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Dynamics of toxic genotypes of *Microcystis aeruginosa* complex (MAC) through a wide freshwater to marine environmental gradient

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ABSTRACT

Bloom-forming species belonging to *Microcystis aeruginosa* complex (MAC) are the most commonly reported worldwide. MAC blooms are composed by toxic and non-toxic genotypes and the environmental conditions favouring the dominance of toxic genotypes are still a matter of debate among the scientific community. In this study, we evaluated the distribution of toxic MAC genotypes along a seasonal cycle and over an environmental gradient spanning 800 km, from a eutrophic freshwater reservoir in Río Uruguay to marine water in the outer limit of Río de la Plata. Abundance of four *mcy* genes, *mcyB*,*mcyD*, *mcyE* and *mcyJ* was determined by qPCR and used as a proxy of abundance of toxic MAC genotypes. All the *mcy* genes were detected through the seasonal cycle at all sampling sites, being systematically higher in the freshwater reservoir and decreasing towards the marine site. The highest toxic genotype abundance was found during the austral summer months. According to generalized linear regressions and random forest models, temperature and conductivity were the most relevant explanatory variables. This suggests that although toxic MAC genotypes grow optimally in freshwater, they are also able to tolerate the high-salinity and low temperature conditions found in estuarine and marine waters. This ability to resist harsh conditions impose a health risk and a management challenge. To our knowledge, this is the first report addressing several *mcy* genes in a broad gradient that includes a wide array of different environmental conditions.

1. Introduction

The frequency and intensity of cyanobacterial blooms has increased in recent decades in freshwater and marine ecosystems (Chorus and Bartram, 1999; Paerl and Huisman, 2008). Cyanobacterial blooms alter ecosystem function, decrease water transparency, oxygen concentration and biodiversity and generate taste and odour problems (Huisman et al., 2006; Ibelings and Mur, 1992). The main concern regarding the presence of cyanobacteria blooms is the ability of some species to produce toxins (cyanotoxins) that can affect humans and animals (Briand et al., 2003; Codd et al., 2005).

Microcystis is the most commonly reported bloom-forming cyanobacterial genus in lakes and reservoirs worldwide (De Leon and Yunes, 2001; González-Piana et al., 2011; Huisman and Hulot, 2005; O'Neil et al., 2012; Paerl and Otten, 2013; Srivastava et al., 2013) *Microcystis aeruginosa* complex (MAC) includes all *Microcystis* species that were unified based on the intergenic transcribed spacer sequences (ITS) (Otsuka et al., 2001, 1999). MAC species produce high density scums and have the potential to produce microcystin, a powerful hepatotoxin with more than 100 variants (Puddick et al., 2014) that can cause serious liver diseases (Azevedo et al., 2002; Dittmann and Wiegand, 2006; Milutinović et al., 2003). Microcystins are one of the most common toxins found in aquatic ecosystems and can be produced by a diverse range of cyanobacteria other than MAC, such as species of *Dolichospermum*, *Nostoc* or *Planktothrix* (Rinehart et al., 1994; Sivonen, 1996).

Despite MAC species share morphological traits and ecological preferences (Kruk and Segura, 2012; Reynolds et al., 2002), their blooms often consist of mixtures of microcystin-producing (hereafter toxic genotype) and non-producing (hereafter non-toxic genotype) populations (Kaebernick and Neilan, 2001;

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Kurmayer and Kutzenberger, 2003; Vezie et al., 1998). Toxic genotypes synthesize microcystins via non-ribosomal peptide synthesis (NRPS) and polyketide synthase (PKS) modules (Dittmann et al., 1997; Tillett et al., 2000). Ten genes spanning 55 kb have been described as involved in microcystin production in several species, *mcyA-J* (Tillett et al., 2000). Multiple mechanisms have been proposed to explain the production of cyanotoxins, such as those related to biological interactions and communication, protection against herbivory and competition (Lei et al., 2015; Schatz et al., 2005). Further, it has been suggested that since microcystin synthesis would involve high energetic costs, toxic genotypes would be favored in environments where resources are highly available (Kardinaal et al., 2007a, 2007b; Lei et al., 2015). The stimulation effect exerted by high temperature and high nutrients concentration on the development of MAC blooms, as well the negative effect of high salinity and high water flux have been thoroughly demonstrated (Harke et al., 2016; O'Neil et al., 2012). It is also clear that within the ranges of environmental variables that enhance MAC, some combinations can differentially affect the relative abundance of different genotypes (O'Neil et al., 2012). However, information regarding the specific variables that are responsible for the predominance of MAC toxic genotypes is still lacking.

Several field studies have addressed the relationship between *mcy* genes and environmental variables. For example, positive effects of total nitrogen and/or phosphorus were found for *mcy* abundance (i.e. *mcyA*, *mcyB*, *mcyE*, *mcyJ*) (Guedes et al., 2014; Joung et al., 2011; Te and Gin, 2011; Xu et al., 2010; Yoshida et al., 2007), while others found a negative effect (Rinta-Kanto et al., 2009). Related to temperature, it has been shown that toxic genotypes, quantified as *mcyD* and *mcyJ* abundance, increased at higher temperature (10–30 °C) (Davis et al., 2009; Joung et al., 2011), although in some cases no temperature effect was found (Guedes et al., 2014; Rinta-Kanto et al., 2009; Te and Gin, 2011; Xu et al., 2010). Moreover, a positive relationship between turbidity and *mcy* genes has been reported (i.e. *mcyE*, *mcyB* and *mcyJ*) (Joung et al., 2011; Te and Gin, 2011; Xu et al., 2010). On the other hand, culture-based studies demonstrated the dominance of toxic genotypes (*mcyB* abundance) under different light intensities (35 and 120 $\mu mol\, m^{-2}\, s^{-1}$) (Lei et al., 2015). Tonk et al. (2007) reported salinity as an environmental variable affecting the toxicity of MAC species in experimental assays; they found that microcystin cell quotas decreased at salinities above 10 $g\, L^{-1}$ and proposed that the presence of toxic genotypes and toxin production would be negatively affected by stress conditions, such as high salinity (Tonk et al., 2007). On the other hand, field studies did not find any association between salinity (measured as conductivity) and *mcyE* gene abundance (Te and Gin, 2011).

The link between the presence of *mcy* genes and their expression in nature is just begining to be explored (i.e. Ngwa et al., 2014). Based on experimental evidence, (Sevilla et al., 2008) reported a positive correlation between *mcyD* expression and microcystin-LR in *M. aeruginosa* PCC7806. On the contrary, Ngwa et al. (2014) found a weak correlation between *mcyE* expression and microcystin concentration in laboratory and field studies.

In summary, although there is an agreement about the influence of environmental variables on the dominance of toxic genotypes in freshwater ecosystems, their specific role and the extent of their influence is still unknown. Further, most of the studies addressing the abundance of toxic genotypes by qPCR of *mcy* genes usually assess just one or two of them, which owing to the bias imposed by primer design may not cover all the possible genetic variability. Thus, responses assessed through a single *mcy* gene can be biased to those populations carrying the genetic variant target by the primers, and this might explain some contradictory findings (Ngwa et al., 2014; Rinta-Kanto et al., 2009; Te and Gin, 2011; Xu et al., 2010).

The aim of this study was to evaluate the effects of environmental variables in the spatial and temporal dynamics of toxic genotypes of MAC in a wide environmental gradient (Rio Uruguay-Rio de la Plata; Fig. 1). The strategy involved bimonthly samplings in an 800 km gradient from freshwater to marine water and quantification of four *mcy* genes (*mcyB*, *mcyD*, *mcyE* and *mcyJ*) by real-time PCR (qPCR) along with in situ measurement of abiotic variables (wind intensity, water temperature, conductivity, turbidity, total nitrogen and phosphorus) and records of phytoplankton community structure.

2. Materials and methods

2.1. Study site

The study area covered an extension of ca. 800 km, from Salto Grande reservoir in Río Uruguay (31° 11′ latitude, 57° 52′ longitude) to Punta del Este (34° 57′latitude, 55° 02′ longitude), at the marine end of Río de la Plata estuary (Fig. 1). Six sites were sampled every two months during one year (from January 2013 to March 2014) (Fig. 1). At each site water samples were taken from two different locations, one next to the coast (0.01–0.5 km) and one at 2.4–7.7 km off shore. A total of 72 water samples were evaluated.

2.2. Samples collection and measurement of environmental variables

Subsurface water samples were collected with Niskin bottles for laboratory analysis of *mcy* genes, phytoplankton community structure and morphology, nutrients and LR-microcystin concentration. *Water* samples for DNA extraction were stored on ice during sampling (ca. 4 h) and once arrived to the laboratory they were immediately filtered and filters were stored at −20 °C. Phytoplankton samples for microscopic analyses were fixed in acid Lugol solution. Air temperature (°C) and wind intensity (ms^{-1}) were measured at each sampling site. Water temperature (°C), conductivity ($mS\, cm^{-1}$) and turbidity (NTU) were measured at the water subsurface using a multiparameter probe (Horiba multiparameter sensor). Total concentration of nitrogen and phosphorus were determined using the ISO/TR 11905-2 and Lachat QuikChem 31–115-01-3-D methods, respectively. Microcystin-LR was quantified by HPLC with diode array detector PEC (ISO, 2005; Lawton et al., 1994). For chlorophyll-a extraction 50–200 ml water were filtered through 0.7 μm Whatman GF/F, and filters were immediately frozen (−20 °C) until processing. Chlorophyll-a concentrations ($mg\, L^{-1}$) were determined after extraction with 95% ethanol for 24 hs at 4 °C in the dark (Jespersen and Christoffersen, 1987). A plankton net with 115 μm-size mesh (opening area ~8000 cm^2), which was dragged for ca. 2 min at a speed of one knot was used to detect the presence or absence of MAC colonies through microscopical inspection based on morphological characteristics. Organisms were assigned as MAC when coccoid cells having a similar size (3.5 to 5 μm diameter) formed large, mucilage-embedded colonies (Komárek and Komárková, 2002). The information about the presence or absence of MAC colonies retrieved from the 115 μm-size mesh was complemented with the data obtained from the Lugol-fixed samples used for phytoplankton determination. Thus, presence or abscence of MAC colonies in the whole dataset was used for further analyses.

2.3. Phytoplankton composition, abundance and biovolume estimations

Phytoplankton abundance (organisms mL^{-1}) was estimated from inverted microscope counts using settling chambers

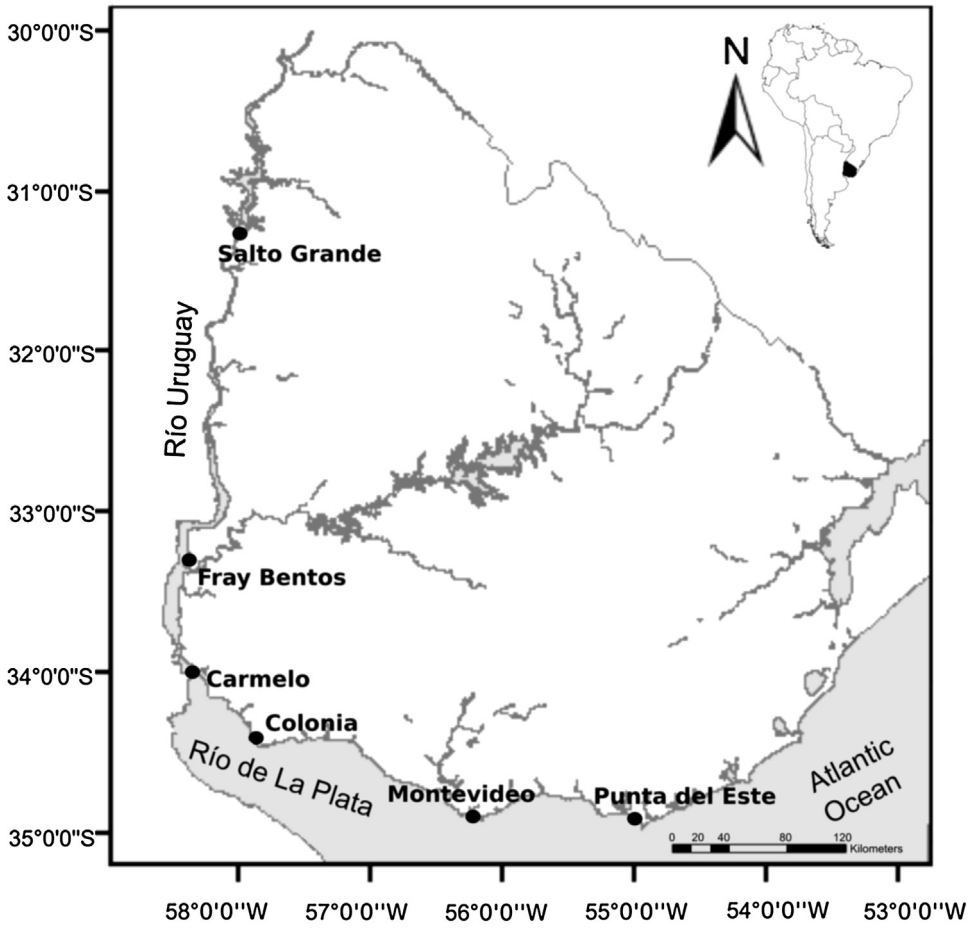

Fig. 1. Map of Uruguay showing the Rio Uruguay–Río de la Plata gradient. Sampling sites are shown as black circles: Salto Grande, Fray Bentos, Carmelo, Colonia, Montevideo and Punta del Este.

(Utermöhl, 1958). Samples were examined at several magnifications (100–1000×) and counted until reaching at least 100 organisms of the most frequent species (Lund et al., 1958). Organism dimensions were measured in order to estimate volume (V, μm^3) using geometrical approximations (Hillebrand et al., 1999). Measurements and calculations were obtained for each organism in each sample. The biovolume of phytoplankton populations at each station was calculated by multiplying mean individual volume in the corresponding sampling station by its abundance. Phytoplankton species were identified and classified into potential microcystin-producers or non microcystin-producers according to their taxonomic affiliation and to literature-based information.

2.4. DNA extraction

For DNA extraction, 250–300 ml of the water sample were filtered through 0.22 μm sterile polycarbonate membrane (Milipore), which were immediately frozen at −20 °C until processing. Procedures for nucleic acid extraction were performed as describe by (Martínez de la Escalera et al., 2014). The concentration and purity of DNA were determined spectrophotometrically at 260 and 280 nm (NanoDrop), and DNA samples were stored at −20 °C.

2.5. Quantitative PCR (qPCR)

Four genes belonging to the two operons involved in the microcystin synthesis were quantified by qPCR, *mcyB* (operon *mcyABC*) (Tooming-Klunderud et al., 2008) and *mcyD*, *mcyE*, *mcyJ* (operon *mcyDEFGHIJ*) (Kaebernick et al., 2002). Since we used primers from the literature, which are based on MAC species from other latitudes, we decided to quantify four *mcy* genes to ensure the detection of most of the toxic genotypes and to avoid losing information. Two microlitres of DNA extracts from each sample (ca. 50 ng DNA) were applied to the Power SYBR Green PCR (Invitrogen) with a final reaction volume of 20 μl. Primers are listed in Table 1. Cycling conditions were 2 min at 50 °C, 15 min at 95 °C and 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, including a last melting step from 65 to 95 °C at increases of 1 °C each 4 s. A 96 FLX Touch TM thermal cycler (Bio-Rad) was used. To quantify the abundance of each *mcy* gene in the water samples, several amplicons obtained from each gene were cloned into a TOPO TA 2.1 vector (Invitrogen) (see below). After identification, each cloned gene was confirmed by sequencing (Institut Pasteur de Montevideo Sequencing Service) and these clones were used to perform the calibration curves. Curves were achieved using five serial dilutions from 1/10 to 1/100,000 of the cloned genes (in quintuplicates) and applied to qPCR in the same PCR plate where the samples were assayed. Samples were run in triplicate.

2.6. Cloning of mcy genes

Four *mcy* genes (*mcyB*, *mcyD*, *mcyE* and *mcyJ*) were cloned using the strain *Microcystis* 140313 as target (kindly provided by Dr. Sylvia Bonilla). DNA extraction and gene cloning were performed as described by (Martínez de la Escalera et al., 2014). PCR of *mcy* genes was done using the qPCR primers (Table 1). Amplicons were run in a 1% agarose gel electrophoresis, purified using the gel-extraction QIAquick PCR purification kit (QIAGEN, Hilden,

Table 1
List of the primers used in this study.

Gene targeted	Primer name	Sequence (5′-3′)	Refs.
mcyB-Microcystis spp.	mcyB_F	AGATTTTAATCCACAAGAAGCTTTATTAGC	Hautala et al. (2013)
	mcyB_R	CTGTTGCCTCCTAGTTCAAAAAATGACT	Hautala et al. (2013)
mcyD-Microcystis spp.	mcyD_F	GGTTCGCCTGGTCAAAGTAA	Kaebernick et al. (2000)
	mcyD_R	CCTCGCTAAAGAAGGGTTGA	Kaebernick et al. (2000)
mcyE-Microcystis spp.	mcyE_F	AAGCAAACTGCTCCCGGTATC	Sipari et al. (2010)
	mcyE_R	CAATGGGAGCATAACGAGTCAA	Sipari et al. (2010)
mcyJ-Microcystis spp.	mcyJ_F	TAGCTAAAGCAGGGTTATCG	Kim et al. (2010)
	mcyJ_R	TCTTACTATTAACCCGCAGC	Kim et al. (2010)

Germany) and ligated into a TOPO 2.1 vector (TOPO-TA cloning kit, Invitrogen). Competent TOP10 *Escherichia coli* cells were transformed and white/blue colony selection was applied. Those clones having right-sized inserts (assessed by PCR using M13 forward and reverse primers) were selected to generate the calibration curves for qPCR.

2.7. Statistic analysis

Spearman bivariate correlations (rs) were used to identify which environmental variables were correlated with toxic genotypes abundance (*mcy* genes). Non-parametric median tests (Kruskal-Wallis) were used to evaluate differences in *mcy* genes abundance based on the presence or otherwise of MAC colonies in 115 μm net. Generalized multiple linear models (GLM) with gamma distribution and a log link function were constructed to evaluate the relation between environmental variables and the abundance of toxic genotypes. In all cases the explaining environmental variables were total nutrients (Total Nitrogen; TN and Total Phosphorus; TP), wind intensity (WI), water temperature (T), turbidity (Turb) and conductivity (K). The models were constructed using backward selection and were compared based on the Akaike criterion (AIC) (Burnham and Anderson, 2002). Pseudo-R^2 for each GLM model were calculated as: (null deviance − residual deviance)/null deviance; where null deviance corresponds to the null model and residual deviance corresponds to the deviance of the estimated model. Random Forest (RF, Cutler et al., 2007) were used to determine the most important environmental variable in effecting toxic genotype abundance. The abundance of *mcy* genes was classified in 2 categories: 1) Low (less than 999 copies ml^{-1}) abundances, that does not present a risk to public health (Falconer et al., 1999; World Health Organization, 2004) and 2) High abundances (more than 1000 copies ml^{-1}) achieved during cyanobacteria blooms and representing public health risk (Falconer et al., 1999; World Health Organization, 2004). The same independent variables as in GLM were included in this analysis and their percentage of explained variability was represented graphically (Kruk and Segura, 2012). We evaluated the accuracy of the RF by exploring missclassification rate in ten fold cross-validation. All statistic analysis were performed with free software R, version 3.0.2 using {randomForest} and {rpart} packages (Liaw and Wiener, 2002; R Core Team, 2013; Therneau et al., 2015).

3. Results

3.1. Environmental and biological gradients

Strong temporal and spatial gradients were observed for the environmental variables (Fig. 2). Suface water temperature varied from 11 to 34 °C, showing higher values in Salto Grande reservoir during summer (January to March) and lower temperatures in the outer marine zone of the Rio de la Plata during winter-early spring (June to October) (Fig. 2A and B). The highest wind intensity (7 ms^{-1}) was observed in winter-early spring (June to October) in Punta del Este. A clear spatial gradient was defined by conductivity and turbidity. Conductivity showed a minimum of 0.023 mS cm^{-1} (freshwater systems: Salto, Fray Bentos and Colonia Carmelo) and a maximum of 51 mS cm^{-1} at the estuary (Montevideo and Punta del Este) (Fig. 2B). The larger conductivity range (9–51 mS cm^{-1}) was observed in the stations of Montevideo (Fig. 2B). Turbidity ranged from 0 to 187 NTU, with higher values in the stations located at the middle of the gradient (Carmelo and Colonia) (Fig. 2B). Higher concentrations of nutrients were measured in Salto Grande reservoir and Montevideo (TP ∼ 60 μg L^{-1}, TN ∼ 0.9 mg L^{-1}).

Microystin-LR was only detected during summer in the Salto Grande reservoir and in Colonia (Table 2). Chlorophyll-a was higher in the station at the freshwater reservoir Salto Grande and at the estuary Río de la Plata (Punta del Este), showing lower maximum values in the middle of the studied gradient (Carmelo and Colonia) (Table 2). A similar pattern was observed for phytoplankton total biovolume (Table 2). Total cyanobacteria biovolume was maximum in Salto Grande reservoir and decreased towards the outer estuary station (Punta del Este; Table 2). Potentially microcystin-producing MAC species were the dominant cyanobacteria in terms of biovolume. reaching higher total percentage of the phytoplankton community in Salto Grande reservoir during summer. However,

Table 2
Mean values and ranges of biological variables: total phytoplankton biovolume (Phy_BV), cyanobacteria biovolume (Cya_BV), chllorophyl-a (Chl-a), and microcystin-LR. BDL = below detection limit.

Sites	Phy_BV ($mm^3 L^{-1}$)	Cya_BV ($mm^3 L^{-1}$)	Chl-a (μg L^{-1})	Microcystin-LR (μg L^{-1})
Salto Grande	361.4 (0.2–2188)	457 (BDL–2175)	7.9 (0.9–29.9)	7.1 (0.4–15)
Fray Bentos	0.5 (0.1–3.4)	0.06 (BDL–0.5)	0.6 (0.1–2.1)	BDL
Carmelo	1.4 (BDL–3.8)	0.4 (BDL–2.9)	0.7 (0.3–1.9)	BDL
Colonia	4.7 (BDL– 42.6)	0.8 (BDL–9.1)	0.8 (0.1–1.4)	0.2 (BDL–0.3)
Montevideo	2.4 (0.1–6.7)	1.4×10^4 ($BDL–8.5 \times 10^{-4}$)	1.2 (0.3–1.8)	BDL
Punta del Este	19.8 (0.6–141.4)	2.8×10^{-4} (BDL–0.0029)	2.4 (0.3–7.9)	BDL

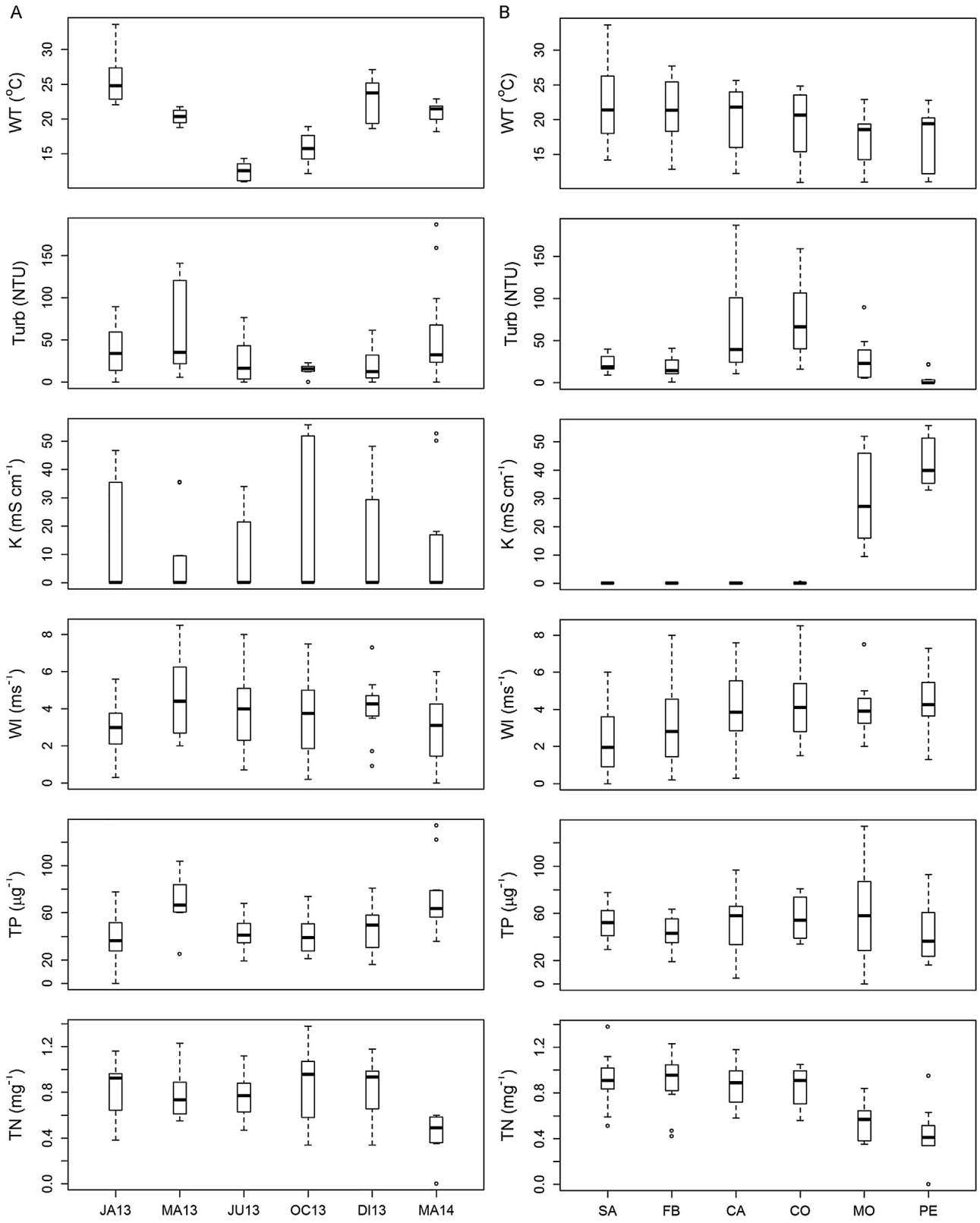

Fig. 2. Temporal (A) and spatial (B) variability of the environmental variables measured during each sampling. Water temperature (WT), turbidity (Turb), conductivity (K), wind intensity (WI), total phosphorus (TP) and total nitrogen (TN). Medians, interquartile ranges, maximum, minimum and outliers are shown. JA13: January 2013, MA13: March 2013, JU13: June 2013, OC13: October 2013, DI13: December 2013 and MA14: March 2014. SA: Salto, FB: Fray Bentos, CA: Carmelo, CO: Colonia, MO: Montevideo and PE. Punta del Este.

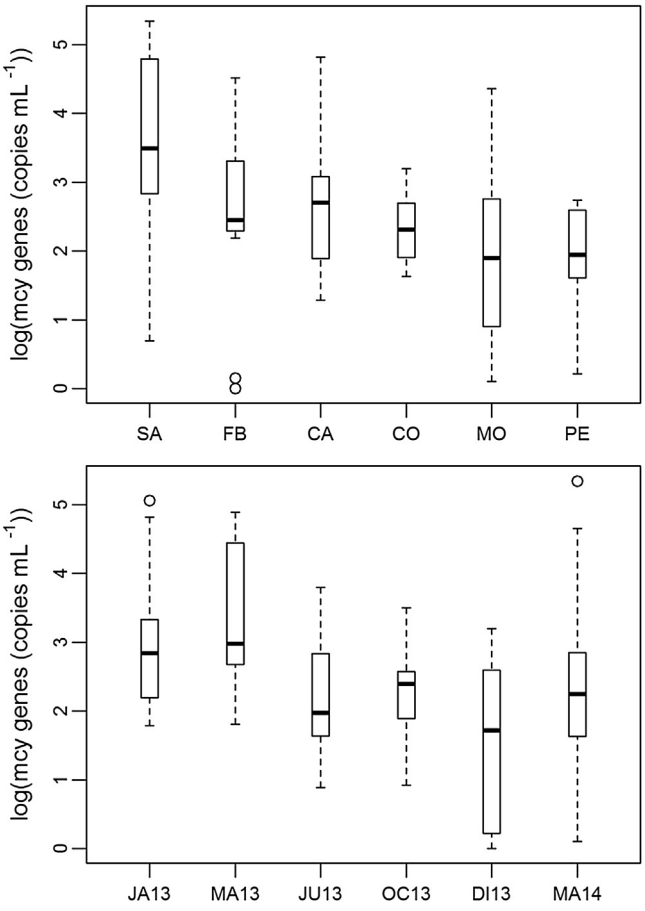

Fig. 3. Temporal (bottom) and spatial (top) variability of the logarithm of maximum abundance of toxic genotypes (copies of mcy ml-1). Medians, interquartile ranges, maximum, minimum and outliers are shown. JA13: January 2013, MA13: March 2013, JU13: June 2013, OC13: October 2013, DI13: December 2013 and MA14: March 2014. SA: Salto, FB: Fray Bentos, CA: Carmelo, CO: Colonia, MO: Montevideo and PE. Punta del Este.

presence of MAC blooms was also registered in winter (Temperature = 9 °C). In addition, the presence of MAC colonies in the 115 μm net was detected along the whole gradient and in most sampling dates.

3.2. Spatial and temporal distribution of mcy genes

The highest abundances of *mcy* genes occurred in freshwater stations in Río Uruguay (Salto Grande reservoir and Fray Bentos) decreasing towards estuarine and marine sites (Montevideo and Punta del Este). The maximum total abundances were observed in summer (Fig. 3). The presence of *mcy* genes (*mcyB*, *mcyD*, *mcyE* and *mcyJ*) was detected in all sampling dates and stations (supplementary Fig. 1 and 2). The individual abundance of all addressed *mcy* genes (*mcyB*, *mcyE, mcyJ* and *mcyD*) showed the same spatial pattern, higher in the freshwater reservoir and decreasing towards the estuary end (supplementary Fig. 1). The temporal distribution of *mcy* genes was similar between *mcyB*, *mcyE* and *mcyJ*, being the maximum abundance observed in summer (supplementary Fig. 2). Surprisingly, the abundance of *mcyD* showed an opposite temporal pattern (higher abundances in winter) (supplementary Fig. 2).

Table 3
Values of Spearman Correlation (rs) between the abundance of mcy genes estimated by qPCR (copies mcy ml-1) and environmental variables: water temperature (WT), conductivity (K), turbidity (Turb), wind intensity (WI), total phosphorus (TP) and total nitrogen (TN). Only significant values are shown ($p < 0.05$).

	WT	K	Turb	WI	TN	TP
mcyB	–	−0.37	–	–	–	–
mcyD	−0.33	−0.28	−0.26	−0.27	–	–
mcyE	0.50	−0.25	0.41	–	–	–
mcyJ	–	−0.31	–	−0.23	–	–

3.3. Relation of toxic genotypes and environmental variables

Conductivity was negatively correlated with all *mcy* genes abundances (Table 3). Temperature and turbidity were positively correlated with *mcyE* abundance and negatively with *mcyD*. Wind intensity was negatively associated with *mcyD* and *mcyJ* abundance. The concentration of nutrients showed no association with the abundance of toxic genotypes. The variables selected in the GLM models differed for each *mcy* gene and the explained variance (pseudo R2) varied from 0.14 to 0.42 (Table 4). Conductivity affected negatively the abundance of *mcyB,* while temperature had a positive effect on the abundance of *mcyE*. Conversely, although the abundance of *mcyD* gene showed the same trend of the other genes for conductivity, turbidity and wind intensity, it was negatively affected by temperature. In the case of *mcyJ* abundance, conductivity, turbidity and TP affected negatively its abundance while the affect of temperature was positive.

Random Forest analyses showed a high predictive accuracy of the two classes (high/low) for each *mcy* gene (between 81 and 93%; Fig. 4). Variable importance differed for each *mcy*. The highest percentage of variance in the *mcyB* gene was mostly related to conductivity (K), wind intensity (WI) and water temperature (WT), while total nutrients (TP and TN) and wind intensity (WI) explained a higher percentage of variance in *mcyD* classes. Variance in *mcyE* classes were accounted by water temperature (WT) and conductivity (K). Finally, *mcyJ* abundance was mostly related to conductivity (K) and water temperature (WT) (Fig. 4).

3.4. Relation of toxic genotypes abundance with other toxic cyanobacteria indicators

Significant correlations were detected among the assessed genes, except between *mcyE* and *mcyD* (Table 5). The abundance of *mcyE* correlated positively with traditional phytoplankton indicators, such as chlorophyll-a, total phytoplankton biovolume and cyanobacteria biovolume (Table 5); while *mcyB* and *mcyJ* abundances had a strong positive relationship only with total cyanobacteria biovolume (Table 5). Also, the abundance of *mcyB*

Table 4
Results of GLM models to explain *mcy* abundances based on six environmental variables: water temperature (WT), conductivity (K), turbidity (Turb), wind intensity (WI), total phosphorus (TP) and total nitrogen (TN). Coefficient and intercept with significant values are shown ($p < 0.05$).

Genes	Environmental variables/coefficients						Parameters	
	WT	K	Turb	WI	TN	TP	Intecept- value	pseudo-R2
mcyB		−0.011					1.770	0.14
mcyD	−0.074	0.020	0.007	−0.100			3.180	0.35
mcyE	0.100						−1.040	0.42
mcyJ	0.047	−0.021	−0.006			−0.010	1.880	0.16

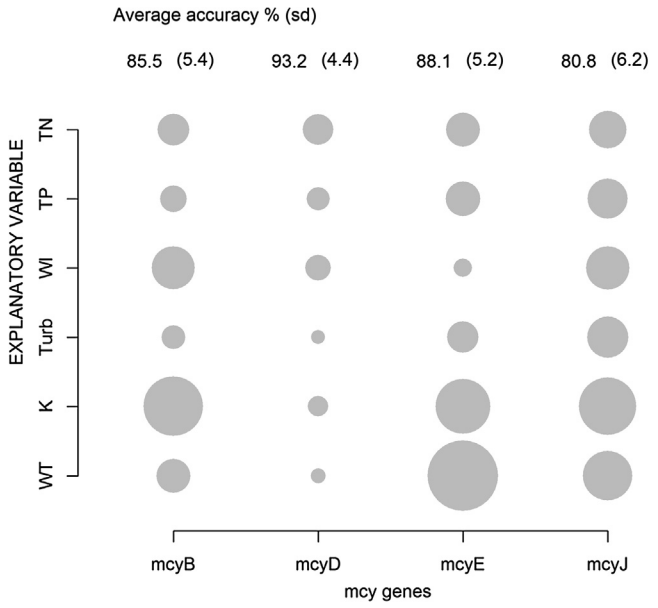

Fig. 4. Variance importance plot on the Random Forest prediction of *mcy* gene classes. The importance of each environmental variable (assessed through Gini index) to explain the abundance of each *mcy* gene is proportional to circle diameter. Six environmental variables were evaluated: water temperature (WT; °C), conductivity (K; μm cm-1), turbidity (Turb, NTU), wind intensity (WI; m s-1), total phosphorus (TP μg L-1) and total nitrogen (TN mg L-1). Average accuracy of the RF model (%) and its standard deviation (sd) are shown.

Table 5
Values of Spearman Correlation (rs) between the abundance of each *mcy* gene estimated by qPCR (*mcy* copies ml-1) and between gene abundances and other indicators of potentially toxic cyanobacteria: chllorophyl-a (Chl-a), total phytoplankton biovolume (Phy_BV), cyanobacteria biovolume (Cya_BV) and MAC biovolume (MAC_BV). Only significant values are shown ($p < 0.05$).

	mcyB	*mcyD*	*mcyE*	*mcyJ*	Chl-a	Phy_BV	Cya_BV
mcyB					–	–	0.57
mcyD	0.28				–	–	–
mcyE	0.71	–			0.25	0.68	0.43
mcyJ	0.38	0.41	0.33		–	–	0.33

and *mcyE* were significantly higher when organisms of MAC were detected in the 115 μm net (Kruskal-Wallis, H = 20 y 25 respectively, $p < 0.05$) (Fig. 5). Differently, *mcyD* and *mcyJ* did not show relationship with the presence of large MAC colonies.

4. Discussion

The environmental mechanisms responsible for the control of toxic populations of MAC are still not well understood and the information about which variables are more relevant to explain their abundance dynamics is sometimes contradictory. Most previous studies focused on one or few lakes and did not include a high degree of environmental variability. Here, we provide insight into these issues by analysing the spatial-temporal distribution of toxic genotypes of MAC species in a wide environmental gradient, from freshwater (Río Uruguay) to estuarine and marine waters (Río de la Plata). Linear correlations, GLM and RF methods revealed that the relevant environmental variables explaining the observed distribution of toxic genotypes were conductivity (hereafter used as indicator of saline stress) and temperature.

Saline stress was the main variable limiting the abundance of population harbouring toxic genotypes. Despite there is scarce evidence on the role of salinity in toxin production by MAC species, it is well known that saline stress affects respiration rates, decreases photosynthesis rates and alters permeability of cell membranes (Chen et al., 2015, 2015; Khomutov et al., 1990; Sudhir and Murthy, 2004; Zhang et al., 2010). It is also well established that cyanobacteria species, and particularly those belonging to MAC, are sensitive to saline environmental conditions (Tonk et al., 2007). Moreover, several studies reported the optimum growth of *Microcystis* spp. at salinity 0 and an upper limit for active growth at salinities close to 6 (estuarine salinity) (Liu, 2006; Nasr et al., 2012; Robson and Hamilton, 2003; Tonk et al., 2007; Wangwibulkit et al., 2008). In agreement with those reports, we found the highest MAC abundance in freshwater (Río Uruguay) and a decrease towards the marine end of Río de la Plata estuary (salinity range 20–30). Besides, the *mcy* genes showed a similar trend related to the freshwater-marine water gradient. Although MAC populations are described as freshwater, the detection of colonies and toxic genotypes in marine waters could be attributed to their transport from headwaters (Río Uruguay) downstream into Río de la Plata. Similar findings have been reported by (Pinckney et al., 1997) for the Neuse River Estuary (North Carolina, USA), who found higher abundance and growth rates of phytoplankton in periods of low salinity, when the river discharged into the estuary, implying that MAC species would be able to survive but not able to grow in estuarine or marine waters (Lehman et al., 2008; Pinckney et al., 1997).

The lack of correlation between the concentration of microcystin-LR and the abundance of *mcy* genes could be due to a methodological limitation to detect other variants. This toxin was only detected during summer in Salto Grande reservoir, while MAC representatives were found at high abundances also in other stations, suggesting that other microcystin variants different than LR could have been synthesized. This lack of correlation between toxin production and gene abundances has been already found for other systems using different approaches to quantify the toxin (Beversdorf et al., 2015; Rinta-Kanto et al., 2009). As in the present work, previous studies only addressed few microcystin variants (usually LR) over more than 100 that have been reported so far, which could mask the actual association between the presence of the toxic genotypes and the toxin synthesis. In this sense, (Vaitomaa et al., 2003) found a positive correlation between total microcystins evaluated by ELISA and *mcyE* copy numbers in two Finland lakes, confirming the reliability of using *mcy* gene copy numbers as surrogates for microcystin-producers.

Mostly, toxic genotypes abundance was higher in summer than in winter, following the same pattern as for water temperature. This corresponds well with the optimal growth temperature described for *Microcystis* spp. (close to 25 °C; Reynolds, 2006). According to GLM and RF water temperature would be one of the main factors affecting the abundance of most toxic genotypes. However, when Spearman correlations are considered, only the abundances of *mcyD* and *mcyE* were significantly related to water temperature (Spearman correlation, $p < 0{,}05$). In this sense, our findings are in agreement with those reported by other authors, who did not found significant correlations between *mcyB* and temperature (Guedes et al., 2014; Xu et al., 2010). The correlation between *mcyJ* and temperature was not significant, differing from the work of (Joung et al., 2011), who found that higher temperatures promoted a rapid growth of *Microcystis* toxic genotypes (assessed as the abundance of *mcyJ* gene), when compared to non-toxic genotypes. (Davis et al., 2009), using *mcyD* gene abundances, demonstrated that temperature increased the growth rate of toxic *Microcystis*. In contrast, in the current study the abundances of *mcyD* showed a relationship with temperature that was opposite to the others *mcy* addressed genes. Since primers used in this study were designed based on sequences of microcystin-producing cyanobacteria from other geographical origins at different

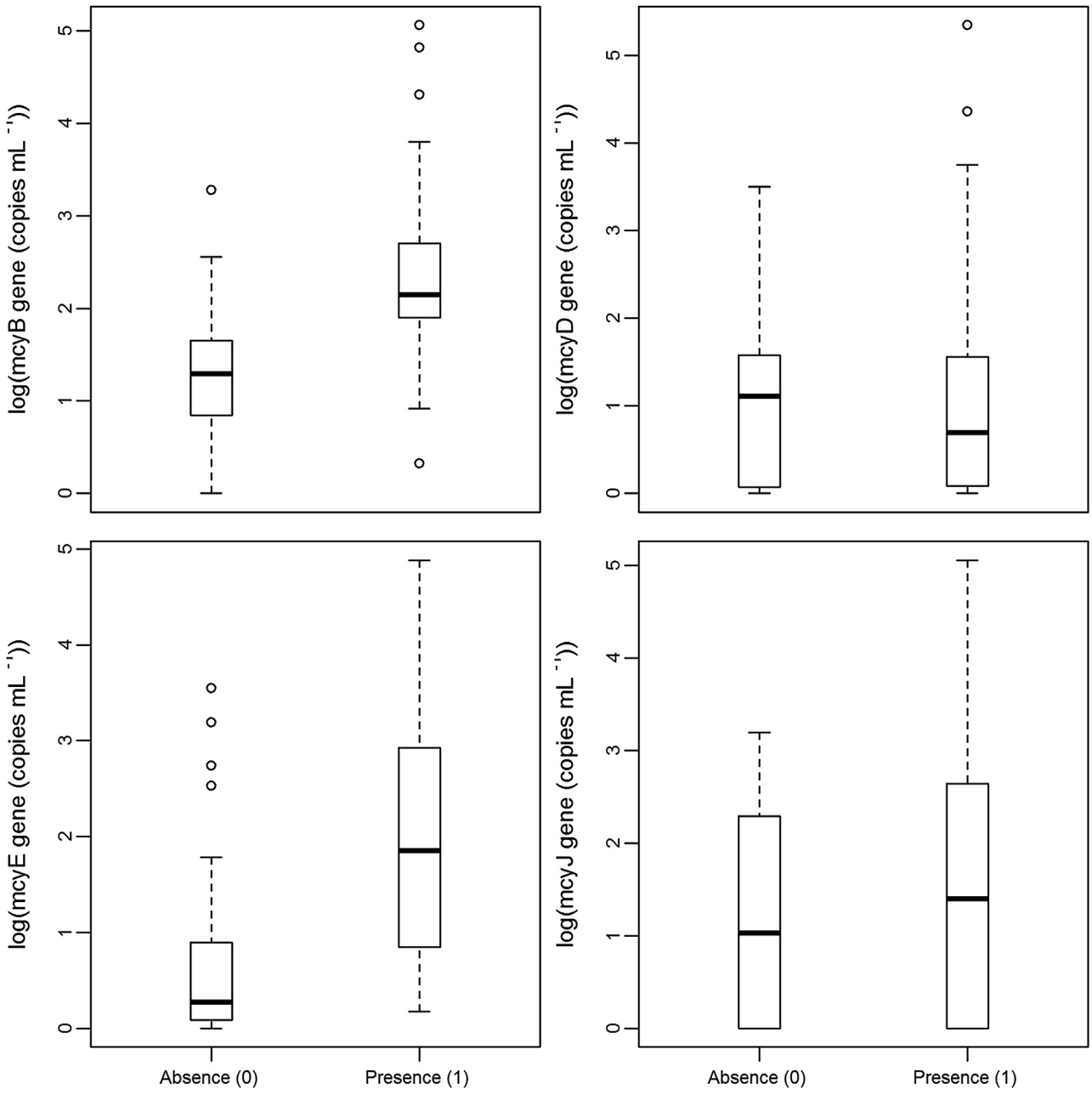

Fig. 5. Box-plots showing the medians, interquartile ranges, maximum, minimum and outliers of *mcy* genes abundance (log copies mcy ml-1) related to the presence (1) or absence (0) of visible MAC colonies in the 115 μm net.

latitudes (Kaebernick et al., 2000), a possible explanation for this behaviour can be attributed to a bias imposed by the *mcyD* primers, which would only detected a genetic variant or sub-population of toxic MAC species that are able to growth at low temperature. Therefore, this finding should be taken with caution and new primers based on local sequences should be designed to confirm the presence of populations of toxic MAC able to thrive in winter conditions in the studied system. In a similar study addressing four lakes, (Beversdorf et al., 2015) found different trends between *mcy* genes and environmental variables for each lake, e.g. that genes were highly variable temporally and correlated with increased temperature and nutrients just in few cases. Therefore, the positive trends found between *mcyE*, *mcyB* and *mcyJ* and temperature and the negative correlation with conductivity obtained in the present work, which comprised a high range of temperature and salinity conditions, are encouraging and suggest that both variables are relevant for the dynamics of toxic populations, at least in aquatic ecosystems involving river-estuary connections.

Turbidity was negatively correlated to the abundance of *mcyD* gene and positively to *mcyE* gene targeted in this study. Also *mcyE* abundance was positively correlated with MAC biovolume, suggesting that observed turbidity could be a consequence of the high MAC biomass. According with these results, (Te and Gin, 2011) reported a positive correlation between *mcyE* gene and turbidity in a tropical reservoir and suggested the use of this variable as an indicator of cyanobacteria biomass. On the other hand, the negative correlation between *mcyD* gene and turbidity would be a consequence of the higher *mcyD* abundances at low temperatures, when the lowest MAC biomass and turbidity were found. Therefore, turbidity would be rather a cyanobacteria biomass indicator and not an intrinsic water property affecting toxic genotypes.

When the effect of wind intensity over the abundance of toxic genotypes was addressed, significant and negative correlations were only found for *mcyJ* and *mcyD*, suggesting that high wind intensities would difficult the growth of populations harbouring these genotypes. It has been described that wind can hamper MAC blooms formation reducing their growth rate due to disturbance of vertical stability and mixing of the water column (Blottière et al., 2014; Harke et al., 2016). However, the mixing effect is applicable to small shallow lakes or ponds (Visser et al., 1996) and would be limited in estuarine waters owing to their large area and volume (Paerl, 2014). This could explain the low contribution of this explanatory variable in RF models.

Surprisingly, we could not find association between the abundance of any *mcy* genes and the concentration of total nutrients (TP or TN). Several studies have shown that there is a positively correlation between nutrients (TN and TP) and the abundances of the *mcy* gene (*mcyB*, *mcyD*, *mcyE* and *mcyJ*) (Guedes et al., 2014; Joung et al., 2011; Rinta-Kanto et al., 2009; Te and Gin, 2011; Xu et al., 2010). This lack of correlation suggest that after a given threshold of nutrient load has been overpassed, temperature and saline stress would be more relevant for the regulation of toxic genotypes abundance than nutrient concentration.

Among the four genes targeted in this study (*mcyB*, *mcyD*, *mcyE* and *mcyJ*), only *mcyD* showed an opposite response to temperature. This implies that the potential to produce microcystin could be achieved under a different combination of conditions (i.e. Guedes et al., 2014). The differential response to the environment found for mcyD e.g. and in order to avoid primers biases, we recommend the use of more than one *mcy* gene to monitor and predict toxin production in aquatic ecosystems. In this work, the qPCR strategy employed showed to be successful to detect toxic genotypes of MAC with high sensitivity ($\leq$2 cells mL-1), outperforming classical methods used to monitor abundance of cyanobacteria (i.e. microscopy). Moreover, the results showed that the abundance of the *mcyB, mcyE* and *mcyJ* genes correlated with cyanobacteria and MAC biovolume, as estimated by classic counting and measuring. In agreement with this, the abundance of *mcyB* and *mcyE* genes were significantly higher when the presence of organisms of MAC was detected using the plankton net. These results together to the high sensitivity displayed by qPCR assay allowed us to suggest that this is a promising tool for the early detection of MAC toxic genotypes. Indeed, due to the strong correlations found between *mcyE* and biologicals variables assessed though a wide environmental gradient, we propose that this *mcy* gene should always be included as an indicator of the presence of MAC toxic genotypes.

5. Conclusions

Toxic genotypes of *Microcystis* spp. were detected along the whole environmental gradient of the studied system, where they were the dominant microcystin-producer group. The analyses involving environmental variables suggested that temperature and salinity were the main variables affecting the abundance of toxic genotypes in Río Uruguay and Río de la Plata. We also suggest the existence of several toxic populations carrying different genotypes, since the addressed *mcy* genes showed differential responses to the assessed environmental variables. A noticeable example was the *mcyD* population that showed its maximum abundance during winter.

Our study is the first work using a molecular-based analysis to detect toxic genotypes in the large environmental and temporal gradient comprised by Río Uruguay-Río de la Plata system. The qPCR strategy showed a high sensitivity and good correlations between toxic-genotypes abundance and the presence of MAC colonies were found, representing a suitable alternative tool for early prediction of toxic MAC blooms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.hal.2016.11.012.

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