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Bacterioplankton Community Shifts during a Spring Bloom of *Aphanizomenon gracile* and *Sphaerospermopsis aphanizomenoides* at a Temperate Shallow Lake

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Abstract: Climate change is enhancing the frequency of cyanobacterial blooms not only during summer but also in spring and autumn, leading to increased ecological impacts. The bacterioplankton community composition (BCC), in particular, is deeply affected by these blooms, although at the same time BCC can also play important roles in blooms' dynamics. However, more information is still needed regarding BCC during species-specific cyanobacterial blooms. The goal of this study was to assess BCC succession in a hypereutrophic shallow lake (Vela Lake, Portugal) during a warm spring using a metagenomic approach to provide a glimpse of the changes these communities experience during the dominance of *Aphanizomenon*-like bloom-forming species. BCC shifts were studied using 16S rRNA gene metabarcoding and multivariate analyses. A total of 875 operational taxonomic units (OTUs) were retrieved from samples. In early spring, the dominant *taxa* belonged to Proteobacteria (mainly Alphaproteobacteria—Rickettsiales) and Bacteroidetes (Saprospirales, Flavobacteriales and Sphingobacteriales). However, at the end of May, a bloom co-dominated by cyanobacterial populations of *Aphanizomenon gracile*, *Sphaerospermopsis aphanizomenoides* and *Synechococcus* sp. developed and persisted until the end of spring. This led to a major BCC shift favouring the prevalence of Alphaproteobacteria (Rickettsiales and also Rhizobiales, Caulobacteriales and Rhodospirillales) and Bacteroidetes (Saprospirales, followed by Flavobacteriales and Sphingobacteriales). These results contribute to the knowledge of BCC dynamics during species-specific cyanobacterial blooms, showing that BCC is strongly affected (directly or indirectly) by *Aphanizomenon-Sphaerospermopsis* blooms.

Keywords: shallow lake; cyanobacterial blooms; *Aphanizomenon gracile*; *Sphaerospermopsis aphanizomenoides*; bacterioplankton community succession; 16S rRNA gene metabarcoding

1. Introduction

Climate change is enhancing the occurrence and persistence of cyanobacterial blooms across the globe, with impacts on aquatic ecosystems but also posing risks to human health. Harmful Cyanobacterial Blooms (HCBs) are caused by cyanobacteria that can produce toxins that can lead to dermatotoxic, hepatotoxic, neurotoxic or cytotoxic effects on water users/consumers [1–4]. Under the global warming scenario, there has been a special concern related to tropical invasive bloom-forming species such as *Raphidiopsis raciborskii* (formerly named *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju [5–8]), *Cuspidothrix issatschenkoi* (previously *Aphanizomenon issatschenkoi* (Usačev) Proshkina-Lavrenko [9]) and *Sphaerospermopsis aphanizomenoides* (also formerly named *Aphanizomenon aphanizomenoides* (Forti) Horecká & Komárek/*Anabaena aphanizomenoides*

Forti [6–8]), which have been spreading north to higher latitudes in Europe, America and Asia [10–14]. These invasive cyanobacteria have been detected in Portuguese water bodies, including *Raphidiopsis raciborskii* [15–18], *Cuspidothrix issatschenkoi* [19,20] and *Sphaerospermopsis aphanizomenoides* [19]. These species include potentially toxic strains that are able to produce cyanotoxins such as cylindrospermopsin, saxitoxins and anatoxin-a [21–24], thus increasing the diversity of potentially HCBs at higher latitudes and subsequently enhancing health risks for water users.

On the other hand, cyanobacterial blooms are being increasingly reported for extended periods not only for tropical but also for subtropical and temperate lakes [25–30], which makes the related potential health hazards from the exposure to HCBs no longer exclusive to the late spring/summer/early autumn seasons but rather occurring during most of the year. Therefore, research and monitoring should be intensified in early spring and late autumn in order to better understand the successional dynamics of blooms and the particular environmental requirements and triggering conditions for a given species (or strain) to bloom.

For each cyanobacterial species, growth conditions can vary greatly [31–34]. This physiological diversity can even occur at the strain level, with impacts on the cyanobacterial growth [19,35,36] but also on the synthesis of cyanotoxins [37,38]. Therefore, infra-specific conclusions retrieved from classical approaches per se have become limited considering the wider and strain-specific information provided by molecular methodologies and which is impossible to track under a microscope. Moreover, the morphological variation and the suggestion of a polyphyletic nature of Nostocales genera such as *Anabaena* and *Aphanizomenon* has been controversial [39–42], and only polyphasic approaches over the past two decades have contributed to put some consensual order onto their taxonomy [6,8,43–45]. However, this long-lasting taxonomic controversy and difficulties related to the correct morphological identification of *Anabaena*/*Aphanizomenon taxa* has led to many microscopic misidentifications, which inevitably have compromised a deeper assessment of biogeography and toxicity patterns of bloom-forming species. Nevertheless, through DNA-based approaches, new insights of biogeographic/phylogeographic patterns of harmful bloom-forming Nostocacean cyanobacteria were revealed [46–49].

High-throughput sequencing (HTS) analysis of the 16S rRNA gene (or 16S rRNA gene metabarcoding), in particular, have provided an in-depth overview of spatial and/or temporal variations in cyanobacterial communities either from different geographical origins [50–52] or from the same waterbody [53,54] as well as the assessment of the potential toxicity risks of a bloom [55]. Moreover, it became possible to track the eventual re-incidence of specific cyanobacterial populations in target water bodies directly from complex environmental samples, with evident major implications for subsequent modelling and water management design strategies. Additionally, this approach can also be useful to simultaneously study the cyanobacterial and heterotrophic bacterial communities from the same sample [56–58]. Bacterioplankton is essential for the biogeochemical cycling of nutrients and the ecological dynamics of waterbodies. The bacterioplankton community composition in HCBs plays an important dual role: (1) as an indicator of potential toxic risks, as it can be strongly affected by cyanobacterial blooms; (2) or as an active player on the bloom dynamics by promoting or inhibiting cyanobacterial growth [59,60] or even lysing bloom-forming cyanobacteria [61] and degrading cyanotoxins [62,63]. These interactions thus have implications for the toxicity potential and health risks of a bloom. However, the bacterial community succession during species-specific cyanobacterial blooms is still a research path that has not been extensively explored in order to hypothesize potential global dynamic patterns, particularly under a global climate change scenario. Therefore, more studies on the dynamics of bacterioplankton communities could bring more insights to this still unravelled topic, particularly under a species-specific scope regarding *Aphanizomenon*-like blooms.

Under a global warming scenario and increasingly extended warmer seasons, the present work aimed to study the bacterioplankton community dynamics during the development of cyanobacterial blooms through a warm and dry spring at a Portuguese temperate shallow lake (Vela Lake). The present study results will contribute to go further in the understanding of the relationships between cyanobacteria and heterotrophic bacteria, particularly during diazotrophic species-specific blooms.

2. Materials and Methods

2.1. Study Area, Sampling and Environmental Parameters

Vela Lake is a shallow eutrophic lake located in Central Western Portugal (Quiaios, Figueira da Foz), surrounded by forest, agriculture fields, livestock farms and urban areas. It has a depth range of 0.9–2.4 m and a flooding area of approximately 70 ha. Agricultural land, in particular, is a source of high amounts of fertilizers and pesticides that are lixiviated into the lake water with rainfall. The lake is mainly used for recreation and irrigation of crop fields. During spring 2006, a total of 12 water samples were collected from April to June, with a sampling interval from 2 to 10 days (Table 1), depending on the development stage of the bloom. Samples were taken sub-superficially at about 1 m from the shore (40°16'24.0" N 8°47'35.0" W) using 2L sterile bottles and were immediately placed at 4 °C in the dark until further treatment in the laboratory for DNA extraction and determination of environmental parameters. Water temperature, conductivity, pH and dissolved oxygen were determined in situ using portable water testing meters (WTW LF 330 conductivity meter, WTW 340-A pH meter and WTW OXI 320 oxygen meter). Preliminary sampling and analyses along several points of the lake showed this sampling site was representative of the BCC at Vela Lake during late spring. In the laboratory, the total suspended solids (TSS), chlorophyll *a* (Chl *a*), soluble reactive phosphorus (SRP), ammonium (N-NH$_4$) and nitrate (N-NO$_3$) concentrations were determined according to standard procedures [64].

Table 1. Environmental data recorded during the study period (April to June 2006) at Vela Lake (Portugal).

Sampling Date	Sample Code	Water Temperature (°C)	pH	O$_2$ (mg L^{-1})	O$_2$ Saturation (%)	TSS (mg L^{-1})	Chl *a* (μg L^{-1})	NO$_3{}^-$	NH$_4{}^+$ (mg L^{-1})	SRP	Conductivity (μS cm^{-1})
10 April	10AP	18.0	8.30	8.40	89.2	19.8	18.2	<0.1	<0.01	<0.01	560
20 April	20AP	18.9	8.45	10.16	110.6	23.7	21.1	<0.1	0.04	<0.01	575
24 April	24AP	20.1	8.33	11.04	120.9	19.5	21.3	<0.1	<0.01	<0.01	566
4 May	4MAY	20.2	8.02	8.35	92.6	14.1	15.4	<0.1	<0.01	<0.01	590
10 May	10MAY	20.6	8,65	9.39	104.8	13.7	10.3	<0.1	<0.01	<0.01	605
22 May	22MAY	20.4	8.81	8.81	97.6	26.0	12.9	<0.1	<0.01	0.01	630
28 May	28MAY	27.5	9.11	12.10	150.7	33.5	67.2	<0.1	<0.01	<0.01	618
1 June	1JN	23.8	9.30	11.06	130.1	39.8	47.5	<0.1	<0.01	<0.01	614
5 June	5JN	26.2	9.82	13.70	173.3	45.5	98.1	<0.1	<0.01	<0.01	605
9 June	9JN	25.9	8.54	8.80	108.3	38.7	60.4	<0.1	<0.01	<0.01	627
11 June	11JN	23.7	8.27	5.99	71.4	51.0	56.3	<0.1	<0.01	<0.01	641
25 June	25JN	22.1	8.49	7.90	79.1	42.0	73.2	<0.1	<0.01	<0.01	647

2.2. 16S rRNA Gene Metabarcoding

Total DNA was extracted as previously described [65]. Briefly, 100 mL water samples were filtered through 0.22 μm polycarbonate sterile filters; collected cells were resuspended in 2 mL of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0), centrifuged and resuspended in 200 μL of TE. Lysis was performed using lysozyme (1 mg mL^{-1}) over 1 h at 37 °C. DNA extraction and purification was performed using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) and DNA was resuspended in TE buffer and stored at −20 °C. Pyrosequencing libraries were obtained using the 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd., West Sussex, UK). Bacterial 16S rRNA gene fragments from the V3V4 hypervariable region were amplified using barcoded fusion primers with the Roche-454 Titanium sequencing adapters A and B (an eight-base barcode sequence), the forward primer 5'-ACTCCTACGGGAGGCAG-3' (338F) and the reverse primer

5′-TACNVRRGTHTCTAATYC-3′ (802R) [66]. PCR amplifications were carried out in 20 µL reactions with Advantage Taq (Clontech) using 0.2 M of each primer, 0.2 mM dNTPs, 1× of polymerase mix, 6% DMSO and 1–2 µL of template DNA. PCR conditions were 94 °C for 4 min, followed by 25 cycles of 94 °C for 30 s, 44 °C for 45 s and 68 °C for 60 s and a final elongation step at 68 °C for 10 min. Amplicons were quantified by fluorometry with PicoGreen (Invitrogen, Carlsbad, CA, USA), pooled at equimolar concentrations and sequenced in the A direction with GS 454 FLX Titanium chemistry, according to manufacturer's instructions (Roche, 454 Life Sciences, Brandford, CT, USA), at Biocant (Cantanhede, Portugal). Pyrosequencing sequence analysis was performed using previously described methods [67].

2.3. Statistical Analysis

A cluster analysis of samples, based on operational taxonomic units (OTUs) distribution, was performed using the unweighted pair group method with mathematical averages (UPGMA). The dendrogram was created using the Pearson correlation coefficient and the PAST software package [68]. Canonical correspondence analysis (CCA) [69] was performed to reveal relationships between environmental variables and the distribution of OTUs across the sampling period, using CANOCO 4.5 (Scientia Software) software. Environmental variables were standardized and OTUs abundance data were log transformed. Furthermore, only OTUs with a contribution of more than 1% (of total sequence reads) in at least one sample were considered. Forward selection was applied to choose the significant ($p \leq 0.05$) environmental parameters for the CCA using a Monte Carlo permutation test (499 unrestricted permutations).

3. Results

3.1. Environmental Parameters

The recorded environmental parameters and sample codes are summarised in Table 1. From early to late spring samples, it was possible to detect a general environmental gradient mainly characterized by water temperature, Chl *a*, TSS, pH and conductivity levels. The lowest values for water temperature (18.0 °C) and conductivity (560 mS cm^{-1}) were recorded on the 10th of April whereas the highest temperature (27.5 °C) and conductivity (647 µS cm^{-1}) were observed on the 28th of May and 25th of June, respectively. The highest Chl *a* (98.1 µg L^{-1}), pH (9.82) and oxygen (13.70 mg L^{-1} and 173.3%) levels were recorded on the 5th of June. The lowest values for pH (8.00) and oxygen (5.99 mg L^{-1} and 71.4%) were detected on the 4th of May and 11th of June, respectively. On the 10th of May, the lowest Chl *a* (10.3 µg L^{-1}) and TSS (51.0 mg L^{-1}) values were recorded whereas maximum concentration of TSS (51.0 mg L^{-1}) was recorded on the 11th of June. Nitrogen inorganic sources (nitrate and ammonium) were undetectable in all samples, except for the 20th of April where ammonium levels (0.04 mg L^{-1}) were slightly above the detection limit (0.01 mg L^{-1}). Soluble reactive phosphorus was only detectable on the 22nd of May (0.01 mg L^{-1}).

3.2. 16S rRNA Gene Metabarcoding

For the 16S rRNA gene metabarcoding analysis, a total of 25,705 sequences corresponding to 875 OTUs were obtained after quality checking. BLAST results for the most abundant OTUs (>3% of total bacterial sequence reads in at least one sample) are presented in Table 2. In general, for Vela Lake samples, a high phylogenetic diversity was recorded at phylum and class levels. By considering the main groups (Figure 1A), it was possible to observe that, from the 10th of April until the 22nd of May, the most abundant bacterial phylum was Proteobacteria (always >50% of total sequence reads), followed by Bacteroidetes (ranging between 18 and 28%). Actinobacteria members were also persistent across samples, although at lower abundances (between 4 and 14%). From the 28th May onwards, Cyanobacteria became progressively dominant, reaching an abundance peak

on the 5th of June (46%), although Proteobacteria remained co-dominant (ranging from 28 to 44%).

Table 2. Phylogenetic denomination, closest relative (after a BLAST search) and corresponding percentage similarity for the 16S rRNA gene partial sequences (408–425 bp) of the most abundant OTUs (above 2% of total reads in at least one sample). Phylogenetic affiliation determined using RDP database is also presented.

OTU	Max Reads in Samples (%)	Sample Code with Max Reads	Closest Relatives (NCBI Accession N)	Origin	Percentage Similarity (%)	RDP Phylogenetic Affiliation
2	34	5JN	*Anabaena* sp. 1LT27S03 (FM177478) *Aphanizomenon* gracile UADFA16 (FJ895128)	Lake Trasimeno, Italy Vela Lake, Portugal	100 99	Cyanobacteria (Nostocaceae)
3	22	25JN	*Aphanizomenon aphanizomenoides* UADFA13(FJ895123), UADFA8 (FJ895122), UADFA3 (FJ895118), UADFA5 (FJ895119), UADFA6 (FJ895120), UADFA7 (FJ895121)	Vela Lake, Portugal	100	Cyanobacteria (Nostocaceae)
1	21	24AP	Alpha proteobacterium SCGC AAA208-M13 (JF488156) Candidatus *Pelagibacter* sp. (JN941972)	Damariscotta Lake, USA Nanhu Lake, China	100 99	Alphaproteobacteria (Pelagibacteraceae)
264	12	22MAY	*Synechococcus* sp. BE0807I (FJ763789), BE0807L (FJ763778), BE0807G (FJ763772), BE0807F (FJ763770)	Eutrophic freshwater, Mazurian Lakes, Poland	100	Cyanobacteria (Synechococcaceae)
6	11	25JN	Uncultured Cyanobacterium sp. clone G4_EH_Jul_2008 (HQ707098) *Synechococcus* sp. Suigetsu-CG2 (AB610891)	Erhai Lake, China Saline Suigetsu Lake, Japan	100 99	Cyanobacteria (Synechococcaceae)
5	9	1JN	Uncultured bacterium clone W-Pla-6 (JX279920)	Wastewater	100	OD1 (Mb-NB09)
4	7	22 and 28MAY	Uncultured bacterium clone DCBP.0912.48 (HQ904840)	Dianchi Lake, China	100	Bacteroidetes (Chitinophagaceae, [Saprospirales])
18	6	10AP	Uncultured Bacteroidetes bacterium clone MEsu06b11A1 (FJ828430)	Mendota Lake, USA	99	Bacteroidetes (Sphingobacteriales)
22	6	25JN	Uncultured bacterium clone FL_03_181 (KC666538)	Villerest Lake, France	100	Bacteroidetes (Sphingobacteriales)
21	6	24AP	Uncultured bacterium clone Filia_2_B12 (HE857172) *Cryptomonas curvata* plastid CCAC 0006 (AM709636)	Lake Filia, Pyrenees, Spain Cornwall, England	100 99	Chloroplast (Cryptophyta)
15	5	4MAY	Uncultured Bacteroidetes bacterium clone BF 021 (KC994701)	Microalgae photobioreactor	99	Bacteroidetes (Chitinophagaceae, [Saprospirales])
14	5	20AP	Uncultured bacterium clone FL_04_148 (KC666711)	Villerest Lake, France	100	Chlorobi (OPB56)
12	5	10AP	Uncultured bacterium clone TH_b88 (EU273054)	Taihu Lake, China	99	Bacteroidetes (Saprospiraceae, [Saprospirales])

Table 2. *Cont.*

| OTU ǀ Max Reads in Samples (%) ǀ Sample Code with Max Reads	Closest Relatives (NCBI Accession N)	Origin	Percentage Similarity (%)	RDP Phylogenetic Affiliation
204 ǀ 5 ǀ 20AP	Uncultured bacterium clone AK1AB1_02E (GQ396807)	Soil	94	Actinobacteria (ACK-M1, Actinomycetales)
13 ǀ 4 ǀ 22MAY	Uncultured bacterium clone DCBP.0912.132 (HQ904919)	Dianchi Lake, China	100	Gammaproteobacteria (Sinobacteraceae, Xanthomonadales)
448 ǀ 4 ǀ 28MAY	Uncultured Bacteroidetes bacterium clone Aug-PC332 (JQ795402)	freshwater shallow lake, China	99	Bacteroidetes (Chitinophagaceae, [Saprospirales])
607 ǀ 4 ǀ 10MAY	Uncultured bacterium clone TSL-50-WB15 (FJ948205)	Tanggula South Lake, Tibet	99	Actinobacteria (Actinomycetales)
38 ǀ 4 ǀ 28MAY	Uncultured bacterium clone AB_03_107 (KC666374)	Villerest Lake, France	100	Alphaproteobacteria
10 ǀ 4 ǀ 10AP	Uncultured bacterium clone DCPA.0912.124 (HQ905382)	Dianchi Lake, China	99	Alphaproteobacteria (Rhizobiales)
35 ǀ 4 ǀ 5JN	Uncultured bacterium clone FFCH12672 (EU134640)	Soil	91	Firmicutes (Paenibacillaceae, Bacillales)
34 ǀ 4 ǀ 28MAY and 1JN	Uncultured Bacteroidetes bacterium clone DE1A6 (FJ916257)	Lake Delton, Wisconsin, USA	98	Bacteroidetes (Saprospiraceae, [Saprospirales])
331 ǀ 4 ǀ 9JN	Uncultured actinomycete clone LJ-29 16S (JX242773)	Salty Beach, China	95	Actinobacteria (C111, Acidimicrobiales)
33 ǀ 4 ǀ 24AP	Uncultured bacterium clone CB27 (KC253298) *Chroomonas caudata* chloroplast (AB591422)	Freshwater pond, China Dinoflagellate symbiont	99 98	Chloroplast (Cryptophyta)
11 ǀ 4 ǀ 28MAY and 1JN	Uncultured bacterium clone XH-4-C-58 (JX075510)	Lake Xihu, China	100	Alphaproteobacteria (Acetobacteraceae, Rhodospirillales)
26 ǀ 3 ǀ 24AP	Uncultured bacterium clone DP10.5.11 (FJ612433) Cryptophyta sp. CR-MAL01 (EU123323)	Dongping Lake, China Gomso Bay, Korea	100 99	Chloroplast (Cryptophyta)
32 ǀ 3 ǀ 10AP	Uncultured bacterium clone dcpa4-43 (HM050711)	Dianchi Lake, China	99	Bacteroidetes (Chitinophagaceae, [Saprospirales])
31 ǀ 3 ǀ 24AP	Uncultured bacterium clone FL_04_46 (KC666611)	Villerest Lake, France	100	Betaproteobacteria (Comamonadaceae, Burkholderiales)
39 ǀ 3 ǀ 20AP	Uncultured bacterium clone 1024 (KC298790)	Lake Taihu, China	99	Bacteroidetes (Sphingobacteriales)
62 ǀ 3 ǀ 22MAY	Uncultured Bacteroidetes bacterium clone KWK12S.43 (JN656869)	Subartic thaw pond KWK12, Canada	100	Bacteroidetes (Chitinophagaceae, [Saprospirales])
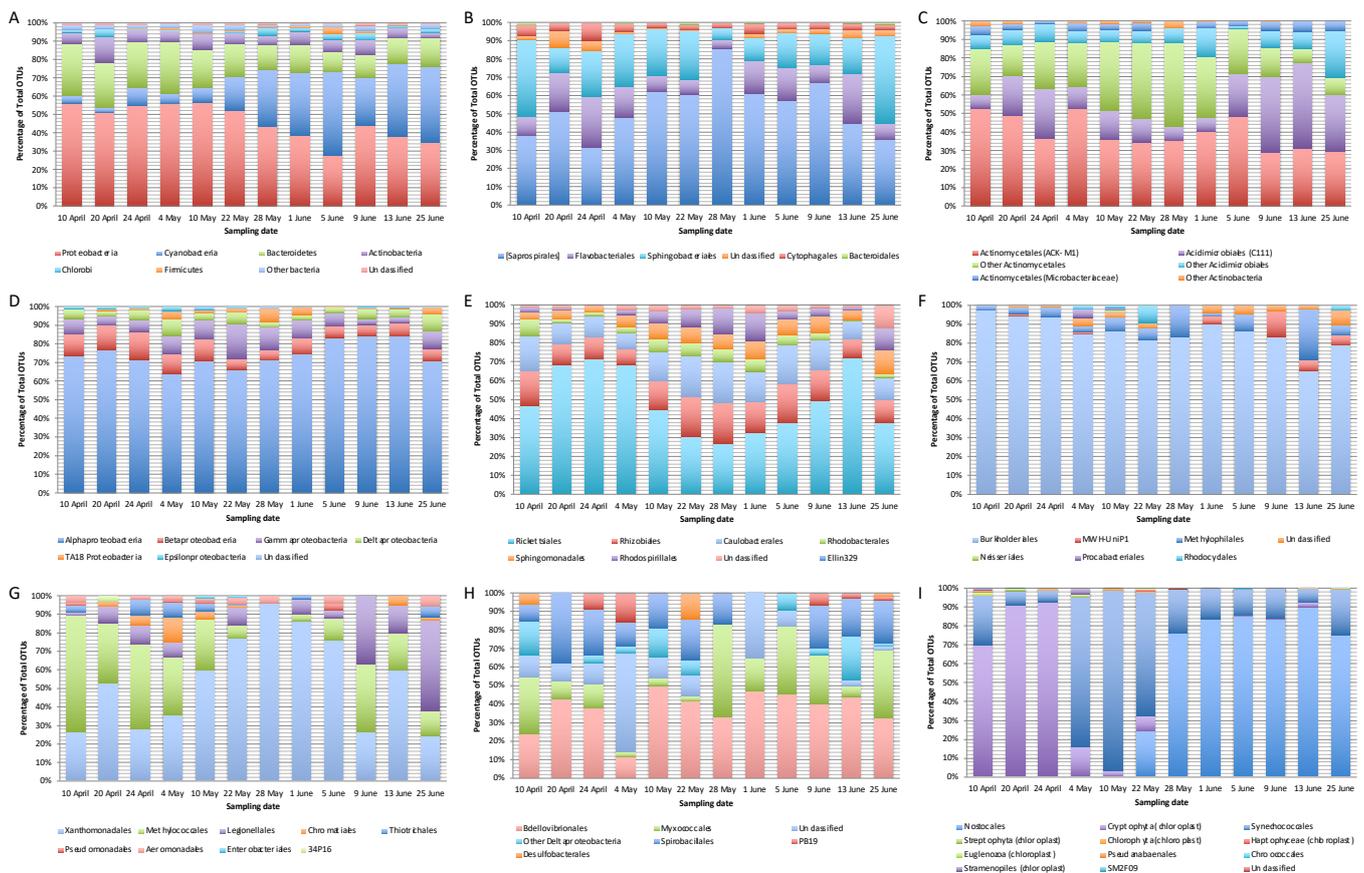
Figure 1. Relative abundance of bacterial OTUs of 12 samples from Vela Lake analysed using 16S rRNA gene metabarcoding: (**A**) total bacteria at the phylum level; (**B**) Bacteroidetes at the order level; (**C**) Actinobacteria at the order/family level; (**D**) Proteobacteria at the class level; (**E**) Alphaproteobacteria at the order level; (**F**) Betaproteobacteria at the order level; (**G**) Gammaproteobacteria at the order level; (**H**) Deltaproteobacteria at the order level; (**I**) Cyanobacteria at the order or similar level; Ribosomal Database Project (RDP) was used for the classification of OTUs. Please view the web version of this article for a better interpretation of the colour gradients.

The major bacterial phylum found in Vela Lake samples was Proteobacteria and it was clearly dominated by Alphaproteobacteria (from 64 to 84% of total sequence reads within the phylum) during the study period (Figure 1D). Alphaproteobacteria OTUs (Figure 1E) mostly belonged to the Rickettsiales order (between 26 and 72% of total reads from the class) and the most dominant and persistent OTU affiliated with Pelagibacteraceae, reaching 21% of total bacterial sequence reads on the 24th of April (Table 2). Between the 10th of May and 1st of June, the relative abundance of Rickettsiales declined and the relative abundance of the orders Caulobacterales (9 to 22% of total class reads) and Rhizobiales (8 to 22%), and Rhodospirales (up to 14%) increased. As seen in Table 2, the dominant Rhizobiales reached 4% of total bacterial sequence reads on the 10th of April, whereas Rhodospirales and the other Alphaproteobacteria OTU reached maximum values of 4 and 3%, respectively, between the 28th of May and 1st of June. The Sphingomonadales order was persistent across samples (with relative abundances varying between 4 and 12% of total Alphaproteobacteria sequence reads and attaining maxima values between the 10th of May and 25th of June). Betaproteobacteria varied from 6 to 14%, Gammaproteobacteria from 2 to 18%, and Deltaproteobacteria from 4 to 10% (Figure 1D). The Burkholderiales order almost completely dominated the betaproteobacterial class, and varied between 66 and 98% of total class sequence reads (Figure 1F). Gammaproteobacteria composition (Figure 1G) oscillated, with the Xanthomonadales order clearly dominating from the 10th

of May until the 5th of June (ranging from 60 to 96% of the class sequence reads) and a dominant Sinobacteraceae (Xanthomonadales, Gammaproteobacteria) OTU reached a peak of 4% of total bacterial reads on the 22nd of May (Table 2). The Methylophilales order was most abundant from the 10th of April until the 10th of May (between 26 and 63% of total class reads) and there was an increase in the Legionellales abundance between the 9th and 25th of June (up to 48%), during the later phase of the bloom (Figure 1G). In all samples, Bacteroidetes were generally dominated by members of the Saprospirales order (from 32 to 86% of total sequence reads within the phylum), followed by Sphingobacteriales (from 8 to 46%) and Flavobacteriales (from 4 to 28%), as shown in Figure 1B. Chitinophagaceae were the most abundant Saprospirales members, with maxima on the 10th of April (3% of total bacterial reads), 4th of May (with 5%), and 22nd of May (3%) or 28th of May (with 7%), depending on the OTU (Table 2). Saprospiraceae (Saprospirales) OTUs exhibited pronounced oscillations in abundance throughout the study period, with peaks on the 10th of April (5%) and from the 28th of May until the 1st of June (4%), depending on the OTU. Different dominant OTUs assigned to the Sphingobacteriales order appeared with different peaks on the 10th of April (6%), 20th of April (3%) and 25th of June (6%).

For Cyanobacteria there was a clear temporal succession in *taxa* (Figure 1I). During early spring, cyanobacterial abundance was low (between 2 and 10% of total bacterial reads) (Figure 1A). From the 10th to 24th of April, cyanobacterial abundance was mostly dominated (from 70 and 93% of cyanobacterial sequence reads) with different sequences affiliating with chloroplasts from the microalga *Cryptomonas* (Cryptophyta) which attained a total maximum of 12% of total bacterial sequence reads on the 24th of April (Table 2). From the 4th until the 22nd of May, the Synechococcales order became dominant (varying between 67 and 96% of cyanobacterial reads) with a *Synechococcus* OTU attaining a maximum of 21% of total bacterial sequence reads on the 22nd of May (Table 2). From the 22nd of May onwards, Cyanobacteria abundance increased and coincided with the rise in the Nostocales, which dominated until the end of the study period (reaching 90% of cyanobacterial sequence reads on the 13th of June). The OTUs that achieved the highest abundance levels were assigned to the cyanobacterial species *Aphanizomenon gracile* (reaching 34% of total bacterial reads on the 5th of June) and *Sphaerospermospis aphanizomenoides* (formerly *Aphanizomenon aphanizomenoides* [7,8,36]) which reached 22% on the 25th of June) (Table 2). Interestingly, the *S. aphanizomenoides* OTU was similar to sequences retrieved from strains isolated from Vela Lake in 2004 and 2006, during the summer season [70]. On the 25th of June, when the *Aphanizomenon gracile* density began to decline (to 15% of total bacterial reads), another *Synechococcus* OTU increased its abundance reaching 11% of total bacterial sequence reads.

Actinobacteria were ubiquitous throughout samples and dominated by the orders Actinomycetales (with different dominant OTUs showing maxima in distinct samples: 4 or 5% of total bacterial reads on the 10th of May or 20th of April, respectively) and Acidimicrobiales (with an OTU showing a peak of 4% of total bacterial reads on the 9th of June).

Other dominant OTUs were assigned to the Candidate division OD1 (with a peak of 9% of total bacterial sequence reads on the 1st of June) and the phyla Chlorobi (present in all samples, with a peak of 5% on the 20th of April) and Firmicutes (also with a maximum relative abundance on the 5th of June at 4%).

3.3. Analysis of BCC vs. Environmental Conditions

Despite the variability of environmental parameters, cluster analysis of samples (Figure 2) according to OTUs distribution revealed two distinct temporal clusters: one from the 10th of April to the 22nd of May (cluster I—before the establishment of the bloom) and another from the 28th of May till the 25th of June (cluster II—during the bloom). The major shifts in BCC coincided with the establishment of the *Aphanizomenon*-like spp. bloom on the 28th of May. CCA (Figure 3) showed that the significant parameters relating to OTUs distribution were total suspended solids and conductivity levels, rather than

temperature (although a gradient could be recorded throughout the study period). The first two canonical axes explained 30.7% of the total variance. The first axis explained 19.3% of total variance and it was significantly related to conductivity (0.92) and TSS (0.86). Samples 11JN and 25JN (and OTUs 9, 45, 79, 100, 75 and 22, belonging to Sphingobacteriales, Flavobacteriales and Cytophagales (Bacteroidetes), as well as to Rhodobacterales and Sphingomonadales (Alphaproteobacteria)) defined the positive side of the axis whereas samples 10AP and 24AP (and a cluster of OTUs from mixed groups) defined its negative side. The second axis explained 11.4% of the total variation in OTU composition and it showed a lower correlation with conductivity and TSS (both with 0.44). Sample 5JN (and OTU 35, a Bacillales (Firmicutes)) defined its positive side and 10MAY and 22MAY (and a cluster of OTUs (e.g., 90, 7, 89) belonging to several groups) defined its negative side.
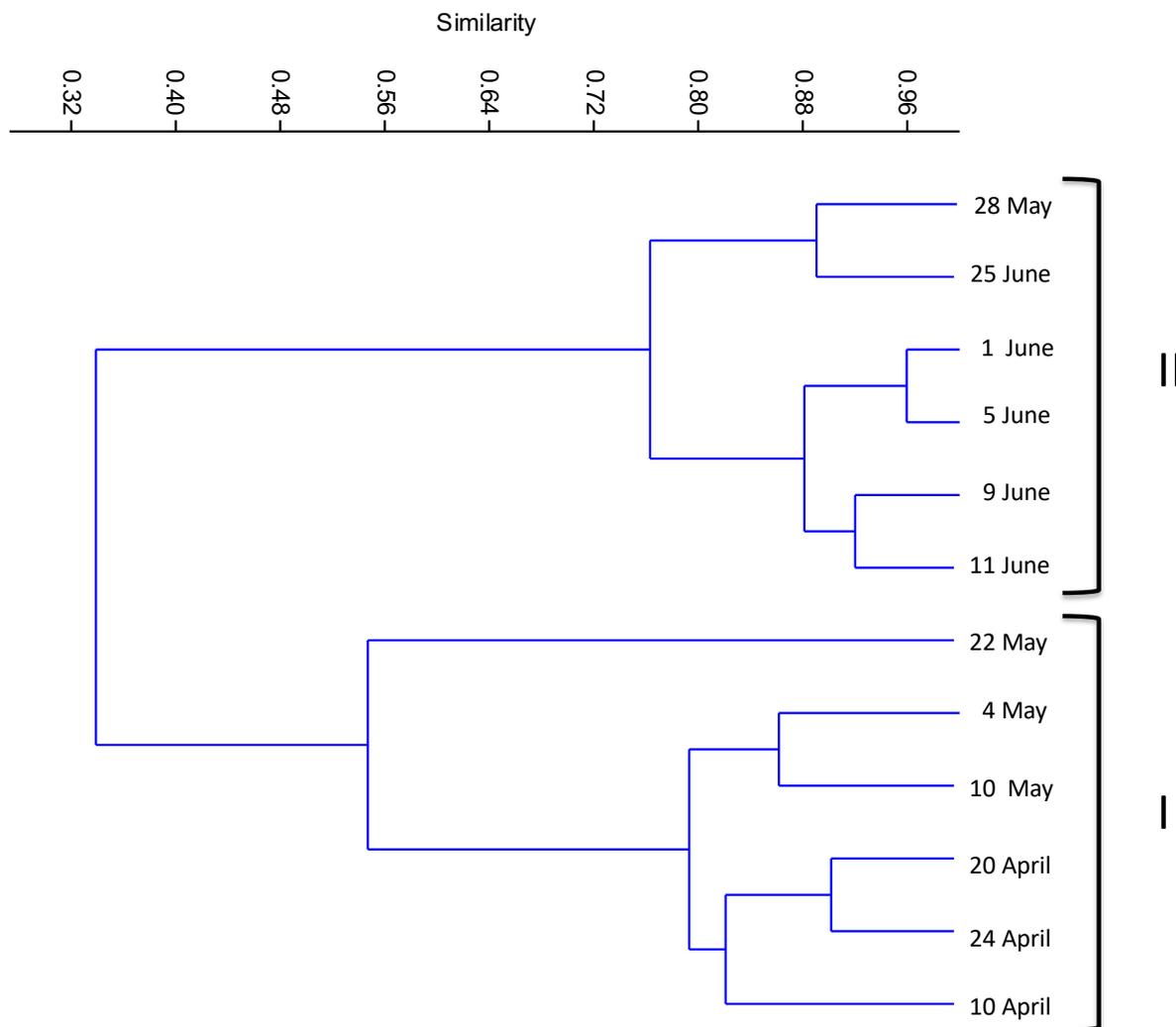

Figure 2. Dendrogram derived from hierarchical cluster analysis based on Pearson correlation between the 12 temporal samples, according to the distribution of OTUs (with a contribution of 1% or more in at least one sampling date).
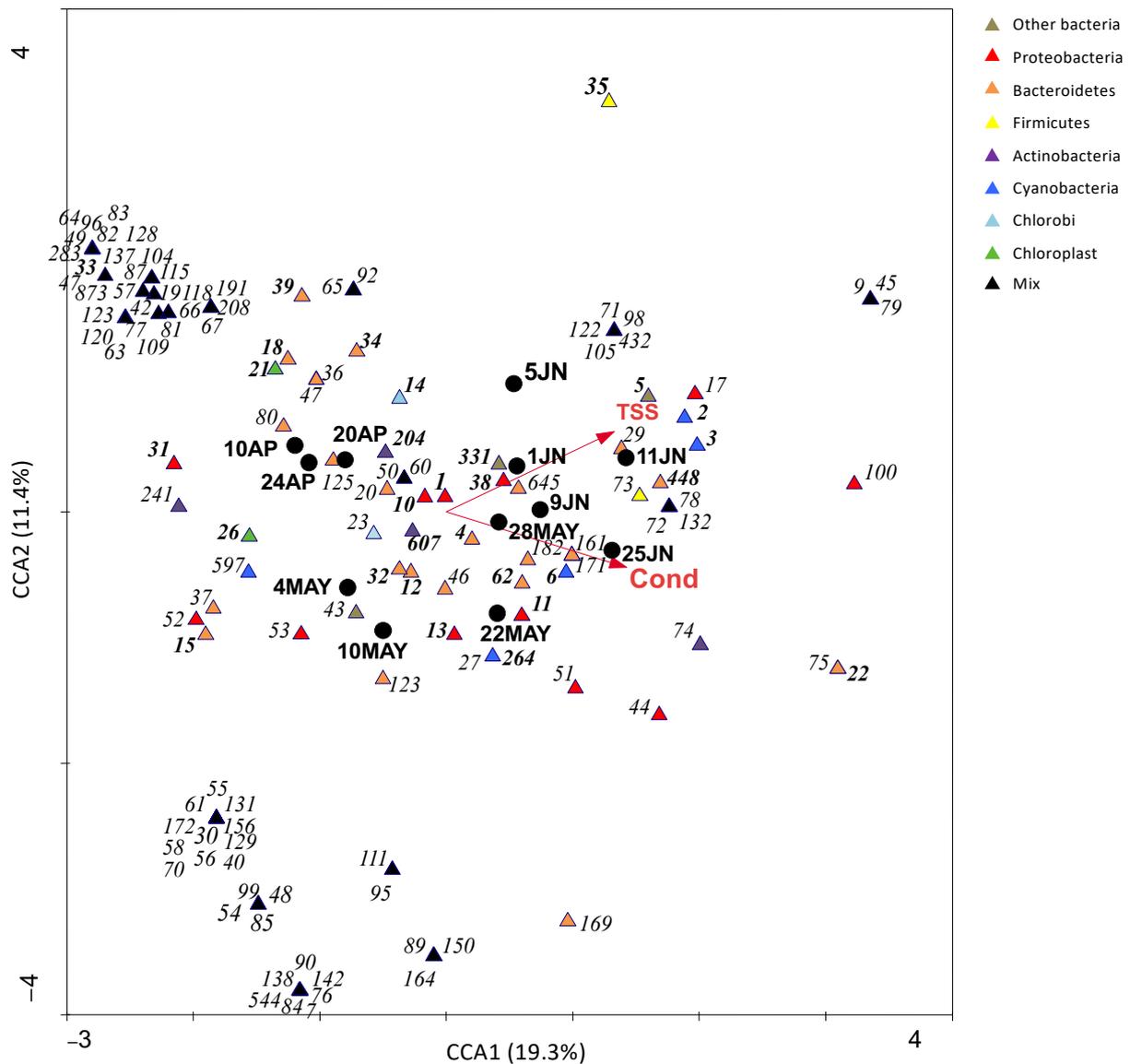
Figure 3. Canonical Correspondence Analysis (CCA) ordination triplot of OTUs (obtained from 16S rRNA gene metabarcoding) and only significant (*p* < 0.05) environmental parameters (TSS: total suspended solids; Cond: conductivity) recorded during the corresponding sampling dates. The circles represent the samples and the triangles represent the OTUs (different colours represent different bacterial groups—please view the coloured web version of this article). OTUs with a contribution of less than 1% in at least one sampling date were not considered for multivariate analysis. Dominant OTUs (corresponding to the ones described in Table 2) are highlighted in bold.

4. Discussion

The occurrence of cyanobacterial blooms has been reported in Portugal for the past 30 years with many references to cyanotoxin production [71–73]. Recurrent cyanobacterial blooms have been recorded at Vela Lake, with the dominance of taxa belonging to genera such as *Microcystis*, *Anabaena*, *Aphanizomenon*, *Cuspidothrix* or *Cylindrospermopsis* [18,19,26,70,74,75]. However, as for most Portuguese freshwater bodies, information on the environmental BCC at Vela Lake is very scarce [76,77].

Vela Lake is a shallow lake characterized as eutrophic decades ago [78], with a trend for increasing eutrophication [76,77]. In fact, conductivity levels recorded in the present study showed a two-fold increase in comparison to data from spring 2001 [26], although temperature, pH and nutrient values fell into or below the range previously reported. More

recent data, from 2012, reported a further intensification of eutrophication in the summer season (achieving a very high value for conductivity (802 µS cm^{-1})) and dense toxic blooms of *Microcystis* sp. with the production of microcystins (MC-LR) [79].

Over the past decades, spring seasons have become warmer in many regions of the world, enhancing the occurrence of cyanobacterial blooms earlier in the year [25,29]. For Vela Lake, the dominance of diazotrophic cyanobacteria (namely *Aphanizomenon* spp.) during late spring was also recorded in 2012 [80], supporting the recurrence of these earlier blooms. In the present study, the peak of the bloom coincided with higher values for temperature, pH, conductivity and TSS. The abundance of the *Aph. gracile* OTU was significantly correlated ($p < 0.05$) to temperature ($r = 0.65$), chlorophyll *a* ($r = 0.79$) and TSS ($r = 0.92$). The *S. aphanizomenoides* OTU also significantly correlated ($p < 0.05$) with chlorophyll *a* ($r = 0.64$) and TSS ($r = 0.81$), but also conductivity ($r = 0.74$), suggesting species-specific conditions to bloom. The *Synechococcus* OTU was only significantly related to conductivity ($r = 0.65$). In eutrophic shallow lakes, the occurrence of cyanobacterial blooms has been also associated with an increase in temperature, conductivity and pH levels, but also scarcity or undetectable nutrient levels, particularly from inorganic nitrogen sources when diazotrophic species are dominant [81–84], such as in the present case. Nevertheless, it should be kept in mind that the increase in conductivity, TSS and chlorophyll *a* levels are certainly a direct consequence of the bloom development.

16S rRNA gene metabarcoding results showed that dominant OTUs were generally assigned to Alphaproteobacteria, Cyanobacteria, Bacteroidetes and Actinobacteria groups, which are usually found dominating bacterial communities in eutrophic shallow lakes [65,77,85,86]. However, the results also revealed a high diversity and successional dynamics below phylum and class levels, which highlights the need to go deeper into the phylogenetic affiliation effort for ecological surveys. Dominant groups belonging to Proteobacteria (Alphaproteobacteria—Rickettsiales, Caulobacterales, Rhizobiales and Sphingomonadales; and Betaproteobacteria—Burkholderiales), Bacteroidetes (Saprospirales, Sphingobacteriales and Flavobacteriales) and Actinobacteria (Actinomycetales) remained abundant throughout the sampling period.

The most abundant bacterial group was Alphaproteobacteria, with the most abundant OTU assigned to "Candidatus *Pelagibacter*" (Pelagibacteraceae, Rickettsiales), and was recorded in all samples. However, its abundance declined during the bloom development—a negative correlation was observed with cyanobacterial OTUs from *Aph. gracile, S. aphanizomenoides* and *Synechococchus* sp. ($r = -0.61$ to -0.59, $p < 0.05$). Strains of *Pelagibacter* spp. are frequent in marine environments but can also dominate in freshwaters where cyanobacterial blooms occur, namely from *Microcystis* spp. [87] or *Planktothrix rubescens* [88]. Caulobacteraceae (Caulobacterales) bacteria have also been found in European lakes with blooms of Cyanobacteria [89], namely co-dominated by *Aphanizomenon* and *Cylindrospermopsis* spp. [90]. Sphingomonadaceae (Sphingomonadales) have been associated with cyanobacterial co-dominance of *Aphanizomenon/Anabaena* [89] and some strains have been shown to degrade cyanobacterial toxins, namely microcystins [63,91–93]. The microcystin-degrading potential has also been reported for Rhizobiales [94], a group well represented during the study period at Vela Lake, particularly during the bloom peak. In fact, *S. aphanizomenoides* was dominant in the bloom and strains of this species have proven to be able to produce microcystins [95].

Burkholderiales (Betaproteobacteria), particularly from the Comamonadaceae family, were less abundant but persistently represented across Vela Lake samples. This family has been related to eutrophic lakes [65,96,97] during blooms dominated by diazotrophic cyanobacteria including *Dolichospermum* spp. [89,96,97]. A *Polynucleobacter* (Oxalobacteraceae, Burkholderiales, Betaproteobacteria) OTU was also persistent throughout the sampling period, although studies reported a decrease in the relative abundances of *Polynucleobacter* OTUs just before blooms co-dominated by *Microcystis* and *Dolichospermum* [98]. Nevertheless, *Polynucleobacter* OTUs have been also recorded during blooms of *Microcystis*,

Anabaena, *Aphanizomenon* and *Dolichospermum* spp. [90,98] and some strains have been related to microcystin degradation [94].

Bacteroidetes (Saprospirales, Sphingobacteriales and Flavobacteriales orders) were recorded in all Vela Lake samples and there was a positive correlation with the *Aph. gracile* OTU ($r = 0.72$, $p < 0.05$). Saprospirales members have been recorded during *Microcystis* spp. blooms [99–101] and Sphingobacteriales have been related to high densities of *Synechococcus* sp. and *Anabaena flos-aquae* [102]. However, in the present study, some OTUs of Saprospirales (Chitinophagaceae) showed a negative correlation with *Aph. gracile*, *S. aphanizomenoides* and *Synechococcus* sp. ($r = -0.72$ to -0.60, $p < 0.05$) while another showed a positive relationship with *S. aphanizomenoides* ($r = 0.65$, $p < 0.05$). This highlights the need for a deeper taxonomic study to distinguish below the family and even genus level. The occurrence of Flavobacteriales (Bacteroidetes) in shallow lakes is also very frequent and has been linked to cell lysis and the declining phase of cyanobacterial blooms of *Microcystis* [87,100] but has also been associated with *Synechococcus* strains in culture [101]. In the present study, Flavobacteriales had high OTU diversity and succession, before and during the *Aphanizomenon*-like spp. bloom, suggesting differential sensitivity to environmental conditions and/or bloom-forming strains. However, no significant positive or negative correlations were recorded for this order while for Sphingobacteriales positive ($r = 0.58$ to 0.76, $p < 0.05$) and negative ($r = -0.67$ to -0.60, $p < 0.05$) correlations were found with the bloom-forming cyanobacteria *Aph. gracile*, *S. aphanizomenoides* and *Synechococcus* sp.

Actinobacteria, particularly from the Actinomycetales order, were also found persistently in samples although there was a clear succession of different OTUs during the study period and several OTUs had significantly negative correlations with *Aph. gracile*, *S. aphanizomenoides* and *Synechococcus* sp. ($r = -0.74$ to -0.66, $p < 0.05$). Actinomycetales are characteristic of shallow lakes and persist across seasons [65,76,86] but negative relationships have been reported with bloom-forming diazotrophic *Dolichospermum* spp. although a positive correlation was found with *Synechococcus* spp. [96]. Moreover, this group also includes strains capable of microcystin degradation [63].

Before the bloom development, we observed peaks of dominant OTUs belonging to Chlorobi (OPB56), Cyanobacteria (Synechococcales) and chloroplasts from Cryptophyta. Chlorobi have been found to be abundant in hypolimnetic waters of lakes and ponds where Cyanobacteria (such as *Synechococcus*) are also present [99]. *Synechococcus* spp. (Cyanobacteria) are ubiquitous in marine and freshwater environments but with different temporal and/or spatial successional strain patterns [102,103]. In the present study, several *Synechococcus* OTUs had different abundance patterns from April to June. Cryptophyta OTUs were negatively correlated with the bloom-forming cyanobacterial OTUs, but only one was significant, with the *Synechococcus* OTU ($r = -0.59$, $p < 0.05$). In eutrophic shallow lakes, the prevalence of microalgae belonging to Cryptophyta during winter and spring seasons is frequent [86,104,105]. In fact, microscopic analysis detected a strong presence of *Cryptomonas* (Cryptophyta) in samples from Vela Lake during spring 2006 [74]. However, 16S rRNA gene metabarcoding analysis did not provide much more relevant information on the remaining phytoplankton.

From the 22nd of May onwards, there were major shifts in BCC, with the increasing dominance of Cyanobacteria over the bacterioplankton community. The successional dominant cyanobacterial OTUs were affiliated with *Aphanizomenon gracile*, *Sphaerospermopsis aphanizomenoides* and *Synechococcus* sp. As already mentioned, these *taxa* can be frequently found in high densities in lentic freshwaters and have been linked to cyanotoxin production [95,106–109]. Strains of *S. aphanizomenoides* and *Synechococcus* spp. can produce microcystins [95,106] and *Aph. gracile* has been reported to produce saxitoxins [23,107,109,110] and cylindrospermopsin [111]. Blooms of *Aph. gracile* and *S. aphanizomenoides* have been previously recorded at Vela Lake during warmer months and both these OTUs were positively correlated ($r = 0.63$, $p < 0.05$). Their growth can be successful under nitrogen depletion [19,26]. In the present study, *S. aphanizomenoides* was also favoured by water temperatures between 24 and 28 °C during undetectable levels of NO_3^- and SRP and

pH values higher than 9.0. The strong dominance of *S. aphanizomenoides* in cyanobacterial blooms under high temperatures (25–28 °C) and very low nutrient levels have also been reported for other shallow lakes [95]. Although *S. aphanizomenoides* is known for its ecological plasticity under different nutrient and temperature levels [112], severe fitness differences may also be observed among different strains under nutrient depletion [19,113]. Interestingly, the *S. aphanizomenoides* OTU was similar to sequences retrieved from strains isolated from Vela Lake from 2004 to 2006 [70] suggesting an interannual re-incidence of this bloom-forming cyanobacterium. This is a major advantage of using DNA-based approaches that enables the matching of sequences from diverse origins and times, either from isolates or environmental samples.

Moreover, in microscopic phytoplankton analyses from samples collected during the 2006 sampling period at Vela Lake, only a Nostocacean bloom-forming species was considered to bloom and identified as *Aph. flos-aquae* [74]. Classical microscopic-based analyses do not allow distinguishing unequivocally between *Aphanizomenon* species [24,114], particularly in the absence of heterocysts and/or akinetes in filaments. Moreover, a bloom of *Aph. flos-aquae* had been also reported during late spring of 2001, through microscopic identification based on phenotype characteristics [26] but it was re-identified as *Aph. gracile* after molecular analysis [70], highlighting the problems related to morphological identification but also taxonomic revisions. Indeed, *Aph. gracile* was previously known as *Aph. flos-aquae* f. *gracile* [40] and *S. aphanizomenoides* that was formerly *Aphanizomenon aphanizomenoides* or *Anabaena aphanizomenoides* [7,8,36]. The taxonomic vs. molecular features of *Anabaena* and *Aphanizomenon*-like cyanobacteria have been controversial for the past decades, but consensus is beginning to arise and bring light on how to identify Nostocales cyanobacteria using polyphasic approaches for new revisions and modern cyanobacterial taxonomy [115–117]. Nevertheless, these differences in nomenclature and potential misidentifications add a considerable variability to the historical ecological data recorded for each bloom-forming cyanobacterial species. Therefore, molecular approaches are essential to overcome these classical taxonomic ambiguities and provide robust information for specific distribution and ecological patterns of cyanobacterial blooms. Next generation sequencing should be considered for monitoring strategies [118], with the advantage of providing species-specific essential information concerning bloom-forming cyanobacteria but also information on the whole community and ribotype re-incidence, which is very important to go deeper in ecological studies and modelling approaches. In fact, an OTU matching the invasive *Cuspidothrix issatschenkoi* (previously reported at Vela Lake in 2004 [70]) was also detected in samples, although less representative within the cyanobacterial group. One of the bloom-forming *Synechococcus* (OTU 6) showed a significant positive correlation ($r = 0.67$, $p < 0.05$) with *S. aphanizomenoides* but not with *Aph. gracile*, emphasising the potential affinities for succession in the cyanobacterial dominance. However, despite *Synechococcus*-like OTUs being recorded at high densities during June, microscopic analyses from this sampling period [74] did not report this genus in the cell counts, probably due to their small size. Nevertheless, *Synechococcus* strains are able to produce cyanotoxins, namely microcystins [106], which makes this genus important to quantify for bloom risk assessment. Once more, 16S rRNA gene-based methodologies can overcome the subjectivity and limitations of classical identification approaches and provide a better basis for health risk assessment.

During the bloom, besides the already mentioned dominance of Alphaproteobacteria and Bacteroidetes orders (in all samples), we also observed high abundance peaks for the groups Candidate division OD1 (Mb-NB09) and Firmicutes (Bacillales) (both with a positive correlation with *Aph. gracile*, $r = 0.72$ and 0.67, respectively, $p < 0.05$). *Bacillus* strains (Bacillales, Firmicutes) have been shown to lyse cells from *Aphanizomenon flos-aquae* [61] and *Anabaena flos-aquae* [116]. This could help explain the highest abundance recorded for Bacillales during the *Aph. gracile* bloom peak. Several *Bacillus* strains can also degrade microcystins [63]. The Candidate division OD1 has been found within *Microcystis* bloom samples [117]. Other bacteria such as Actinobacteria (C111, Acidimicrobiales) had negative correlations with the bloom-forming cyanobacterial abundances, namely *Synechococcus*

sp. ($r = -0.63$, $p < 0.05$). Members of Acidiomicrobiales, namely C111, have also been observed during bloom events dominated by *Microcystis* spp. [118]. Although negative correlations between Gammaproteobacteria (Xanthomonadales) and the cyanobacterial bloom-forming OTUs were not statistically significant, references report Xanthomonadales to be associated with blooms of *Microcystis* spp. [100] but also with blooms of *Planktothrix* spp. or co-dominated by strains of *Microcystis, Dolichospermum, Cylindrospermopsis* and *Synechococcus* [119]. Moreover, Gammaproteobacteria comprise many pathogens, and can potentially increase health risks during cyanobacterial blooms [120–122].

5. Conclusions

This study provides new insights into bacterial succession during a cyanobacterial bloom co-dominated by *Aphanizomenon gracile* and *Sphaerospermopsis aphanizomenoides*, but also *Synechococcus* sp. at a later phase. The dominance of *Aph. gracile* was correlated with high water temperatures, and *S. aphanizomenoides* with high conductivity levels. The bloom occurred during NO_3^- and SRP concentrations below detection levels, highlighting the ability of these diazotrophic species to bloom under low-nutrient conditions. A clear shift in BCC coincided with the establishment of the cyanobacterial bloom, suggesting an impact on the bacterioplankton succession dynamics. In fact, significant negative correlations were recorded between the bloom-forming cyanobacteria and some OTUs of Saprospirales and Sphingobacteriales (both Bacteroidetes), Rhizobiales (Alphaproteobacteria) and Burkholderiales (Betaproteobacteria). Nevertheless, bacterial groups such as Rickettsiales, Caulobacterales, Rhizobiales and Sphingomonadales (Alphaproteobacteria); Saprospirales, Flavobacteriales and Sphingobacteriales (Bacteroidetes); and Actinomycetales (Actinobacteria) remained dominant throughout the study period. Actually, these groups have been frequently associated with cyanobacterial blooms and some strains are capable of degrading cyanotoxins such as microcystins, highlighting the potential BCC influence on the dynamics and impacts of blooms. Nevertheless, only during the bloom event did we observe an increase in Mb-NB09 (Candidate division) and Bacillales (Firmicutes) members (both with a positive correlation with *Aph. gracile*); Acidimicrobiales (Actinobacteria), in addition to Xanthomonadales (Gammaproteobacteria).

There is still the need to go further in our understanding of HCBs' dynamics by considering the occurrence patterns of species-specific cyanobacterial blooms and the whole BCC dynamics. The repeatability of patterns of the bacterioplankton dynamics is probable to occur during species-specific blooms, which makes it important to record every possible temporal and spatial dataset from waterbodies prone to HCBs. Next-generation sequencing (NGS) approaches can thus provide important species-specific bloom information for that purpose: (i) considering shifts in the overall community and (ii) avoiding the constraints of culture-dependent approaches and classical ambiguous microscopic identifications. In the near future, modelling, and ultimately management strategic plans, will rely on robust time-series data provided by such technological support.

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