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Application of real-time PCR in the assessment of the toxic cyanobacterium *Cylindrospermopsis raciborskii* abundance and toxicological potential

Cristiana Moreira · António Martins · Joana Azevedo · Marisa Freitas · Ana Regueiras · Micaela Vale · Agostinho Antunes · Vitor Vasconcelos

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Abstract Cyanobacteria are prokaryotic photosynthetic microorganisms that pose a serious threat to aquatic environments because they are able to form blooms under eutrophic conditions and produce toxins. *Cylindrospermopsis raciborskii* is a planktonic heterocystous filamentous cyanobacterium initially assigned to the tropics but currently being found in more temperate regions such as Portugal, the southernmost record for this species in Europe. Cylindrospermopsin originally isolated from *C. raciborskii* is a cytotoxic alkaloid that affects the liver, kidney, and other organs. It has a great environmental impact associated with cattle mortality and human morbidity. Aiming in monitoring this cyanobacterium and its related toxin, a shallow pond located in the littoral center of Portugal, Vela Lake, used for agriculture and recreational purposes was monitored for a 2-year period. To accomplish this, we used the real-time PCR methodology in field samples to quantify the variation of specific genetic markers with primers previously described characterizing total cyanobacteria (16S rRNA), *C. raciborskii* (*rpoC1*), and cylindrospermopsin synthetase gene (*pks*). The results report the high abundance of both cyanobacteria and *C. raciborskii* in Vela Lake, with *C. raciborskii* representing 0.4% to 58% of the total cyanobacteria population. Cylindrospermopsin synthetase gene was detected in one of the samples. We believe that with the approach developed in this study, it will be possible to monitor *C. raciborskii* population dynamics and seasonal variation, as well as the potential toxin production in other aquatic environments.

Keywords *C. raciborskii* · Monitoring · Real-time PCR · Cylindrospermopsin genes

C. Moreira · A. Martins · J. Azevedo · M. Freitas · A. Regueiras · M. Vale · A. Antunes · V. Vasconcelos (✉)
CIMAR/CIIMAR, Laboratory of Ecotoxicology, Genomics and Evolution, University of Porto,
Rua dos Bragas, 289,
4050-123 Porto, Portugal
e-mail: vmvascon@fc.up.pt

C. Moreira · V. Vasconcelos
Departamento de Biologia, Faculdade de Ciências,
Universidade do Porto,
Rua do Campo Alegre,
4169-007 Porto, Portugal

J. Azevedo
Escola Superior de Tecnologia da Saúde do Porto,
Rua Valente Perfeito, 322,
4400-330 Vila Nova de Gaia, Portugal

M. Freitas
Centro de Investigação em Saúde e Ambiente,
Escola Superior de Tecnologia de Saúde do Porto,
Rua de Valente Perfeito, 322,
4400-330 Vila Nova de Gaia, Portugal

Introduction

Cylindrospermopsis raciborskii (Woloszyńska, 1912) Seenayya and Subba Raju (1972) is a filamentous, heterocystous, freshwater planktonic cyanobacterium belonging to the Nostocales order (Neilan et al. 2003). First classified a tropical species due to its affinity to warm water temperatures, *C. raciborskii* has currently a worldwide distribution with records in all continents. Its recent and increasing occurrence in Europe reveals its spreadness and invasive character to more temperate environments (Padisák 1997). Italy, France, Spain, and Germany are some of the examples, with the last being the northernmost record for this

species in Europe to date (Romo and Miracle 1994; Briand et al. 2002; Fastner et al. 2003; Manti et al. 2005). Similarly, its proliferation to other more temperate regions, for instance in the American continent, was reported recently by Hamilton et al. (2005) that described its presence in a shallow lake in Ontario (Canada).

In Portugal, the first record of presence of *C. raciborskii* was documented by Saker et al. (2003) where they isolated four strains from the south part of the country. Later Valério et al. (2005) isolated another five strains of this species also from the same location. More recently, Freitas (2009) isolated *C. raciborskii* from the Vela Lake. The study of this species is of particular importance due to its ability to produce dense blooms, as a result of eutrophication, decreasing the water quality. Australia, Brazil, Europe, and USA are some of the places where blooms of this species have been known to occur (Schembri et al. 2001). Another interesting feature associated with this species is its capability to biologically produce toxic secondary metabolites such as cylindrospermopsin and the "paralytic shellfish toxin" (Lagos et al. 1999). Cylindrospermopsin is, after microcystin, the most widely occurring hepatotoxin produced by cyanobacteria (Falconer 2005). It is known to cause damage in the liver, kidneys, adrenal glands, lungs, and intestines in mouse toxicity assays (Humpage and Falconer 2003). Its cytotoxicity, carcinogenic potential, and genotoxic nature has already been reported (Runnegar et al. 1994; Humpage et al. 2000; Falconer and Humpage 2001), and cases of cattle mortality (Thomas et al. 1998; Saker et al. 1999) and human morbidity (Bourke et al. 1983) have been attributed to this toxin. Though the occurrence of cylindrospermopsin in European countries has already been reported (Fastner et al. 2003; Quesada et al. 2006; Spoof et al. 2006; Brient et al. 2009), in Portugal its presence remains undetected.

Traditionally, cyanobacteria identification and enumeration are based on light microscopy. This common methodology for monitoring cyanobacteria fails to differentiate toxic from non-toxic species or strains and requires the skills of an experienced observer for its reliable identification. Other methodologies, such as chemical and immunological assays, can be used to quantify toxic cyanotoxins but are time consuming and require personnel specialized especially for the chemical assays. Therefore, the development and the implementation of more accurate and faster methodologies, namely molecular methods, are highly recommended. In this sense, the advent of molecular methods brought a new perspective for cyanobacterial studies given its ability to detect genes involved in toxin production and the advantage of their direct detection from environmental samples (Saker et al. 2006). Also, the development of primers that target specific DNA sequences added not only specificity but also resulted in an important tool for field studies avoiding the need of an extensive sample processing. Recently, real-time PCR methodologies allowed the quantification of cyanobacteria permitting the evaluation of the proportion and abundance of a given species and also in establishing the proportion of toxic and non-toxic genotypes within a cyanobacterial population (Kurmayer and Kutzenberger 2003; Rinta-Kanto et al. 2005). This technique is faster and more accurate than conventional PCR, allowing the reliable detection of only a few cells per reaction (Pearson and Neilan 2008). A real-time PCR assay for *C. raciborskii* and cylindrospermopsin in field samples was previously developed by Rasmussen et al. (2007) using primers already applied for conventional PCR. In their work, they showed a good correlation between quantification of *C. raciborskii* using *rpoC1* gene with the respective cell numbers, by microscopy counting, with a detection limit of 100 copies per reaction or 1,000 cells mL^{-1}. An evaluation of cylindrospermopsin-producing cyanobacteria in field samples was also developed based on the molecular detection of the *pks* gene with the number of copies of this cylindrospermopsin biosynthesis gene matching the results of conventional toxin detection (HPLC). However, no establishment of the proportion and abundance of *C. raciborskii* within a cyanobacterial population were so far conducted. Therefore, in this study, we applied the real-time PCR technique to assess the temporal distribution of *C. raciborskii*, establish its abundance within the total cyanobacterial population, and finally evaluate the toxic potential regarding cylindrospermopsin production. To perform this aim, the 16S rRNA gene was used to quantify the total cyanobacteria population using primers previously described (CYA primers) by Nübel et al. (1997). For *C. raciborskii* and cylindrospermopsin, a real-time PCR assay developed by Rasmussen et al. (2007), which we applied and adapted for the SYBR Green I technology, was used. The ultimate goal of our study will be the application of this methodology as a future tool for monitoring the *C. raciborskii* ecological and toxicological population dynamics. To achieve this goal, we studied the seasonal variation of the cyanobacterial communities in the Vela Lake, a freshwater system with a previous report of toxin-producing cyanobacteria (microcystins) (Vasconcelos et al. 1993).This is a shallow eutrophic freshwater body located in the littoral center of Portugal used for recreation activities, sports fishing, and agricultural purposes (Vasconcelos et al. 1993; de Figueiredo et al. 2006).

Materials and methods

Sampling Water samples up to a maximum of 2 L were collected at the Vela Lake between June and October of

2008 and 2009. All samples were collected from the surface and shore part of the lake and subsequently brought to the laboratory under refrigerated conditions.

DNA extraction Fifty milliliters to 2 L of water was filtered with a GF/C membrane (Whatman, Kent, UK). The obtained biomass was frozen for DNA extraction. Total genomic DNA was obtained using the PureLink™ Genomic DNA Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol for Gram-negative bacteria, and the eluted DNA was diluted 1/50 in elution buffer solution for the real-time PCR quantification assays to avoid any possible presence of amplification inhibitors.

Primers and sensitivity Primers used in this study were based on previously published primers from conventional PCR. For the quantification of total cyanobacteria, the CYA primers 359F/781R (a)+781R (b) (16S rRNA) were applied (Nübel et al. 1997); for the quantification of *C. raciborskii*, the cyl2/cyl4 primers (*rpoC1*) were used as previously described by Wilson et al. (2000); and for the detection of cylindrospermopsin biosynthesis gene, the K18/M4 primers (*pks* gene) (Fergusson and Saint 2003) were used. Sensitivity limits for 16S and *rpoC1* were determined using DNA obtained from estimated cell numbers of a *C. raciborskii* cylindrospermopsin-producing strain (LEGE 97047) cell culture. In general, cell numbers were obtained by counting the culture under microscope and the concentration per milliliter calculated. DNA per cell was estimated through quantification of the total genomic DNA extracted from the culture and dividing by the total number of cells. For each 4 μL of a real-time PCR reaction, the equivalent cell numbers were determined and a series of 10-fold dilutions of the extracted DNA ranging from 10^7 to 10^0 cells mL^{-1} were used to assess sensitivity limits.

Preparation of standards for real-time PCR assays Standards used to determine cell numbers were prepared using 1-mL aliquots from a pure culture of *C. raciborskii* (cylindrospermopsin producer) LEGE 97047 strain. Cells were counted under a microscope and cell numbers of the initial culture per milliliter estimated. DNA from that culture was then extracted and quantified and the amount of DNA per cell calculated. A series of 10-fold dilutions were prepared and used for real-time PCR analyses (4 μL per reaction). Linear regression equations for the obtained cycle threshold (Ct) values were calculated as a function of known cell numbers of each real-time PCR template reaction value.

Real-time PCR assay for cyanobacteria Four microliters of extracted diluted DNA was amplified for the 16S rRNA gene. All real-time PCR reactions were performed in a volume of 20 μL containing 1× iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and 10 pmol of each primer. Amplification was performed with the iQ™5 Real-Time PCR (Bio-Rad) as follows: an initial denaturation of 5 min at 95 °C, followed by 50 cycles with one cycle consisting of 15 s at 95 °C, 15 s at 60 °C, and 30 s at 72 °C. Data acquisition was monitored after each extension step with temperature increments of 0.5 °C between 55 °C and 95 °C. Melting temperatures of the 16S rRNA products were determined with the Bio-Rad iQ™5 Optical System Software (version 2.0) (Bio-Rad). Cell numbers per milliliter were estimated in function of the linear regression equation and the Ct values obtained multiplying by the initial DNA dilution factor and dividing by the volume of water per sample used.

Real-time PCR assay for C. raciborskii Four microliters of extracted diluted DNA was amplified for the *rpoC1* gene. All real-time PCR reactions were performed as previously described for cyanobacteria. Amplification was performed with the iQ™5 Real-Time PCR (Bio-Rad) as follows: an initial denaturation of 8 min at 95 °C, followed by 50 cycles with one cycle consisting of 15 s at 94 °C, 15 s at 55 °C, and 50 s at 72 °C. Data acquisition was monitored after each extension step with temperature increments of 0.5 °C between 55 °C and 95 °C. Melting temperatures of the *rpoC1* DNA products were determined with the Bio-Rad iQ™5 Optical System Software (version 2.0) (Bio-Rad). Cell numbers per milliliter were estimated as previously described for cyanobacteria.

Real-time PCR assay for cylindrospermopsin gene An end-point analysis was performed with 4 μL of extracted diluted DNA for the *pks* gene. All real-time PCR reactions were performed as initially described. Amplification was performed with the iQ5™ Real-Time PCR (Bio-Rad) as follows: an initial denaturation of 8 min at 95 °C, followed by 50 cycles with one cycle consisting of 15 s at 94 °C, 15 s at 55 °C, and 50 s at 72 °C. Data acquisition was monitored after each extension step with temperature increments of 0.5 °C between 55 °C and 95 °C. Melting temperatures of the *pks* DNA products were determined with the Bio-Rad iQ™5 Optical System Software (version 2.0) (Bio-Rad). The presence or absence of the amplified product was determined by a melting curve analysis and compared with that of the standard strain (LEGE 97047).

DNA sequencing One hundred microliters of PCR reaction of a *pks* positive sample was amplified and the product purified using NucleoSpin® Extract II (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) in accordance to the manufacturer's protocol and sent for direct sequencing. Nucleotide sequence was obtained and submitted

to the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST) for identification (JN053048).

ELISA for cylindrospermopsin Fifteen milliliters of sampled water was frozen and sonicated on ice for 5 min at 60 Hz. The obtained solution was filtered with a 0.45-μm-pore-size filter (Whatman, Kent, UK). Presence of cylindrospermopsin was evaluated using the ABRAXIS Cylindrospermopsin ELISA Kit (Biosense Laboratories AS, Bergen, Norway) following the manufacturer's protocol. The absorbance was measured using a Well-Scan ELISA plate reader (Denley Corp., Billinghurst, UK) at a wavelength of 450 nm.

High pressure liquid chromatography (HPLC-PDA) analysis All solvents used in HPLC analysis were high-purity chromatography grade (LiChrosolv, Merck). Aqueous solutions were prepared with ultrapure water supplied from a Millipore water purification system (0.0054 μS cm^{-1}). Reagents like sodium 1-heptanesulfonate monohydrate and trifluoroacetic acid (TFA) were of spectrophotometric grade. The cylindrospermopsin used as the reference standard was gently provided by the Laboratory of Prof. Kevin James, Cork University, Ireland. The Vela lake sample (1 L) was prepared according to Welker et al. (2002) with minor modifications. As soon as it arrived, the environmental sample was immediately GFC filtered (VWR, Portugal) in order to separate particulate and dissolved matter. The filtrate was concentrated to dryness on a rotary evaporator (30 °C; 50 mbar), re-suspended in 1 mL of distilled water acidified with 0.1% TFA, and injected in the HPLC. The filter was macerated in a mechanical crusher (Silent Crusher M, Heidolph) with 10 mL of distilled water acidified with 0.1% TFA until complete homogenization, treated by ultrasound on ice bath at 60 Hz for 5×1 min (VibraCell 50-sonics & Material Inc., Danbury, CT, USA) and the resulting slurry centrifuged (4,995×*g*; 15 min; 4 °C). The extraction procedure was repeated and both supernatants pooled together, dried by rotary evaporation (30 °C; 50 mbar), the residue dissolved in 500 μL ultrapure water and injected in the HPLC. Cylindrospermopsin quantification in both filtrate and filter extracts were carried out in a HPLC-PDA system from Water Alliance e2695 coupled with a photodiode array detector 2998. The toxin analytical assay was performed using a HILIC phase column (Waters Atlantis® HILIC Silica; 25 cm×4.6 mm, 5 μm) kept at 30 °C. The isocratic elution utilized 5% methanol containing 2 mM of sodium 1-heptanesulfonate monohydrate (99%) with a flow rate of 0.5 mL/min. All HPLC solvents were filtered (Pall GH Polypro 47 mm, 0.2 μm) and degassed by ultrasound bath. The injected volume was 10 μL, up to 100 μL when necessary. The PDA range was 210–400 nm, with a fixed wavelength at 262 nm. The system was calibrated by using a set of seven dilutions of cylindrospermopsin standard (25, 20, 10, 5, 2, 1, and 0.5 μg mL^{-1}) in ultrapure water. Empower 2 Chromatography Data Software was used for calculation and reporting peak information. The minimum amount of cylindrospermopsin that can be detected in water is 0.3 μg mL^{-1}, based on a signal-to-noise ratio of 3. The retention time of the cylindrospermopsin peak was 7.35 min.

Liquid chromatography/mass spectrometry (LC/MS) The same standard solution, the filtrate, and filter extracts were also analyzed by LC/MS, following the method of Bláhová et al. (2009) with minor modifications. The LC/MS system was a LCQ Fleet ion trap MS^n (ThermoScientific, USA) with electrospray (ESI) interface. Separation was achieved on C18 Hypersil Gold column 100×4.6 mm ID, 5 μm (ThermoScientific, USA) kept at 25 °C, with a flow rate of 0.8 mL/min. The injected volume was 25 μL. A gradient elution was used with mobile phase A, methanol/water 1:99 v/v, and B, methanol/water 90:10 v/v, both acidified with 0.1% formic acid (0% B 0–2 min, gradient 0–60% B during 2 min, 60% B held for 10 min, then ramped from 60% to 90% B in 15–16 min and equilibrated for further 2 min). The mass spectrometer was operated in a multiple reaction monitoring mode (MRM) with collision energy of 35 eV. The capillary voltage and fragmentation energy were 5.5 kV and 120 V, respectively. The cylindrospermopsin transition *m/z* 415.7 ($M+H^+$) to 193.7 and *m/z* 416.2 to 175.8 was monitored for 1 microscan time. The retention time of the cylindrospermopsin peak was 6.30 min.

Statistical analysis One-way ANOVA test was performed for mean average abundance between sampled years for both total cyanobacteria and *C. raciborskii* population. Proportion in the environment was estimated by dividing the total number of *C. raciborskii* cells numbers by the total number of cyanobacteria cells for each sampled month. All statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS Inc.) software version 17.0 and significance estimated at the 95% confidence level.

Results

Primers specificity and sensitivity

Sensitivity of the chosen primers revealed to be high for both quantification assays. The CYA primers were able to detect up to 11 cells of cyanobacteria per reaction and the *cyl* primers up to 258 cells of *C. raciborskii* per reaction (Fig. 1a, b). Amplification efficiencies slope and intercept of the linear regression and the R^2 and also the threshold

value used to set the starting copy number (number of cells per concentration of template DNA) were also calculated for both DNA markers used. Therefore, for the 16S rRNA, amplification efficiency was 86.7% while for the *rpoc1* it was 98%. Slope and intercept were respectively −3,688 and 34,881 for the 16S rRNA and −3,372 and 45,066 for the *rpoC1*. R^2 was 0.998 and 0.996 for 16S rRNA and *rpoC1*, respectively, while the threshold value used to set the starting copy number was 16.37 and 23.68 for the 16S rRNA and *rpoc1* markers, respectively. Specificity of all primer sets was determined previously in other studies and these revealed to be highly suitable for our SYBR Green I real-time PCR assay, especially the CYA primers for the quantification of total cyanobacteria using the already published amplification conditions since they were firstly used for cyanobacteria quantification by a real-time PCR methodology. In regard to the cyl2/cyl4 and K18/M4 for *C. raciborskii* and cylindrospermopsin biosynthesis gene, respectively, amplification conditions proved to be more specific at an annealing temperature of 55 °C rather than at 45 °C, as previously described, for all the tested field samples (data not shown).

Quantification of total cyanobacteria

Cyanobacteria were determined in both sampled years and in all the sampled months. In the first sampled year, the highest value was obtained in the month of June with 6.62×10^7 cells mL^{-1} and the lowest in the month of September with 1.84×10^7 cells mL^{-1}. Throughout all the sampling period, the number of cyanobacteria remained elevated with little variation (Table 1).

During the second year, we observed an overall small reduction in the cyanobacteria population at Vela Lake with the highest value registered in the first sampling date (June) with 2.2×10^5 cells mL^{-1} and the lowest with 8.46×10^3 cells mL^{-1} in the last sampling date (October). In contrast to what was observed in the first year, the total cyanobacteria population at Vela Lake showed some variation throughout the sampling period, with the total cell numbers decreasing overtime. Mean values obtained in the first sampled year were significantly higher than those found in the second sampled year ($p<0.05$) (Table 2).

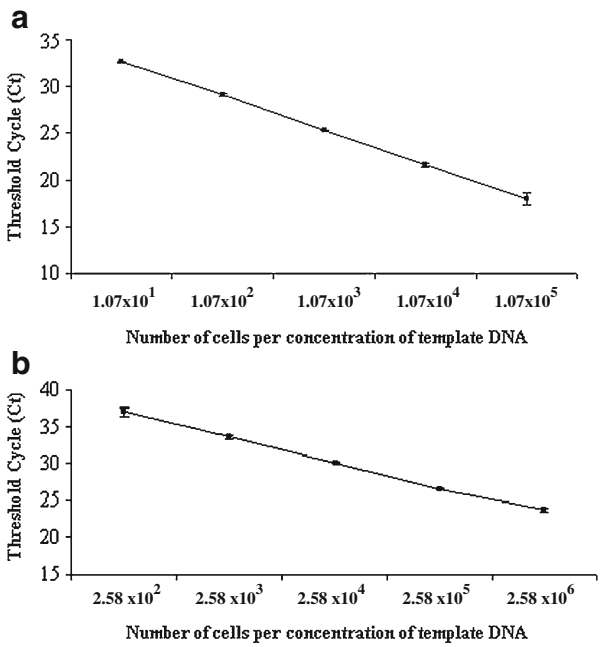

Fig. 1 Sensitivity limits for both 16SrRNA (**a**) and *rpoC1* (**b**) genes. Ct values are represented as a function of number of cells per concentration of template DNA of cyanobacteria and *C. raciborskii*, respectively. *Error bars*, which are hidden by the symbols in almost all cases, give the standard deviations for three independent amplifications

Quantification of *C. raciborskii*

C. raciborskii was present at Vela Lake in all the sampled months between 2008 and 2009. In 2008, the highest value recorded was obtained in the last sampling month (October) with 1.34×10^7 cells mL^{-1} and the lowest value in July with 1.99×10^5 cells mL^{-1} (Table 1). In the first year, *C. raciborskii* cell numbers had a continuous increase in the population throughout the sampling period recording the highest values in the end of summer and beginning of autumn (Fig. 2).

In 2009, *C. raciborskii* population was lower than that observed in 2008 reaching the lowest value with 2.32×10^2 cells mL^{-1} in the last sampling month (October) and the highest value with 2.51×10^4 cells mL^{-1} in the previous month (September). When we compared both years, a significant decrease was observed in 2009 in the *C. raciborskii* population ($p<0.05$) (Table 2). In terms of proportion in the environment, in 2008 the population increased with time; however, in 2009 it had a small decrease between June and July increasing then until September and finally decreasing in October (Fig. 2).

Real-time PCR for cylindrospermopsin gene

The potential production of cylindrospermopsin through the screening of the *pks* gene was assessed by a real-time qualitative analysis (end point). In 2008, all sampled months showed negative results for the presence of *pks* gene while in 2009 one sample was positive, specifically the water sample from the last sampling month (October). DNA from that sample was further amplified and sent for direct sequencing in order to identify the cyanobacterium species it belonged to. The BLAST results showed that the gene sequence have a 99% homology with the *pks* gene sequence from an *Aphanizomenon ovalisporum*. The

Table 1 Cyanobacteria and *C. raciborskii* cell numbers, qualitative results for *pks* gene, and respective ELISA cylindrospermopsin concentration in the water

	Cyanobacteria abundance (cells mL^{-1})		*C. raciborskii* abundance (cells mL^{-1})		*pks* gene		Cylindrospermopsin ($\mu g\ L^{-1}$)	
Sampling month	2008	2009	2008	2009	2008	2009	2008	2009
June	6.62×10^7	2.20×10^5	1.18×10^6	1.92×10^4	−	−	−	−
July	3.90×10^7	1.11×10^5	1.99×10^5	1.40×10^3	−	−	−	−
August	4.27×10^7	6.94×10^4	7.68×10^4	1.01×10^4	−	−	−	−
September	1.84×10^7	8.42×10^4	3.40×10^6	2.51×10^4	−	−	−	−
October	2.33×10^7	8.46×10^3	1.34×10^7	2.32×10^2	−	+	−	0.28

obtained environmental DNA sequence was submitted to the GenBank database.

ELISA

Quantification of total cylindrospermopsin in all the collected water samples was performed through an ELISA. The data obtained showed negative results in all the sampling dates for the presence of this toxin at Vela Lake with the exception for the last sampling date (October) in 2009 where the value of 0.28 $\mu g\ L^{-1}$ was obtained (Table 1).

HPLC-PDA and LC/MS

The ELISA positive sample from October 2009 was also analyzed by HPLC-PDA and LC/MS. In either of the performed chemical assays, cylindrospermopsin was not detected.

Discussion

In this study, we assessed the population dynamics and seasonal variation of *C. raciborskii* in a Portuguese aquatic system using for the first time a real-time PCR methodology. This technology allowed the detection and quantification of both total cyanobacteria as well as *C. raciborskii* with a reliable sensitivity limit of only a few cells per milliliter. While in cyanobacteria, detection was achieved in up to 11 cells mL^{-1}, for *C. raciborskii* a limit of 258 cells mL^{-1} was required. Nevertheless, for *C. raciborskii* we were able to obtain a higher sensitivity limit than that of 1,000 cells mL^{-1} previously described by Rasmussen et al. (2007). This could be due to the higher specificity obtained by the higher annealing conditions of 55 °C rather than with the 45 °C as reported by these authors.

Quantification of the total cyanobacteria population and in particular of *C. raciborskii* was assessed at Vela Lake. This system is well known for being eutrophic due to the high nutrient levels and high productivity and also by the occurrence of toxic blooms (microcystins) that can cause high fish kills (Abrantes et al. 2006; de Figueiredo et al. 2007). The high values obtained in our study for either total cyanobacteria and *C. raciborskii* well highlights this eutrophic nature of Vela Lake and are in agreement with the values, in terms of total cyanobacteria, determined by de Figueiredo et al. (2006) (10^5 to 10^7 cells mL^{-1}) though 16S rRNA is repeated in the genome in more than one copy and an overestimation may possibly have occurred for our results.

Though its presence has been previously reported, the total and relative abundance of *C. raciborskii* in this aquatic ecosystem has never been conducted. Population composition at Vela Lake has already been described being the main dominant species *Microcystis aeruginosa*, *Aphanizomenon flos-aquae*, *Oscillatoria* sp., and *Pseudanabaena* sp. (Abrantes et al. 2006; de Figueiredo et al. 2006). However,

Table 2 One-way ANOVA for variation of total cyanobacteria and *C. raciborskii* at Vela Lake between 2008 and 2009

	F test	*p* value
Cyanobacteria	130.6	0.000
C. raciborskii	32.8	0.000

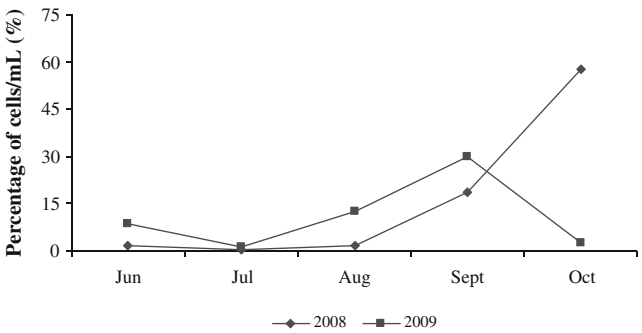

Fig. 2 *C. raciborskii* relative abundance at Vela Lake in 2008 and 2009. Quantification values obtained by real-time PCR assay

in neither of these studies was *C. raciborskii* described, making this work the first to document the presence of *C. raciborskii* at Vela Lake. In this sense, we were able with this study to establish *C. raciborskii* population dynamics and seasonal variation applying the estimated values obtained by its relative abundance in comparison with the total cyanobacteria population. Therefore, in terms of *C. raciborskii* seasonal variation, the data from our work does not seem to reveal a common pattern, meaning that while in the first year *C. raciborskii* population showed an increase in cell numbers in the end of the sampling period (autumn), in the following year the obtained result revealed a decrease in the population in the same date. These results highlight the high variation in the *C. raciborskii* population dynamics in this aquatic system. In contrast, total cyanobacteria population dynamics and seasonal variation resulted in a common pattern with only a significant decrease in cell numbers from 2008 to 2009. Though cyanobacteria populations were stable, the *C. raciborskii* population, as previously stated, appeared highly variable especially in the second sampled year preceded by a more stable population variation in the first sampled year with a continuous population increase throughout the sampling period. In terms of cell numbers, we observed a significant decrease in *C. raciborskii* population from 2008 to 2009. A possible explanation for this reduction could be attributed to the nutrient availability such as phosphorous that is a limiting factor for *C. raciborskii* growth (Chiswell et al. 1997) in the last sampled year.

Since Vela Lake is used for agriculture in terms of water source and recreational purposes, it is to be expected that proper management policies should be implemented at this aquatic system given the high values of cyanobacteria detected (up to 10^7 cells mL^{-1}).

Apart from the estimated abundance of total cyanobacteria and *C. raciborskii* population, a toxicological approach was conducted. Cylindrospermopsin is a hepatotoxic alkaloid that was first described in a *C. raciborskii* species (Hawkins et al. 1985), but it is now being found in seven other different species, such as *Aphanizomenon flos-aquae* (Preußel et al. 2006), *Anabaena lapponica* (Spoof et al. 2006), *Aphanizomenon ovalisporum* (Banker et al. 1997), *Umezakia natans* (Harada et al. 1994), *Anabaena bergii* (Schembri et al. 2001), *Raphidiopsis curvata* (Li et al. 2001), and *Lyngbya wollei* (Seifert et al. 2007). Cylindrospermopsin has been reported in all continents with the exception of the African continent. In the European continent, its occurrence has been attributed to all other genera but *Cylindrospermopsis*. In our work, a *pks* gene was found in a sample from the second sampling year in the end of summer. This gene had a high similarity to an *Aphanizomenon* species rather than to *C. raciborskii* as initially would to be expected. This result, however, is not surprising because in the European continent reports on the presence of cylindrospermopsin have never been attributed to this species. In our study, a positive result was obtained for both the presence of the *pks* gene and the presence of cylindrospermopsin (ELISA assay) for the same date. Nonetheless, in our work, quantification of the *pks* gene was not performed much due to the absence until now of cylindrospermopsin in Portugal. However, further analysis of the same positive sample by HPLC-PDA and LC/MS revealed the absence of cylindrospermopsin. This lack of agreement between the ELISA and both HPLC-PDA and LC/MS data for this toxin has already been previously reported (Yilmaz et al. 2008; Bláhová et al. 2009; Berry and Lind 2010). In all these previous studies, they stated that the difference in performance between both methods may possibly be due to the presence of an unidentified cylindrospermopsin isomer or congener or even some cross-reactivity in the ELISA due to environmental interferences. This may have occurred in our tested sample. Nevertheless, cylindrospermopsin remains until now undetected in Portugal; however, due to its spreadness character, continuous monitoring will be carried out.

In summary, the real-time PCR proved to be a valuable tool in monitoring *C. raciborskii* population distribution and seasonal dynamics at Vela Lake. Cylindrospermopsin is to date still undetected in Portugal; however, future monitoring would be needed to further assess its presence in our aquatic systems and namely at Vela Lake. With this work, the real-time PCR methodology was validated as a tool to be used in an ecological approach and toxicity evaluation for *C. raciborskii* and cyanobacteria in any given environment.

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