception in the laboratory, 1.5 L of water was filtered on a Whatman GF/C filters and the filter was stored at -20°C for further analysis.

### 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). The abundance of cyanobacterial cells was determined in accordance with Utermöhl (1958), using 50 mL sedimentation chambers, thus detection limit was 20 cells L<sup>-1</sup>. Transects were counted and the variation coefficient was always kept under 20%. Phytoplankton abundance is presented in number of cells L<sup>-1</sup>.

### Molecular detection

DNA was extracted using the protocol described in Atashpaz *et al.* (2010) for Gram negative bacteria, after slicing the filters with a sterile scalpel. In order to identify cyanobacteria and potentially MC-producing cyanobacteria we used two different sets of primers, respectively: the 16S 27F (5'-AGAGTTTGATCCTGGCTCAG-3') /16S 1494R (5'- TACGGTTACCTTGTTACGAC -3') primer pair which amplifies a 1367-bp fragment of 16S rDNA in all cyanobacteria (Neilan *et al.*, 1997) and the *mcyA* CD1F (5'- AAAATTAAGCCGTATCAAA-3')

/*mcyA* CD1R (5'- AAAAGTGTTTTATTAGCGGCTCAT-3') primer pair (Hisbergues *et al.*, 2003), which was designed to amplify a 297-bp fragment of the *mcyA* gene from MC-synthesizing cyanobacteria 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 positive results in this assay have been shown to have a high probability of producing MCs (Hisbergues *et al.*, 2003; Vasconcelos *et al.*, 2010). We chose to detect a gene target known to be involved in the biosynthesis of MC, as this is the most frequent cyanotoxin in Greece (Gkelis and Zaoutsos, 2014; Gkelis *et al.*, 2015b) and worldwide.

PCR was carried out on the DNA extracts using the primer pairs presented previously. All PCR reactions were prepared as described in Gkelis and Zaoutsos (2014). Thermal cycling was carried out using an Eppendorf MasterCycler Pro (Eppendorf). Amplification was performed according to the protocols described by Neilan *et al.* (1997) and Hisbergues *et al.* (2003) for 16S rRNA and *mcyA*, respectively. DNA extracted from *Microcystis aeruginosa* M6 strain (see Vasconcelos *et al.*, 2010) was used as positive control for the amplification of *mcyA* gene target and water as negative control. PCR products were separated by 1.5% (w/v) agarose gel in 1X TAE buffer. The gels were stained with ethidium bromide and photographed under UV transillumination.

## RESULTS AND DISCUSSION

Cyanobacteria were detected by microscopy only in the bottled sample BF/A, collected on the 5<sup>th</sup> of July 2012 (Tab. 1). A colonial form of *Microcystis*-like cyanobacteria was found, which could not be further identified (Fig. 1). Cells were spherical with an average diameter

**Tab. 1.** Microscopic and molecular detection of cyanobacteria in the water samples collected during the study.

Sampling date	Sample	Microscopic analysis		Molecular analysis		
		Cells L <sup>-1</sup>	DNA	Cyanobacteria 16S rRNA	<i>mcyA</i> gene	
05-07-12	SUP (1)	-	-	-	-	-
	SUP (2)	-	-	-	-	-
	BF/ A*	50,000	+	+	-	-
	BF/ B	-	+	+	-	-
18-09-12	SUP (1)	-	-	-	-	-
	SUP (2)	-	-	-	-	-
	BF	-	-	-	-	-
31-10-12	SUP (1)	-	+	+	+	+
	BF	-	+	+	-	-
29-11-12	SUP (1)	-	-	-	-	-
	SUP (2)	-	+	-	-	-
	BF	-	+	-	-	-

BF, Bottled filtered water; SUP (1), source underground water (old drill); SUP (2), source underground water-(new drill); \*sample BF/ A is from the same production line with BF/ B but sampled at a different time of the day.

of 8.53  $\mu\text{m}$  ( $n=30$ , min 6.54  $\mu\text{m}$ , max 11.6  $\mu\text{m}$ ). Cyanobacterial abundance in this sample was 50,000 cells  $\text{L}^{-1}$ , whereas in BF/B, collected on the same day and drilling source but at a different time, no cyanobacteria cells were found (Tab. 1). No presence of cyanobacteria was observed by microscopy in any of the other samples, both bottled and from the underground water source. Although the presence of algae and cyanobacteria in underground habitats (Reisser, 2007) and the ability of soil and deep subsurface cyanobacteria to actively follow a moving water pocket in order to exploit it (Garcia-Pichel and Pringault, 2001) has been shown, to the best of our knowledge this is the first report of planktonic cyanobacteria found in an underground drinking-water source. Recently, Pazouki *et al.* (2016) demonstrated that cyanobacteria can be found in water filtered through bank filtration, especially species with cell size  $<10 \mu\text{m}$ , such as the *Microcystis* cells. In our case the source of the cyanobacterial colony we found remains unknown; however, it has been found that in the nearby River-Reservoir System of Aliakmon-Polyphytos, *Microcystis aeruginosa* can be dispersed in short distances through the wind (Chrisostomou *et al.*, 2009). Furthermore, airborne cyanobacteria have been found in the nearby city of Thessaloniki (Genitsaris *et al.*, 2011). Nevertheless, since the source of the cyanobacterial colony was not found the possibility of opportunistic airborne contamination during the bottling cannot be excluded.

DNA was below the detection limit in seven out of the twelve samples analyzed (Tab. 1). A PCR product of about 1370 bp was obtained using the 16S 27F/16S 1484R primer pair in four samples (including sample BF/A of 05-07-2012, where cyanobacteria were identified by mi-



**Fig. 1.** Microphotograph of the *Microcystis*-like colony found in the bottled filtered water BF/A sample.

croscopy). The 300 bp *mcyA*-Cd 1F/*mcyA*-Cd 1R primer pair PCR product, indicating the presence of *mcyA* gene, was identified only in one out of the twelve samples assayed (Tab. 1). In the studied water source the highest cyanobacterial abundance of 50,000 cells  $\text{L}^{-1}$  was observed in July without the simultaneous presence of the *mcyA* gene responsible for MC production. At the end of October, however, the presence of cyanobacteria was only traced through the detection of 16S rDNA as well as traces of the gene *mcyA*. Similar results were reported by Davis *et al.* (2009) in Lake Agawam in July where the presence of toxic *Microcystis* cells did not coincide with the presence of MC and also in Lake Champlain where MCs were detectable in October but without the presence of toxic *Microcystis* cells at the same time. Gkelis and Zaoutsos (2014) found that the *mcyA* region was amplified only where *Microcystis* spp. were dominant and MC concentrations were  $>40 \mu\text{g L}^{-1}$ . Conjointly, Gkelis *et al.* (2014) in a 14-month monitoring of Lake Pamvotis found that *mcyA* was amplified only in two samples, where *Microcystis aeruginosa* was dominant, whereas *mcyB* and *mcyE* regions were amplified in almost all samples.

Several studies report that cyanobacteria can produce toxins at low temperatures but in combination with other important factors such as presence of light and nutrients' availability (Quiblier *et al.*, 2013). The low temperatures (max. 18°C) of the studied groundwater source together with the absence of one of the genes responsible for cyanobacterial toxicity (*mcyA*) in most of the samples suggest that the detected cyanobacteria do not probably constitute an important hazard for this specific source, also due to the limited input of nutrients and the absence of light. Nevertheless, since traces of the *mcyA* gene were detected in one sample, there is still a possibility that MCs could have been produced in the studied water source, since cyanotoxins are intracellular toxins contained within living cells (Sivonen and Jones, 1999) and cyanobacterial cells were found in high abundances in one sample. In this case, the biggest problem in bottled drinking natural mineral waters arises from the fact that the only treatment option in order to maintain the product type 'Natural Mineral Water', is filtering, as filters can retain a big proportion of the microalgae but not the potential toxins produced by them resulting in human intoxication.

Our finding, although scarce, raises the question of monitoring algae in underground water sources. At present, none of the European countries have established monitoring program for cyanotoxins in potable mineral waters so far, and only some countries have done so for drinking water such as Spain, France, the Czech Republic and Poland (Burch, 2008). In Greece, there are very few official monitoring programs in place (Kaloudis *et al.*, 2013; Gkelis *et al.*, 2015a) for cyanobacterial blooms and toxins produced in freshwaters, whereas there is also no legisla-

tion with regard to monitoring of these quality parameters (Cook *et al.*, 2005). Moreover, there are no legally established maximum allowable concentrations for cyanotoxins in potable mineral water. The only relevant reference is in the National Hygienic Regulation A1β/4841/1979 (FEK 696/B'/1979), where the absence of microalgae is required for bottled waters intended for human consumption. A very small number of water treatment plants in Greece control cyanobacterial growth, measure cyanotoxins or use water treatments for toxin removal: The Athens Water Supply and Sewerage Company implements some measures for control and monitoring of cyanobacteria and cyanotoxins (Kaloudis *et al.*, 2013). Other water utilities are small, at a local level, and they do not implement such measures, with the possible exception of the Thessaloniki Water Supply and Sewerage Company (Thessaloniki) (Kaloudis, *personal communication*).

## CONCLUSIONS

The results of this small scale monitoring program demonstrated for the first time the presence of cyanobacteria in bottled natural mineral drinking water. While it seems unlikely that the use of bottled water would constitute any major hazard with regard to cyanotoxin exposure, our findings call for further research to investigate the presence, heterotrophic growth and significance of cyanobacterial colonies and/or biofilms in water distribution systems, such as wells.

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