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Diversity and temporal shifts of the bacterial community associated with a toxic cyanobacterial bloom: An interplay between microcystin producers and degraders

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ABSTRACT

The biodegradation of microcystins (MCs) by bacteria constitutes an important process in freshwater ecosystems to prevent the accumulation of toxins. However, little is known about the diversity and the seasonal dynamics of the bacterial community composition (BCC) involved in the degradation of MCs in nature. To explore these BCC shifts, high-throughput sequencing was used to analyse the 16S rRNA, *mcy*E and *mlr*A genes during a year in a freshwater reservoir with a toxic cyanobacterial bloom episode. The analysis of the *mcy*E and *mlr*A genes from water samples revealed the coexistence of different MC-producing and MC-degrading genotypes, respectively. The patchy temporal distribution of the *mlr*A genotypes (from the families *Sphingomonadaceae* and *Xanthomonadaceae*) suggests their dissimilar response to environmental conditions and the influence of other factors besides the MCs that may control their presence and relative abundance. During the maximum toxic cyanobacterial biomass and cell lysis, other bacterial taxa that lack *mlr* genes increased their relative abundance. Among these bacteria, those with a recognized role in the degradation of xenobiotic and other complex organic compounds (e.g., orders *Myxococcales*, *Ellin6067*, *Spirobacillales* and *Cytophagales*) were the most representative and suggest their possible involvement in the removal of MCs in the environment.

1. Introduction

Freshwater cyanobacterial blooms are common natural phenomena, but their increasing frequency and intensity worldwide are considered undesirable consequences of the eutrophication and global change on aquatic systems (De Senerpont Domis et al., 2013; Paerl et al., 2011). As part of the phytoplankton community, the cyanobacteria are essential to biogeochemical cycles and constitute the basis of aquatic food webs (Cho and Azam, 1990). However, their excessive growth entails significant disruption to ecosystem function (Codd et al., 2005), especially when the toxin-producing cyanobacteria dominate blooms (Moustaka-Gouni et al., 2006). The most widespread and frequent toxins are the microcystins (MCs), which are synthesized non-ribosomally by large multienzyme complexes encoded by the *mcy*A-J gene cluster (Börner and Dittmann, 2005). The MCs are potent inhibitors of protein phosphatases, which cause liver damage, promote tumour activity (MacKintosh et al., 1990; Zhou et al., 2002) and pose serious harm to a broad group of organisms from the ecosystem (Bláha et al., 2009; Sivonen and Jones, 1999).

In the region surrounding cyanobacteria, the so-called the phycosphere (Bell and Mitchell, 1972), other bacterioplankton communities play an important role in the mineralization and turnover of the organic matter. Some of these bacteria, especially those from the family *Sphingomonadaceae* (phylum *Proteobacteria*), are able to degrade the MCs (Ho et al., 2007; Valeria et al., 2006) using an enzymatic pathway encoded by the *mlr*A-D gene cluster (hereafter, *mlr*$^+$ genotype) (Bourne et al., 2001). However, bacterial isolates lacking *mlr* genes (hereafter, *mlr*$^-$ genotype) from the families *Burkholderiaceae*, *Micrococcaceae*, *Nocardiaceae*, *Brevibacteriaceae*, *Xanthomonadaceae* and *Aeromonadaceae*, have also been reported as MC degraders (Kormas and Lymperopoulou, 2013; Lezcano et al., 2016; Manage et al., 2009; Mankiewicz-Boczek et al., 2015; Rapala et al., 2005; Yang et al., 2014). The different MC-degrading bacterial families isolated from field samples (both *mlr*$^+$ and *mlr*$^-$ genotypes), together with the diverse array of

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bacterial taxa associated with cyanobacterial blooms (Berg et al., 2009; Li et al., 2012) and responsive to MCs in microcosm experiments (Mou et al., 2013), point towards the existence of alternative-*mlr* degradation pathway(s) and reveals the largely undetermined MC-degrading bacterial diversity in nature.

Despite the well-studied multiple interactions between cyanobacteria and the associated bacterial assemblage (Ramanan et al., 2016), there is a gap of knowledge about the composition and the seasonal dynamics of the specialized MC-degrading bacterial community, considering both *mlr*$^+$ and *mlr*$^-$ genotypes, under environmental conditions. To unravel this diversity and the seasonal patterns, a high-throughput sequencing experiment of a water reservoir with periodic toxic cyanobacterial blooms was designed. Therefore, in the present work, we studied the following: i) the composition and the seasonal dynamics of the MC-producing cyanobacteria and the fraction of the MC-degrading bacteria with *mlr* genes by analysing the *mcy*E and *mlr*A genes, respectively, and ii) the composition and the seasonal dynamics of the bacteria lacking *mlr* genes that are responsive to the release of MCs during the cyanobacterial bloom decay. This study is the first to analyse the diversity and the seasonal dynamics of both the *mlr*$^+$ and *mlr*$^-$ fractions of the potentially MC-degrading bacterial community in a water reservoir with a toxic cyanobacterial bloom episode.

2. Materials and methods

2.1. Sampling site and sample collection

The San Juan reservoir is a water body located in the Iberian Central Plateau (Madrid, Spain) with a granitic catchment and a history of persistent toxic cyanobacterial blooms. It was periodically sampled at the shoreline (40° 22′ 44.10″ N and 4° 19′ 40.95″ W) from 29 January 2014 to 2 December 2014. Water samples were collected every two months during winter and spring, which was changed to a monthly basis from August to November and to a fortnightly basis throughout the massive cyanobacterial bloom episode until December. Each sample was comprised of a total of 2 L of subsurface water collected with sterilized polyethylene bottles that were rinsed three times on site with reservoir water and stored in dark and cold (4 °C) conditions during transport (less than 2 h) to the laboratory. Samples were split in two aliquots, and each aliquot was treated with a different protocol: 1 L of water was sequentially filtered through 2-μm (25 mm, Whatman, Maidstone, United Kingdom) and 0.22-μm pore size (25 mm, Millipore, Darmstadt, Germany) polycarbonate filters to collect the fractions containing the larger and smaller cyanobacteria and other aquatic bacteria (both those attached to the cyanobacterial mucilage and those living free). Filter membranes were stored at −20 °C for further analysis. The remaining 1 L of water was filtered through fibreglass filters (0.7-μm approx., Millipore) to collect the sestonic fraction and then stored at −20 °C for subsequent MC and chl-*a* analysis. The flow-through was also preserved at −20 °C for quantification of the dissolved MCs.

2.2. Bacterial community DNA extraction

Genomic DNA from 2-μm and 0.22-μm polycarbonate filters was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with several modifications in the cell disruption step, as described in Lezcano et al. (2016). The extracted DNA was dissolved in sterilized Milli-Q water and quantified using an Epoch spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). Equal genomic DNA concentrations from both filtered fractions and the sampling events were used for the subsequent high-throughput sequencing analysis.

2.3. Amplicon sequencing and analysis

The bacterial community composition (BCC) was examined using a bar-coded 16S rRNA amplicon sequencing strategy with primers 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and 805R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013) which include the V3–V4 hypervariable regions of the 16S rRNA gene with specific linking sequences and sequencing adaptors. To study the potentially MC-producing cyanobacterial community and the fraction of the MC-degrading bacterial community possessing the *mlr* gene cluster (Bourne et al., 2001), *mcy*E and *mlr*A were used as the targeted genes due to their essential function in the synthesis (Jungblut and Neilan, 2006) and degradation of MCs (Saito et al., 2003), respectively. Both *mcy*E and *mlr*A, although functional genes, have been reported to possess conserve and variable regions that are used in this study to distinguish between different OTUs and to tentatively identify them into different MC-producing and MC-degrading genera (Chen et al., 2010; Jungblut and Neilan, 2006; Rantala et al., 2003). The primer pairs used for the *mcy*E gene were HEPF and HEPR (Jungblut and Neilan, 2006), due to their wide application on the different MC-producing genera; for the *mlr*A gene, the primer pairs were MF and MR (Saito et al., 2003), which are the most widely used. The quantity and quality of the resulting PCR products were verified using the Epoch spectrophotometer and a 1.5% agarose gel. Equal concentrations of each PCR product were pooled, and the resulting amplicon pool was pair-end sequenced (2 × 250 bp) on an Illumina MiSeq platform (Foundation for the Promotion of Health and Biomedical Research, FISABIO, Valencia, Spain).

Denoising, filtration of low-quality reads and the removal of chimaeras were performed using QIIME software v.1.8.0. (Caporaso et al., 2010), following the 16S Profiling Data Analysis Pipeline from the Brazilian Microbiome Project (Pylro et al., 2014). The sequences were paired-end aligned using the Trimmomatic tool (Bolger et al., 2014). The sequencing reads were also quality filtered to minimize the effects of random sequencing errors. Singletons were discarded to avoid overestimations and clustering bias using UPARSE (Edgar, 2013). The reads were clustered into OTUs with 97% sequence similarity using the UPARSE algorithm. The OTU sequences of the 16S rRNA, *mcy*E and *mlr*A genes were aligned in-house with sequences obtained from the NCBI GenBank (Altschul et al., 1990: accession 05/07/2016) and used as seed sequences in the MUSCLE algorithm. The final alignment lengths were 460 bp for 16S rRNA, 470 bp for *mcy*E and 807 bp for *mlr*A. Using this method, a total of 2679 bacterial OTUs were obtained (2642 for 16S rRNA genes, 19 for *mcy*E genes and 18 for *mlr*A genes). The taxonomic assignations of OTUs from the 16S rRNA gene were performed with 97% similarity. A profile of OTU similarities from 90 to 99% for *mcy*E and *mlr*A genes was generated to study the match differences among samples. Finally, the taxonomic assignations were performed with 97% similarity for *mcy*E genes and 95% similarity for *mlr*A genes, since both values were the highest percentages that accounted for the highest number of different OTUs. To confirm that the gene sequence of each OTU correspond to the McyE and MlrA enzymes, an analysis of the amino acid composition was performed using the ExPASy Translate Tool (http://web.expasy.org/translate/). Then, a multiple alignment was performed with other McyE and MlrA sequences available in the NCBI GenBank using the MUSCLE algorithm from MEGA6 software (Tamura et al., 2013). OTUs that were unable to align and protein BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) did not match with any available McyE or MlrA enzymes from the GenBank were suspected of being different proteins and discarded from the analysis.

Sequences from the 16S rRNA gene that were assigned to non-

bacterial entities (Archaea and chloroplasts from Eukaryote) were removed (Logue et al., 2016). Since the analysis of the 16S rRNA gene is focused on the exploration of the specific MC-degrading bacterial community that lack *mlr* genes, OTUs represented by fewer than 15 sequences among all the water samples were discarded to avoid biased conclusions due to underrepresented bacteria from the ecosystem. Thus, the threshold considered OTUs with a minimum of 0.001% of representation in the total bacterial community and discarded 0.55% of the total sequences.

2.4. MCs and chl-a extraction

Duplicates of sestonic MCs and chl-*a* from fibreglass filters were double-extracted by sonication (P-Selecta Ultrasons, Barcelona, Spain) with 90% aqueous methanol and evaporated at 40 °C under vacuum in a multiple evaporator (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany). Chl-*a* was quantified before evaporation by spectrophotometric measurement at 665 nm (Shimadzu Multispec-1501, Kyoto, Japan) and the final extracts were stored at −20 °C for MC quantification. Dissolved MCs from 600 mL of filtered water was extracted by solid phase extraction using C18 cartridges (200 mg, 6 cc) (OASIS HLB, Waters, Milford, MA, USA) and equilibrated with 10% methanol. The elution of the MCs was performed in 90% methanol. Both sestonic and dissolved MCs extractions were filtered through 0.22-μm syringe filters (Acrodisc GHP, Pall Corporation, Port Washington, NY, USA) before analysis. Quantification of the MCs was performed on an HPLC system (Agilent series 1100, Agilent Technologies, Santa Clara, CA, USA) coupled to a time-of-flight (TOF) mass spectrometer (Agilent 6230 accurate mass TOF Agilent Technologies, Santa Clara, CA, USA) by plotting calibration curves from commercial MC-LR, MC-RR and MC-YR pure standards (Sigma-Aldrich, St. Louis, MO, USA). The chromatographic separation and the gradient profile of the mobile phase were described in Lezcano et al. (2016).

2.5. Statistical analysis

A Pearson correlation analysis was performed with STATGRAPHICS Centurion XV v.15.1.02 software (Statpoint Technologies, Inc. Warrenton, VA, USA) using the whole phylum, order and family datasets to study the relationship between the log-transformed total MC concentrations and the log-transformed relative abundances of each bacterial taxon. Significant positive or negative correlations were assumed when the calculated *p*-value was lower than 0.05. Moreover, a principal component analysis (PCA) was also performed using CANOCO5 v.5.04 software (Microcomputer Power, Ithaca, NY, USA) to explore the relationship between the log-transformed total MC concentrations and the log-transformed relative abundances of the whole bacterial phylum, order and family datasets over the period of study. Shannon-Wiener and Chao1 diversity and evenness indices were calculated as α-diversity estimators for the BCC for all sampling dates.

3. Results

3.1. MCs and chl-a concentrations

The chlorophyll *a* concentrations from water samples ranged from the 1.93 ± 0.53 μg L^{-1} detected in December to the 349.82 ± 54.78 μg L^{-1} detected in autumn (19 November), coincident with a *Microcystis* spp. bloom episode (Fig. 1). Sestonic MC concentrations (the sum of the most frequent variants: MC-LR, MC-RR and MC-YR) varied considerably from undetectable levels (detection limit: 0.2 μg L^{-1}) during the first semester of 2014 to 9857.90 ± 1051.36 μg L^{-1} on 19 November. The dissolved fraction of

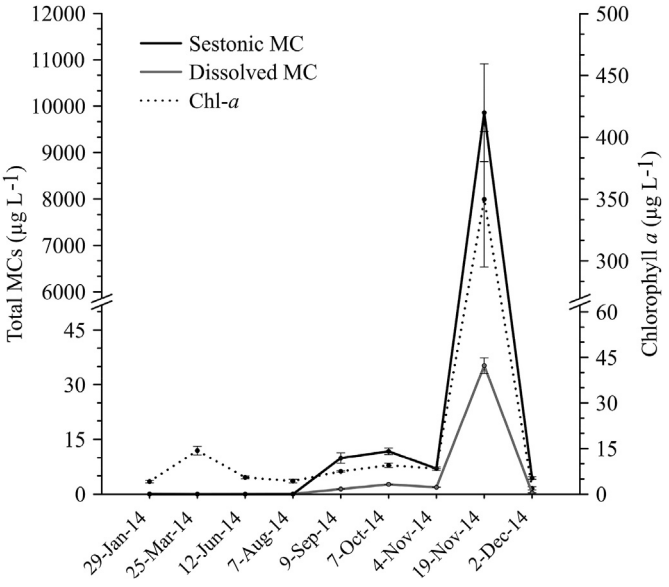

Fig. 1. Temporal changes of chl-*a* (dotted line) and both sestonic (black line) and dissolved MC (grey line) concentrations in the San Juan reservoir. The error bars for the sestonic MC and chl-*a* concentrations represents the standard errors of two replicates and from the dissolved MC concentrations represent the standard errors of two technical replicates.

the MCs was detected in all water samples, with concentrations below 1 μg L^{-1} in the first semester of 2014 and reaching 35.25 ± 2.14 μg L^{-1} at the cyanobacterial bloom peak (19 November). Both sestonic and dissolved MC concentrations decreased approximately 2230-fold and 90-fold, respectively, after the bloom collapsed (2 December), suggesting biological and/or physico-chemical processes for the decrease in the MC concentrations. The toxicity of the cyanobacterial bloom increased over time, as reported by the increasing MCs:Chl-*a* ratios (from 1.52 the 9 September to 28.51 the 19 November, Table S1 in supplementary material).

3.2. Diversity and seasonal dynamics of potentially MC-producing cyanobacteria and MC-degrading bacteria with mlr genes

To identify the cyanobacterial genera/species potentially producing the MCs, a high-throughput sequencing analysis of the *mcy*E gene was performed. A total of 602,703 sequences were recovered from the gene amplicons after quality filtering and the removal of artificial reads. The number of sequence reads varied between samples, ranging from 13,810 (25 March) to 109,102 (4 November) (Table 1), reflecting the common increase of cyanobacteria in summer and autumn seasons. A total of 19 different OTUs of the *mcy*E gene were identified in the water samples over the period of study; 7 of them belonged to the species *Microcystis aeruginosa*, 1 to *Microcystis viridis* and 11 to the genus *Microcystis* (Fig. 2). The analysis showed the dominance of two OTUs in all water samples, which were assigned to *Microcystis* sp., both accounting for more than 98.9% of the relative abundance on all sampling dates, with variations from 16 to 96% (OTU 1) and from 3 to 83% (OTU 2) over the period of study. The rest of the cyanobacterial OTUs were present in significantly less relative abundance (maximum of 0.4%). Some of the OTUs reached their maximum relative abundance during the cyanobacterial bloom episode (e.g., OTUs 11, 12, 14, 15, 18 and 19), while others thrived outside the period of the massive growth (the remainder of the OTUs). The Shannon-Weiner (H′) and the Chao1 indices showed variations in the diversity among

Table 1
Number of filtered sequences (S), number of OTUs, Shannon-Wiener index (H') and Chao1 index from the high-throughput sequencing data of the bacterial 16S rRNA, *mcy*E and *mlr*A genes from the water reservoir. The mark "n.d." means "no data" (absence of doubletons).

Date	16SrRNA gene				*mcy*E gene				*mlr*A gene			
	S	OTUs	H′	Chao1	S	OTUs	H′	Chao1	S	OTUs	H′	Chao1
29-Jan-14	149831	1006	5.07	1091	35783	17	0.76	18.00	3253	14	0.88	20.25
25-Mar-14	134377	715	4.41	805	13810	11	0.18	11.13	1393	12	1.09	14.25
12-Jun-14	178872	626	4.55	698	69145	16	0.65	16.00	182	4	0.68	n.d.
7-Aug-14	144443	803	4.37	920	60244	17	0.46	n.d.	2784	12	1.17	n.d.
9-Sep-14	166150	761	4.53	886	96185	17	0.62	19.00	936	12	1.02	13.50
7-Oct-14	160511	926	4.66	1042	100781	17	0.68	17.00	8127	13	0.87	15.00
4-Nov-14	199534	1075	4.97	1144	109102	19	0.74	20.00	5441	14	0.99	14.25
19-Nov-14	166009	1065	4.67	1123	85942	19	0.58	19.67	2766	12	0.91	20.00
2-Dec-14	68535	1023	5.14	1135	31712	16	0.51	16.17	1861	10	0.88	12.00

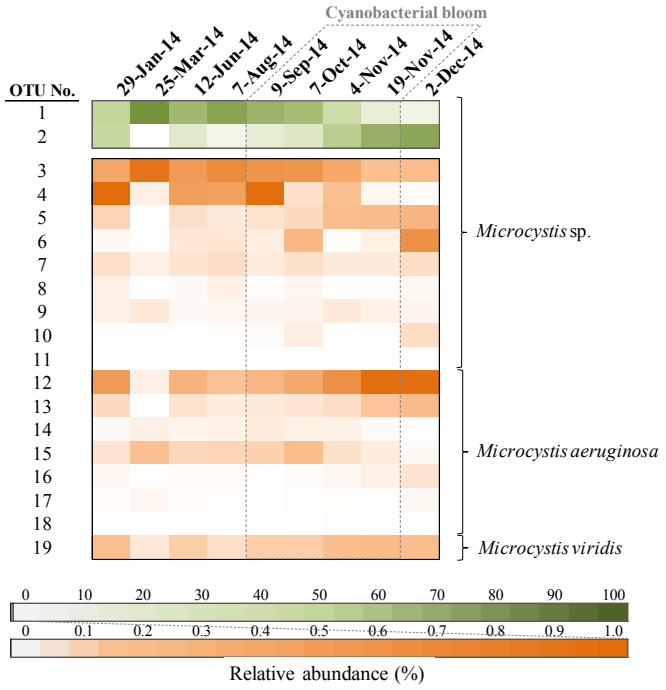

Fig. 2. Temporal shifts of potentially toxic cyanobacterial OTUs during the period of study. The colours indicate the relative abundance of each OTU over the total MC-producing cyanobacterial community at each sampling date. Two colour scales are displayed for an enhanced view of the higher and lower relative abundances. The taxonomic assignations were performed with gene similarities equal or above 97%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

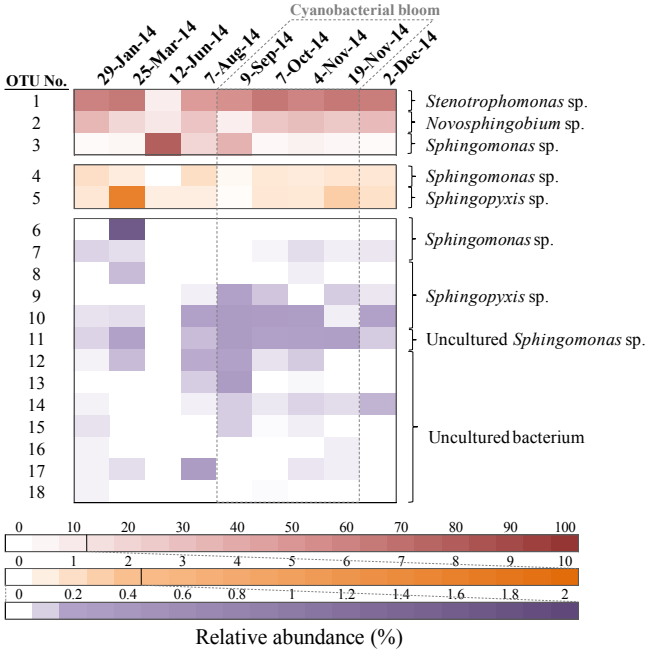

Fig. 3. Temporal shifts of potentially MC-degrading bacterial OTUs with *mlr*A genes during the period of study. The colours indicate the relative abundance of each OTU over the total MC-degrading bacterial community at each sampling date. The three scales are displayed for an enhanced view of the higher and lower relative abundances. The taxonomic assignations were performed with gene similarities equal or above 95%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sampling dates, 25 March being the less diverse (H′ = 0.18 and Chao1 = 11.13), and both 29 January (H′ = 0.76 and Chao1 = 18.00) and 4 November (H′ = 0.74 and Chao1 = 20.00) being the most diverse.

To identify the potentially MC-degrading bacteria encoding the already described enzymatic pathway for the degradation of MCs, the *mlr*A gene was analysed. This analysis showed a total of 26,743 sequences after quality filtering. The number of reads ranged from 182 (12 June) to 8127 (7 October) (Table 1), which may reflect the seasonal shifts of this bacterial community. A total of 18 OTUs from the genera *Stenotrophomonas* sp., *Novosphingobium* sp., *Sphingomonas* sp., *Sphingopyxis* sp. and uncultured bacteria were identified over the period of study (Fig. 3). The results showed that most of the OTUs (2–11) belonged to the family *Sphingomonadaceae*, followed by OTUs 12–18, which were assigned to uncultured bacterium, and OTU 1, which belonged to the family *Xanthomonadaceae*.

The three most abundant OTUs (1–3) accounted for more than 89% of the relative abundance in all water samples, followed by OTUs 4 and 5, which accounted for between 0.32 and 8.54%. Except for the four most abundant OTUs (1–4), which were present in all samples, the rest (5–18) showed a patchy temporal distribution over the period of study; some of them showed their maximum relative abundance during the cyanobacterial bloom episode (e.g., OTUs 1, 9–13, 15, 16) while others peaked outside the period of the massive growth (rest of OTUs). Regardless of the temporal evolution of the cyanobacterial bloom and the variations in the MC concentrations over the period of study, on most of the sampling dates, a similar number of OTUs (between 10 and 14) coexisted. 12 June was an exception, in that only the 4 most abundant OTUs were present (1–4). The Shannon-Weiner and the Chao1 indices confirm the observed slight variations in the diversity, considering samples before and after 12 June (H' = 0.98 ± 0.11 and Chao1 = 15.61 ± 3.22).

3.3. Shifts in the BCC over the period of study

A total of 1,368,262 sequences were recovered from the 16S rRNA gene amplicons from all water samples after quality filtering. The sequence reads was highly similar in all samples, from 134,377 to 199,534. The sample collected on 2 December was lower (68,535 sequences) but enough to cover the composition of the library (Table 1). A total of 1332 different bacterial OTUs, classified in 27 phyla, were observed among all samples (Fig. 4), and only 15 of them showed relative abundances higher than 0.25% in at least one sample. *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* dominated the sampling profiles with slight variations and accounted for more than 60% of the relative abundance in all water samples. Phyla *Verrucomicrobia* and *Cyanobacteria* were the next most abundant, with temporal shifts in their relative abundances ranging from 3.54 to 19.81% and from 0.31 to 17.87%, respectively. The 22 remaining phyla represented between 6 and 12% of the total BCC in all water samples.

3.4. Relationships between the BCC and the MC concentrations

To identify possible relationships between the BCC and the toxic cyanobacterial bloom, a Pearson correlation analysis (Table S2 in supplementary materials) and a set of PCA plots at the phylum, order and family levels (Fig. 5) were performed considering the temporal shifts of the MC concentrations and the relative abundances of each bacterial taxon.

All PCA plots showed a seasonal cyclic trend of the BCC, as shown with the clearly separated sampling dates at all the studied taxonomic levels. For example, winter and summer bacterial assemblages were well differentiated from each other since their sampling dates were opposed in all PCA plots. Despite of the cyclic trend of the BCC as a whole, some specific phyla such as *Actinobacteria*, *Gracilibacteria* and *Chloroflexi* barely showed seasonal variations, based on their central location in the PCA plot (Fig. 5A). Descending to lower taxonomic levels, a higher number of bacterial orders and families were found to be related to the maximum and subsequent decay of the cyanobacterial bloom (late autumn) compared to those present before (spring) or at the early stage (summer) of the massive cyanobacterial growth (Fig. 5B and C). The Shannon-Wiener and Chao1 diversity indices confirmed this increasing diversity during the cyanobacterial bloom episode, from its start on 9 September ($H' = 4.53$, Chao1 = 886) to its decay and the release of toxins on 2 December ($H' = 5.14$, Chao1 = 1135). The toxicity of the bloom appeared to be mainly caused by the cyanobacterial family *Microcystaceae*, as demonstrated a high and significant correlation with the MC concentrations ($r = 0.9581$, $p < 0.05$) and was consistent with the identification of the genus *Microcystis* in the analysis of the *mcy*E gene.

To determine the bacterial families (if absent, their orders (Or.) or classes (Cl.)) closely related with the temporal shifts of the toxic *Microcystis* bloom episode, a Pearson correlation analysis was performed. Fig. 6A represents the core bacteria with a statistically significant positive correlation with the toxin concentrations ($r > 0.5$; $p < 0.05$). This group is represented by 6 families (apart from *Microcystaceae*), 4 orders and 3 classes from 7 different phyla, among which, the relative abundance of 9 increased more than 1.5 times during the cyanobacterial bloom peak (asterisks in Fig. 6A). These bacteria that are highly responsive to the release of MCs are comprised in the phyla *Proteobacteria* (*Coxiellaceae*, *0319-6G20*, Or. *Ellin6067*, Or. *PHOS-HD29*, Or. *Spirobacillales*), *Bacteroidetes* (*Amoebophilaceae* and Cl. *SM1A07*), *Acidobacteria* (*RB40*) and *Gemmatimonadetes* (Or. *KD8-87*), which are potential candidates for the degradation of MCs during the bloom collapse and depict an important interplay with the toxic cyanobacterial bloom. In addition, the families previously described in other studies as MC degraders were also included in this analysis, so that their seasonal dynamics could be followed (Fig. 6B). These MC-degrading families were present in our water samples, with the exceptions of *Nocardiaceae*, *Brevibacteriaceae* and *Bifidobacteriaceae* (Table S3 in supplementary material). Within this group, the relative abundance of only *Methylophilaceae* increased more than 1.5 times at the cyanobacterial bloom peak. However, its higher occurrence in winter led to a low correlation with the MC concentrations ($r = 0.0089$, $p > 0.05$). In the same line, the rest of the families also showed absence of significant positive or negative correlation with the MCs concentrations ($r < 0.40$, $p > 0.05$).

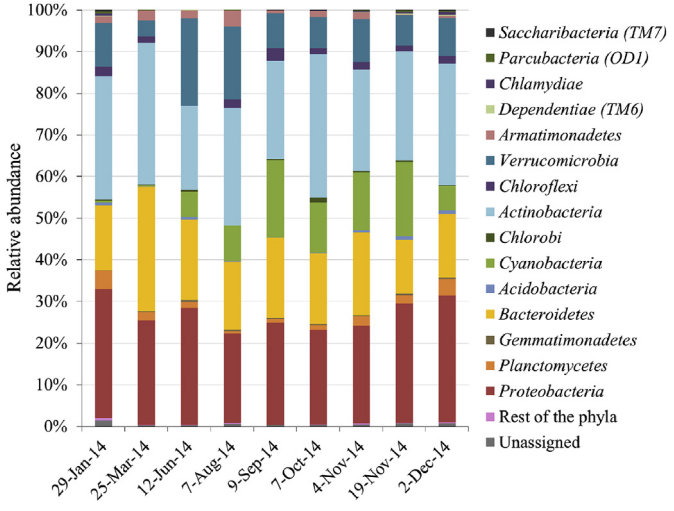

Fig. 4. Temporal shifts of the total bacterial community composition at the phylum level in terms of relative abundance. The phyla with relative abundances higher than 0.25% on at least in one sampling date are represented in the graph. The remaining phyla and the unassigned sequences are included the "rest of the phyla" and the "unassigned" groups, respectively.

4. Discussion

In the present study, extremely high sestonic and dissolved MC concentrations were recorded in the water reservoir due to a toxic cyanobacterial bloom dominated by *Microcystis* spp. that formed thick scum on 19 November 2014. Total MC concentrations (the sum of sestonic and dissolved MCs) from the start of the bloom until its decline were above the WHO guideline value of 1 μg L^{-1} for drinking water (WHO, 1998). The large decrease of the sestonic and dissolved MC concentrations after the bloom collapse (2230-fold and 90-fold, respectively) suggests the contribution of the aquatic natural bacterial community for the degradation of MCs, as reported in previous studies (Dziga et al., 2013; Ho et al., 2007; Lezcano et al., 2016). The lower MC photodegradation rates by the natural sunlight observed in previous studies (Welker and Steinberg, 2000; Wörmer et al., 2010) and the few variations in the water volume of the San Juan reservoir over the sampling period (Table S4 in supplementary material) supports this observation although by no means excludes other possible factors for the decrease in the MCs concentrations (grazing, sedimentation, etc.).

Considering the coexistence of MC-producing and MC-degrading activities in the water reservoir, a high-throughput sequencing analysis was performed to explore the composition and the seasonal dynamics of both bacterial communities over a year. A season-specific BCC was found at all the studied phylogenetic levels (phylum, order and family), according to the PCA analysis. This seasonality is consistent with other studies (Allgaier

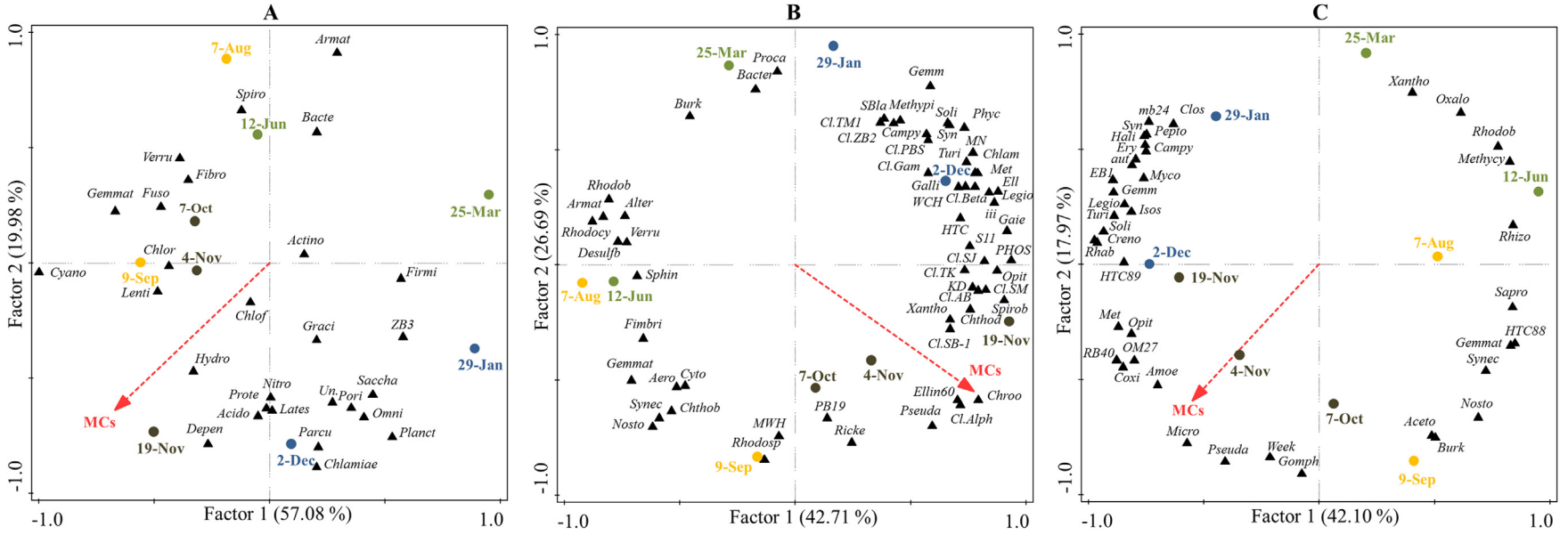

Fig. 5. PCA ordination triplots of data obtained from the water reservoir over a year. The data included the bacterial community composition (16S rRNA gene) at the phylum (A) order (B) and family (C) levels (triangles), the total MC concentrations (red vectors) and the sampling dates (coloured points indicating winter (blue), spring (green), summer (yellow) and autumn (brown)). Plot A shows all the bacterial phyla and, for an enhanced view, plots B and C only show the 60 orders and the 40 families (in absence, the upper taxonomic level) with the greatest relationships to the PCA factors. AB: ABY1; Aceto: Acetobacteraceae; Acido: Acidobacteria; Actino: Actinobacteria; Aero: Aeromonadales; Alph: Alphaproteobacteria; Alter: Alteromonadales; Amoe: Amoebophilaceae; Armat: Armatimonadetes/Armatimonadales; Aut: auto67-4W; Bacte: Bacteroidetes; Bacter: Bacteroidales; Beta: Betaproteobacteria; Burk: Burkholderiales/Burkholderiaceae; Campy: Campylobacterales; Chlam: Chlamydiales; Chlamiae: Chlamydiae; Chlof: Chloroflexi; Chlor: Chlorobi; Chroo: Chroococcales; Chthob: Chthoniobacterales; Clos: Clostridiaceae; Coxi: Coxiellaceae; Creno: Crenotrichaceae; Cyano: Cyanobacteria; Cyto: Cytophagales; Depen: Dependentiae; Desulfb: Desulfobacterales; EB1: EB1017; Ell: Ellin329; Ellin60: Ellin6067; Ery: Erysipelotrichaceae; Fibro: Fibrobacteres; Fimbri: Fimbriimonadales; Firmi: Firmicutes; Fuso: Fusobacteria; Gaie: Gaiellales; Galli: Gallionellales; Gemm: Gemmatales/Gemmataceae; Gemmat: Gemmatimonadetes/Gemmatimonadales/Gemmatimonadaceae; Gomph: Gomphosphaeriaceae; Graci: Gracilibacteria; Hali: Haliangiaceae; HTC: HTCC2188; HTC88: HTCC2188; HTC89: HTCC2089; Hydro: Hydrogenedentes; iii: iii1-15; Isos: Isosphaeraceae; KD: KD8-87; Lates: Latescibacteria; Lenti: Lentisphaerae; Legio: Legionellales/Legionellaceae; mb24: mb2424; Met: Methylococcales/Methylococcaceae; Methycy: Methylocystaceae; Methypi: Methylophilales; Micro: Microcystaceae; MN:MND1; MWH: MWH-UniP1; Myco: Mycobacteriaceae; Nitro: Nitrospirae; Nosto: Nostocales/Nostocaceae; Omni: Omnitrophica; Opit: Opitutales/Opitutaceae; Oxalo: Oxalobacteraceae; Parcu: Parcubacteria; PBS: PBS-25; Pepto: Peptostreptococcaceae; PHOS: PHOS-HD29; Phyc: Phycisphaerales; Planct: Planctomycetes; Pori: Poribacteria; Proca: Procabacteriales; Prote: Proteobacteria; Pseuda: Pseudanabaenales/Pseudanabaenaceae; Rhab: Rhabdochlamydiaceae; Rhizo: Rhizobiaceae; Rhodob: Rhodobacterales/Rhodobacteraceae; Rhodocy: Rhodocyclales; Rhodosp: Rhodospirillales; Ricke: Rickettsiales; S11: S1198; Saccha: Saccharibacteria; Sapro: Saprospiraceae; SBla: SBla14; SJ: SJA-4; SM: SM2F11; Soli: Solibacterales/Solibacteraceae; Sphin: Sphingomonadales; Spiro: Spirochaetes; Spirob: Spirobacillales; Syn: Syntrophobacterales/Syntrophaceae; Synec: Synechococcales/Synechococcaceae; TK: TK17; TM1: TM7-1; Turi: Turicibacterales; Turicibacteraceae; Un.: Undetermined; Verru: Verrucomicrobia/Verrucomicrobiales; WCH: WCHB1-50; Week: Weeksellaceae; Xantho: Xanthomonadales/Xanthomonadaceae. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

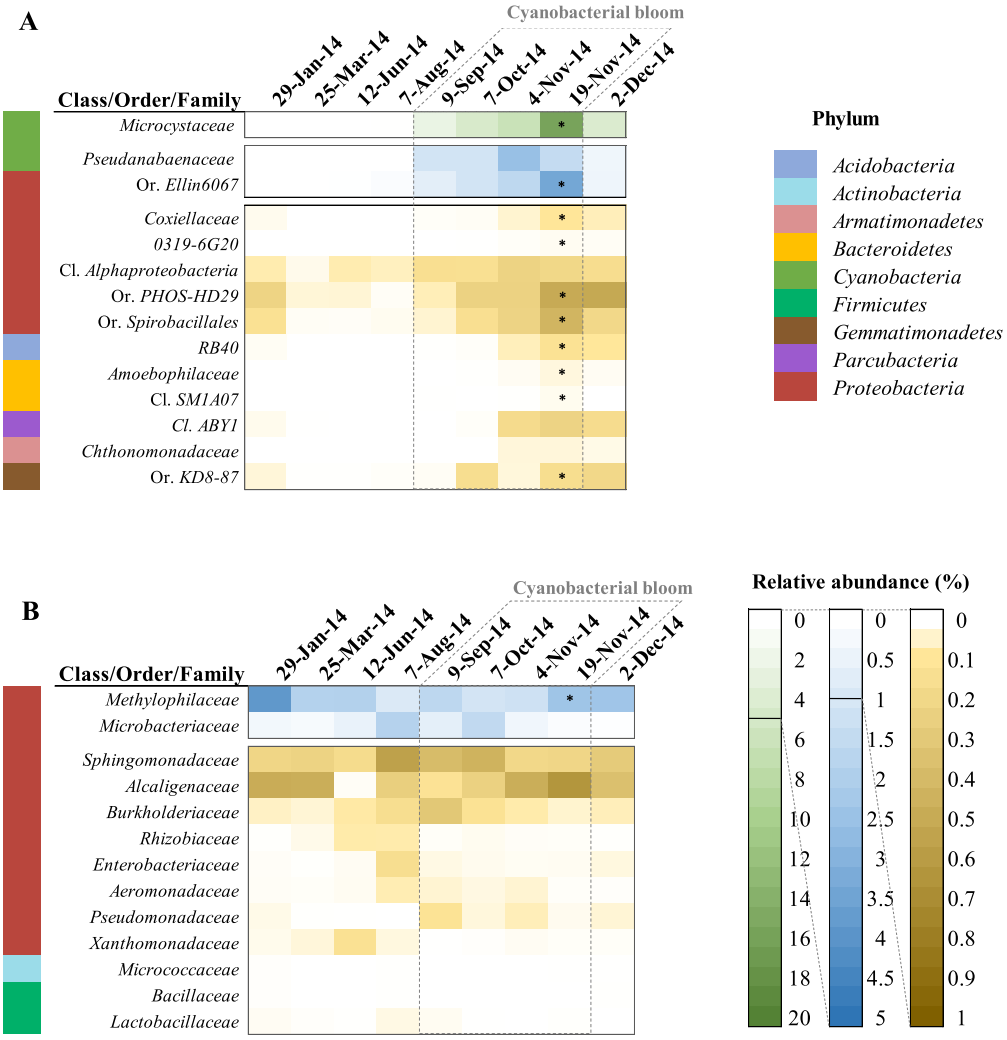

Fig. 6. Temporal shifts of the bacterial families associated with the toxic cyanobacterial bloom during the period of study. In the absence of families, the upper taxonomic level is represented ("Or." indicate "Order" and "Cl." indicate "Class"). Plot A represents the temporal shifts of the bacteria with a positive ($r > 0.5$) and significant correlation (p-value < 0.05) with the MC concentrations, and plot B represents the temporal shifts of the bacterial taxa known from other studies as MC degraders. For an enhanced view of the higher and lower relative abundances of each taxon, three different colour scales (green, blue, brown) are displayed. Asterisks indicate the taxa whose relative abundance increased more than 1.5-fold at the maximum MCs concentrations. The related phyla for each taxon are indicated in colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and Grossart, 2006; Dai et al., 2015) and supports the existence of variable temporal ecological niches (Jaspers et al., 2001), indicating certain environmental factors (e.g., phytoplankton succession, grazing, virus lysis, temperature) may trigger the BCC shifts. The occurrence of the toxic cyanobacterial bloom in the late summer and autumn seasons likely offered new niche opportunities and may result in the observed BCC changes. The higher bacterial diversity at the order and family levels during the development and decay of the toxic cyanobacterial bloom, compared to samples collected before or during the early state of the massive cyanobacterial growth, supports this idea. Moreover, previous studies on phytoplankton have shown that the overgrowth and the subsequent decay cause changes in the dissolved organic matter (DOM) concentrations (Søndergaard et al., 2000) and, thus, induce shifts in the BCC (Dinasquet et al., 2013; Li et al., 2012; Shao et al., 2014), favouring growth of those bacterial taxa able to degrade not only the amount, but also the quality of the dissolved organic matter (low- or high-molecular-weight carbon) (Logue et al., 2016). Therefore, the breakdown of the toxic cyanobacterial bloom described here may lead to a release of labile and commonly biodegradable molecules from the cell as well as complex compounds, such as toxins with high molecular weights (>600 Da), that cannot be readily taken up by microorganisms across the membrane (Weiss et al., 1991) and require specialized bacterial communities that used specific enzymatic cleavages (Logue et al., 2016). Thus, the maximum diversity observed during and after the toxic cyanobacterial bloom episode is most likely the answer of a specialized bacterial community to the increase of complex organic matter in the ecosystem. Among these bacteria, those with a role on MC degradation may represent an important fraction, considering the fast decrease in MC concentrations after the bloom peak. Previous studies also reported shifts in the BCC in response to the addition of MCs in a microcosm experiment (Mou et al., 2013) and as consequence of the historical presence of MC in the reservoir (Giaramida et al., 2013), supporting the idea that despite the multiple factors governing the *in situ* environmental samples, the MCs released during the cyanobacterial bloom constitute an important factor driving BCC changes.

The analysis of the *mcy*E gene disclosed a diverse potentially MC-producing cyanobacterial community. The coexistence of these

different *mcy*E genotypes, their different seasonal dynamics and the increase of bloom toxicity over time reveal the existence of *Microcystis* subpopulations exhibiting *mcy* gene heterogeneity (genotypes) and producing different MC variants and content (chemotypes). This genotype and chemotype diversity has been reported previously for other *mcy* genes (Kurmayer et al., 2002; Milkalsen et al., 2003) and in different cyanobacterial genera (Rohrlack et al., 2008; Welker et al., 2007), suggesting dissimilar ecological traits and different responses to the environmental conditions (Agha et al., 2014).

The identical analysis performed on the potentially MC-degrading bacterial community possessing the *mlr* gene cluster also revealed an *mlr*A gene heterogeneity (genotypes), especially within the genera *Sphingomonas* and *Sphingopyxis*. The coexistence, the patchy temporal distribution and the variations in the relative abundance of the observed *mlr*A genotypes in the period of study revealed the formation of subpopulations that respond differently to the environmental conditions. This ubiquitous temporal distribution point toward the existence of other environmental factors, apart from the concentrations of MCs, that determine their presence and provide evidence for other relevant roles alternatives to the degradation of MCs that they may play in the ecosystem. The recognized ability of the families *Sphingomonadaceae* and *Xanthomonadaceae* to degrade other complex organic compounds, including xenobiotics (Bosso and Cristinzio, 2014; Leng et al., 2016), supports this idea and suggests that only a fraction of these families were the responsible for the MC degradation after the bloom decay. Similar conclusions were also pointed by Kormas and Lymperopoulou (2013), which observed that the majority of the taxa with MC-degradation ability possess other multiple biodegradation traits.

In addition to the two families *Sphingomonadaceae* and *Xanthomonadaceae*, that represented the fraction of the MC-degrading bacteria using specific Mlr enzymes, higher diversity of other bacterial taxa identified from the 16S rRNA gene analysis responded markedly to the toxic cyanobacterial bloom. So far, many bacteria have been reported to have different types of interaction with cyanobacteria, such as parasitism (Kim et al., 2008; Rashidan and Bird, 2001), commensalism (Briand et al., 2016; Li et al., 2011) and mutualism (Ramanan et al., 2016). Here, different relationships were observed within the bacterial community associated with the toxic cyanobacterial bloom. The candidate phylum *Parcubacteria* has been described as an ectosymbiont or parasite of other microorganisms to obtain nutrients and energy sources (Nelson and Stegen, 2015), which may explain their presence within the associated bacterial community. The order *Legionellales*, a potential pathogen represented in this study by the family *Coxiellaceae*, has been previously related to toxic cyanobacterial blooms (Li et al., 2011) and highlights massive cyanobacterial growths as reservoirs for opportunistic pathogens and waterborne diseases (Mediannikov et al., 2010; Tison et al., 1980). Moreover, cyanobacteria provides protection to other bacteria. The cyanobacterial family *Pseudanabaenaceae*, highly represented in this study, is one example of bacteria that is usually associated with the *Microcystis* mucilage (Vasconcelos and Pereira, 2001), which benefits from the protection it offers.

Apart from the above-mentioned relationships, the release of the high amount of cyanobacterial exudates to the environment during the cyanobacterial growth and lysis may offer nourishment and encourage the association of bacterial scavengers in the phycosphere, which plays a key role in the turnover of the organic matter. The physical and chemical stability of the MCs (Tsuji et al., 1995, 1994) and the need of specialized enzymes for their biodegradation would explain the presence of *mlr*$^+$ bacteria during the toxic cyanobacterial overgrowth. However, the greater relationship of the toxic cyanobacterial bloom to the *mlr*-lacking bacteria compared with that of the bacterial families with *mlr* genes (*Sphingomonadaceae* and *Xanthomonadaceae*), suggests an important role played by the *mlr*-lacking bacteria for the degradation of MCs and/or other cyanobacterial exudates. Most of these bacteria, especially from the phyla *Proteobacteria* and *Bacteroidetes*, reported abilities to degrade complex organic compounds (Muangchinda et al., 2014). The uptake of nitrogen-rich peptides and amino acids have also been described as the top functions of their ABC membrane transporters (Penn et al., 2014), indicating that the peptides are important sources of organic carbon and supporting the possible intake and catabolism of MCs in both phyla. Furthermore, the order *Myxococcales* (family *0319-6G20* belongs to this order) is able to produce several degradative enzymes and to decompose a number of biomacromolecules (Reichenbach, 2015). The order *Ellin6067* has been reported to be ammonia-oxidizing bacteria (Xia et al., 2005), and the classes *Alphaproteobacteria* and *Betaproteobacteria* (the order *Spirobacillales* belongs to this class) have been described as degraders of complex organic compounds (Debroas et al., 2009). The class *TA18* (represented by the order *PHOS-DH29* in this study) has been found in oxygenic sediments enriched with genes associated with xenobiotic metabolism (Robinson et al., 2016). Moreover, the well-known capability of the order *Cytophagales* (represented by the family *Amoebophilaceae* in this study) to degrade large complex organic molecules (Van Hannen et al., 1999), the capacity of the family *Chthnomonadaceae* (phylum *Armatimonadetes*) to transport and use a wide range of carbohydrates (Lee et al., 2014) and the metabolically flexible members of the phylum *Acidobacteria* (represented by the family *RB40* in this study) suggest the contribution of these taxa to the successfully removal of the MCs. Consistent with these results were those found in some previous studies. In Berg et al. (2009), several cultivable cyanobacteria-associated bacteria were affiliated with taxa able to degrade persistent organic compounds, and in the study conducted by Mou et al. (2013), an overrepresentation of genes from xenobiotic metabolism and higher abundance of bacteria from orders other than *Sphingomonadales* were observed in a microcosm experiment amended with MCs (Mou et al., 2013). The higher correlation found in this study between *mlr*-lacking bacteria and the MC concentrations compared to those with *mlr* genes suggest an important role played by bacteria with the ability to degrade persistent organic compounds and absence of the *mlr* gene cluster on the degradation of MCs in the environment.

5. Conclusions

This study shows that toxic cyanobacterial blooms drives BCC changes and encourage the association of bacteria with dissimilar ecological traits and various biodegradation capacities. Beyond the fraction of the MC-degrading bacterial community with *mlr* genes, represented here by the families *Sphingomonadaceae* and *Xanthomonadaceae*, other bacteria with the ability to degrade xenobiotic and other complex organic compounds were closely related with the toxic cyanobacterial bloom. The effective removal of MCs from the environment during and after bloom collapse point toward, not only to the activity of the *mlr*$^+$ bacteria, but also suggest an important role played by other bacterial taxa lacking the *mlr* genes, as judged from their relative abundance, correlation with the MC concentrations and their seasonal dynamics.

Accession numbers: All the genetic data generated in this study have been submitted to the GenBank under the accession numbers SAMN06826931, SAMN06826932, SAMN06826933, SAMN06826934, SAMN06826935, SAMN06826936, SAMN06826937, SAMN06826938, SAMN06826939.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2017.08.025.

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