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Short communication

First occurrence of cylindrospermopsin in Portugal: a contribution to its continuous global dispersal

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ABSTRACT

Cylindrospermopsin (CYN) was found to occur in Portugal for the first time. In this study CYN values varied from a minimum of 1.4 μg L^{-1} to a maximum of 12 μg L^{-1} detected through HPLC technique and confirmed by LC-MS method. Amplification of the *cyrC* gene was done and was confirmed to be from the genera *Aphanizomenon*. This study is therefore an important contribution to the knowledge on the dispersal and biogeography of CYN.

Since the Palm Island mystery disease that occurred in the late 1970's in Queensland, Australia, cylindrospermopsin (CYN) reports have increased throughout the world. Described so far in four of the main continents, where Africa is still the exception, new reports have however showed that this cyanotoxin is already present in the Polar Regions, namely in Antarctica (Moreira et al., 2012; Kleinteich et al., 2014). Data from all these regions shows that temperature does not limit the presence of CYN since it has been known to occur in both tropical, subtropical as well as in temperate environments. CYN was for the first time observed in the Australian continent (Byth, 1980) followed by Asia where it has been so far described in Thailand and Japan (Chonudomkul et al., 2004). In the American continent reports include Canada, USA, Mexico and Brazil (Kinnear, 2010). In Europe these reports began more recently around the year 2000 which in summary includes Germany, Spain, Greece, Poland, France, Czech Republic, Italy, Finland, Israel and Turkey (Banker et al., 1997; Akcaalan et al., 2014; Rzymski and Poniedziałek, 2014). The established frontier between the northernmost reports of CYN in European freshwaters is from Finland to Spain with the latter constituting until now its southernmost record (Quesada et al., 2006; Spoof et al., 2006). Being produced so far by 11 distinct species on a total of the four continents, which include *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae*, *Aphanizomenon gracile*, *Aphanizomenon klebahnii*, *Umezakia natans*, *Raphidiopsis curvata*, *Anabaena bergii*, *Anabaena planctonica*, *Anabaena lapponica* and *Lyngbya wollei*. This wide species distribution of CYN is therefore a well-established feature (Boopathi and Ki, 2014). Though *C. raciborskii* was the first cyanobacterium species to be associated with the production of CYN (Hawkins et al., 1985) in Europe the main CYN-producing genera are *Anabaena* sp. and *Aphanizomenon* sp. the latter being the most prevalent one (Rzymski and Poniedziałek, 2014). Exhibiting cytotoxic, genotoxic and with a carcinogenic potential CYN is currently one of the most studied cyanotoxins on a worldwide scale with a proposed guideline value of 1 μg L^{-1} in drinking water (Moreira et al., 2012; Humpage and Falconer, 2003). In CYN several methods can be used in its environmental monitoring. Generally techniques such as the High-performance liquid chromatography (HPLC) and Liquid chromatography–mass spectrometry (LC-MS) are the mostly frequently used and enable us to quantify and confirm the toxin, respectively (Welker et al., 2002; Bláhová et al., 2009). Along with these methods are the biochemical ones, namely the immunoassays such as the commercially available

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ELISA kits. More recently the molecular methods based in the screening of the genes associated with the biosynthesis of cyanotoxins have been applied. CYN molecular screening consists mainly in the PCR detection of the toxin associated genes that direct the production of CYN. CYN molecular detection involves nowadays a total of four distinct genetic markers (*cyrA*, *cyrB*, *cyrC* and *cyrJ*) and these are available in the literature (Fergusson and Saint, 2003; Kellmann et al., 2006; Mihali et al., 2008).

In Portugal, a previous study detected through a molecular screening using the Real-time PCR methodology, a *cyrC* gene fragment belonging to the *Aphanizomenon* sp. genera, in a freshwater lake, the Vela Lake, but without showing the presence of CYN (Moreira et al., 2011). In light of these results the screening for the presence of CYN was conducted precisely at this specific water system. The samples were collected at Vela Lake, a recreational water system located in the Center region of Portugal (40°16′23.9″N, 8°47′35.1″W), between May and September of 2012 and May and October of 2013. Monthly water samples were collected from the surface and shore part of the water system using sterilized plastic bottles for chemical analysis and DNA extraction and falcon tubes for the ELISA assays. The pH values of the water were measured *in situ* using a WTW Multiline P3 meter (WTW, Germany). Upon laboratory arrival water samples were immediately filtered with Munktell® MGC (Falun, Sweden) micro-glass fiber paper filters. The filtered biomass was kept at −20 °C for DNA extraction. Total genomic DNA from each filter was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen, CA, USA) according to the manufacturer's protocol for Gram-negative bacteria. PCR amplifications were performed using Promega GoTaq® Flexi DNA Polymerase kit (Promega, WI, USA) and in all the PCR reactions was used a final volume of 20 μL. Each reaction contained 5× Green GoTaq® Flexi Buffer, 25 mM $MgCl_2$ solution, 10 pmol μL^{-1} of each primer forward and reverse, 2.5 mM of dNTP's solution, BSA (Bovine Serum Albumin) (10 mg mL^{-1}), GoTaq® DNA Polymerase (5U μL^{-1}) and 5–10 ng of DNA. All PCR reactions were performed on a Biometra Professional Thermocycler apparatus (Biometra, Germany). To confirm the presence of the genes responsible for CYN biosynthesis, the primer set K18/M4 (*cyrC*) were used and the cycling conditions applied according to the literature (Schembri et al., 2001; Fergusson and Saint, 2003). As a positive control for all the PCR amplifications DNA from a pure culture of a CYN-producing *C. raciborskii* culture collection strain LEGE 97047 was used. For DNA sequencing all PCR reactions were purified using the Cut & Spin Gel Extraction kit (GRiSP, Portugal), according to the manufacturers protocol and subsequently sent for direct sequencing. DNA sequence retrieved was submitted to the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST) for sequence confirmation and species identification. In parallel 15 mL of water samples were sonicated, on ice, for 5 min at 60 Hz for ELISA quantification and the obtained solution, was filtered using 0.45 μm Whatman™ filters (Whatman, Kent, UK). MC quantification was performed using Microcystins/Nodularins ADDA ELISA kit (Microtiter Plate) (Abraxis, PA, USA) and CYN using the Cylindrospermopsin ELISA kit (Microtiter Plate) (Abraxis, PA, USA) in accordance with the manufacturer's protocol. CYN concentrations were determined by using the HPLC with a photodiode array detector (HPLC-PDA). Filtered water samples were extracted with H_2O+0.1% TFA (trifluoroacetic acid) and centrifuged (4995 *g*, 10 min, 4 °C). Supernatant collected and treated by solid phase extraction (SPE) following Bláhová et al. (2009). HPLC-PDA analysis was performed with both particulate and dissolved matter, in order to quantify the presence of CYN in biomass cells and in the media, with the exception of the samples from July and August of 2012 that were only from filter content. Samples were quantified in a HPLC system coupled with a photodiode array (PDA) detector Waters Alliance 2695 (Waters, MA, USA), containing a reverse phase column Lichrospher C18 (250 mm × 4.6 mm i.d., 5 μm) (Merck, Lisbon, Portugal) maintained at 40 °C and using a scanning between 210 and 400 nm with a fixed wavelength at 262 nm. Isocratic elution used was 5% of methanol acidified with 0.1% (v/v) of TFA with a flow of 0.9 mL min^{-1} and an injection volume of 10 μL. LC-MS analysis was performed in order to confirm the presence or absence of CYN in environmental samples from Vela Lake previously analyzed by HPLC-PDA. Samples were injected in a Liquid Chromatograph Thermo Finnigan Surveyor HPLC System (Thermo Scientific, MA, USA), coupled with Mass Spectrometry LCQ Fleet™ Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA), with a column Hypersil GOLD (100 × 4.6 mm i.d., 5 μm) (Thermo Scientific, MA, USA). Isocratic elution used was 5% methanol acidified with formic acid at 0.1% (v/v). The injection volume was 10 μL with a flow of 0.8 mL min^{-1}. CYN was identified by comparing the NRC-CRM standard solution spectra obtained considering the precursor ion $[M+H]^+$ *m/z* 415.6 and characteristic fragment ions *m/z* produced for an collision energy of 25.

Sampling was done from May to September of 2012 and from May to October of 2013 on a monthly basis and the pH values measured *in situ*. These were generally higher in the 2012 sampling period (8.1–9.67) than during the same period of 2013 (6.92–8.18). The presence of positive amplicons associated with CYN biosynthesis was detected in only one of the samples belonging to the month of September of 2012 using the *cyrC* K18/M4 primer set (Table 1). In order to confirm the positive amplification from the positive PCR sample, DNA of the amplicon was sent for direct sequencing. BLAST results showed that the gene sequence had a 100% homology with the gene sequence from *Aphanizomenon ovalisporum* FAS-AP1. Quantification of both CYN and microcystins (MC) at Vela Lake samples was performed through the use of ELISA immunoassays commercially available kits for each of the two cyanotoxins. CYN data had negative results in overall the sampling months, with the exception of the samples from August and September of 2012, which quantified CYN with the respective values of 0.3 and 0.6 μg L^{-1}, respectively (Table 1). In contrast, MC were detected in all of the sampled months of 2012 and in May of 2013 (Table 2). In regards to the year 2013 all the sampled months were negative for CYN and only the month of May was positive for MC (Tables 1 and 2). Presence of CYN was also detected by HPLC-PDA and later confirmed by LC-MS (Fig. 1), in the water samples from May, June and September of 2012. Values for CYN present in the water samples from Vela Lake varied between a minimum of 1.4 μg L^{-1} in September 2012 to a maximum of 12 μg L^{-1} in June 2012 (Table 1). Presence of CYN in the water samples of 2012 was further confirmed by performing the MS/MS method, which shows the precursor ion (*m/z* 415.6) and fragment ions at *m/z* 336, 274, 194 and 176 (Fig. 1).

In our study we reported for the first time the occurrence of CYN in a Portuguese freshwater system, the Vela Lake. A study

Table 1
Results for CYN by HPLC-PDA, ELISA and *cyrC* amplification during 2012 and 2013 at Vela Lake. μg L^{-1}.

Sampling	HPLC (μg L^{-1})		ELISA (μg L^{-1})		*cyrC*	
	2012	2013	2012	2013	2012	2013
May	3.9	0	0	0	–	–
June	12	0	0	0	–	–
July	0	0	0	0	–	–
August	0	0	0.3	0	–	–
September	1.4	0	0.6	0	+	–
October	n.d.	0	n.d	0	n.d.	–

n.d. - not determined.