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Seasonal dynamics and sedimentation patterns of *Microcystis* oligopeptide-based chemotypes reveal subpopulations with different ecological traits

Ramsy Agha,[1] María Ángeles Lezcano,[1,2] María del Mar Labrador,[1] Samuel Cirés,[1,3] and Antonio Quesada [1,*]

[1] Universidad Autónoma de Madrid, Department of Biology, C. Darwin 2, 28049 Cantoblanco, Spain
[2] Instituto Madrileño de Estudios Avanzados del Agua, Parque Científico Tecnológico de la Universidad de Alcalá, 28805 Alcalá de Henares, Spain
[3] James Cook University, School of Marine and Tropical Biology, 4811 Townsville, Queensland, Australia

Abstract

The patchy distribution of oligopeptide production abilities in cyanobacterial populations enables the classification of strains into different oligopeptide-based chemotypes. In order to evaluate the ecological significance of chemotypes in natural systems, we tracked the seasonal dynamics and sedimentation losses of *Microcystis* chemotypes in the eutrophic Valmayor reservoir (Spain). Fifty-three distinct chemotypes were identified throughout the season, six of them only present as benthic colonies. There was no correlation between chemotype affiliation and taxonomic morphospecies or colony size. The succession of chemotypes in the water column occurred synchronically in pelagic and littoral habitats and at different depths. Shifts in chemotype assemblages successfully explained temporal fluctuations in biomass-standardized microcystin contents of the bloom. The dynamics of chemotypes were driven both by asynchronous proliferation in the water column and significantly different sedimentation rates among chemotypes. While differential settling was the most important loss process shaping chemotype succession, the existence of alternative processes selectively inflicting massive losses to individual chemotypes was observed. Together, the significant differences in sedimentation and pelagic net growth rates among chemotypes, their segregation among pelagic and benthic habitats, as well as the existence of chemotype-selective loss processes, show that *Microcystis* oligopeptide chemotypes interact differently with their environment and represent commonly overlooked ecologically functional intraspecific linages.

Studies on phytoplankton ecology typically focus on the species at the lowest taxonomic level studied. However, there is increasing evidence for the existence of intraspecific polymorphisms and their relevance in the ecology of a number of organisms. These findings challenge approaches that consider the species as the ecologically relevant unit to understand the complex ecology of phytoplankton. A notable example of such intraspecific polymorphisms has been reported in picoplanktonic cyanobacteria of the genera *Prochlorococcus* and *Synechococcus,* which subdivide into different ecotypes with dissimilar niche preferences (Huang et al. 2012). The composition of these subpopulations in natural systems is variable and allows the whole population to rapidly adapt to environmental fluctuations. This ecological versatility has been pointed out as one of the reasons for their widespread distribution and ecological success (Johnson et al. 2006). The identification of similar ecologically distinct subpopulations in other cyanobacteria is an important need for current research on phytoplankton. Of special interest are cyanobacteria of the bloom-forming genus *Microcystis,* whose massive proliferations in freshwater lentic systems are a matter of concern from both ecological and public health perspectives (Codd et al. 2005).

Microcystis is among the most important producers of microcystins (MCs) worldwide, which is a diverse group of hepatotoxic peptides. In addition to MCs, *Microcystis* can produce a wide range of non-ribosomal secondary metabolites of peptidic nature, commonly referred to as oligopeptides. Oligopeptides display a number of bioactive properties and are chemically very diverse, accounting for > 600 chemical variants that can be classified into major oligopeptide classes (for a comprehensive review *see* Welker and Von Dohren 2006). In analogy to MCs, many oligopeptide classes are synthesized non-ribosomally by single multi-enzyme complexes that are in turn encoded in large gene clusters (although the synthesis of some oligopeptides, such as microviridins and cyclamides, follows ribosomal pathways). Oligopeptides synthesis at the individual level is hence determined by the presence or absence of their respective gene clusters, whose distribution among strains is remarkably heterogeneous because of frequent horizontal gene transfer, recombination, and gene loss events (Christiansen et al. 2003; Mikalsen et al. 2003; Kurmayer and Gumpenberger 2006). Their patchy distribution gives rise to the coexistence of strains with different oligopeptide production abilities. The analysis of single *Microcystis* colonies by Matrix Assisted Laser Desorption Ionization—Time of Flight Mass Spectrometry (MALDI-TOF MS) yields a rapid determination of qualitative oligopeptide compositions at the individual level, enabling the delimitation of oligopeptide-based subpopulations or chemotypes (Erhard et al. 1997). Oligopeptide compositions are suitable markers of subpopulations, because their synthesis is constitutively regulated and their cellular

* Corresponding author: antonio.quesada@uam.es

contents vary within a narrow range (1–5 fold) under different environmental conditions (Repka et al. 2004; Rohrlack and Utkilen 2007; Halstvedt et al. 2008).

Chemotypes are found to coexist in natural populations of several cyanobacterial genera such as *Microcystis* or *Nodularia* (Welker et al. 2007; Fewer et al. 2009). Moreover, in the case of genus *Planktothrix,* differences among chemotypes with regard to seasonal dynamics and depth distribution led to the proposal that chemotypes represent subpopulations with different ecological traits (Rohrlack et al. 2008). However, additional quantitative field data (i.e., differential proliferation and losses of individual chemotypes in nature) seem needed to generalize these findings in order to include other important bloom-forming cyanobacteria such as *Microcystis*. Understanding the dynamics of cyanobacterial chemotypes is particularly relevant from an ecological perspective; differences among chemotypes in terms of growth, susceptibility to grazers and/or pathogens, as well as sedimentation losses, can in principle shape the composition and succession of strains and thereby dynamically affect the properties of the whole population. Furthermore, the study of cyanobacterial populations focusing on differential interactions of chemotypes with their environment provides important insights into the as yet unclear biological function(s) of oligopeptides. It also helps us understand how such a vast metabolic diversity evolved, considering the high energetic cost linked to their biosynthesis. Secondly, the phenology of chemotypes in the water column arguably constitutes an important factor exerting a direct effect on the relative toxicity of cyanobacterial populations. Cyanobacterial blooms often display variations in microcystin concentrations of several orders of magnitude in both spatial (Fastner et al. 1999) and temporal scales (Sanchis et al. 2002). Fluctuations in bloom toxicity cannot be explained by physiological changes in microcystin production at the individual level (Orr and Jones 1998); therefore, shifts in the composition of chemotypes in the population with different toxic properties have to be regarded as critical factors modulating bloom toxicity (Welker et al. 2007; Agha et al. 2012).

Here, we study the dynamics of *Microcystis* oligopeptide-based chemotypes in Valmayor reservoir, a typical Mediterranean reservoir located in central Spain, paying special attention to their participation in sedimentation fluxes and differences in pelagic net growth. Previous studies in Valmayor reservoir revealed a high diversity of MC variants and marked temporal fluctuations in MC contents of settled seston during the season, suggesting differential sedimentation of chemotypes (Wörmer et al. 2011; Cires et al. 2013). In this study, we evaluate whether (1) the distribution and dynamics of *Microcystis* chemotypes are habitat-dependent (i.e., display differences in littoral and pelagic areas), (2) shifts in the composition of *Microcystis* chemotypes are responsible for temporal fluctuations in microcystin : *Microcystis* biomass ratios (i.e., relative toxicity of the population), (3) individual chemotypes display differences in pelagic net growth rates, (4) individual chemotypes differ in their temporal sedimentation patterns, and (5) chemotypes can be selectively affected by alternative loss processes other than sedimentation.

Table 1. List of samples collected at Valmayor reservoir at each sampling date. D—Deployment of sediment traps.

Date	Discrete depths (0.5 m; 3 m; 5 m; 10 m; 20 m)	Integrated net samples		Sediment traps (20 m)
	Pelagic	Pelagic	Littoral	
07 Jul	X	—	—	—
14 Jul	X	—	—	—
20 Jul	X	—	—	—
27 Jul	X	X	X	—
11 Aug	X	X	X	—
24 Aug	X	—	—	—
09 Sep	X	—	—	—
28 Sep	X	—	—	—
15 Oct	X	X	X	D
19 Oct	X	—	—	—
25 Oct	X	X	X	X
03 Nov	X	X	X	X
11 Nov	X	X	X	X
24 Nov	X	X	X	X

Methods

Sampling setup—The study was performed in the eutrophic Valmayor reservoir (40°31′39″N 04°03′19″W), a typical monomictic Mediterranean reservoir located in central Spain used for drinking water supply and recreational activities. Limnological features of the reservoir are described in detail elsewhere (Wörmer et al. 2011; Cires et al. 2013). Valmayor reservoir was sampled during summer and autumn 2010, from 07 July until 24 November (Table 1). In order to address the distribution of chemotypes among reservoir habitats, two sampling points were defined: (1) A pelagic sampling point located in the central part of the reservoir (z_{max} = 34 m) and (2) a second point in the littoral area (z_{max} = 4 m). Vertical profiles of temperature, dissolved oxygen, and chlorophyll *a* (Chl *a*) were obtained using a Yellow Springs Instruments (YSI) 6920 multi-parameter probe at each sampling date. Light irradiance along the water column was measured using a LiCor 2π photosynthetically active radiation sensor attached to a LiCor data logger (LI-1000). Thermal structure of the water column was also monitored by installing a line of 6 TidbiT temperature data loggers (Onset Computer Corporation) at the pelagic sampling point at depths 0.5 m, 3 m, 6 m, 12 m, and 18 m, which recorded water temperature at 30 min intervals throughout the whole study period. Additionally, three sediment cores were collected at the beginning of the study period (07 Jul) at both pelagic and littoral sampling points using a gravity corer (Uwitec), in order to analyze the composition of chemotypes in the benthic *Microcystis* population. After a prolonged clear phase (24 Aug–29 Sep) and a subsequent significant appearance of *Microcystis* colonies in the water column, three sediment traps were installed in the pelagic

Table 2. Number of *Microcystis* colonies collected for chemotyping for each sample type and sampling date. Numbers in brackets stand for colonies providing unknown oligopeptide compositions (*see* text).

	Discrete-depth samples							
	Pelagial					Net samples		Sediment traps
Date	0.5 m	3 m	5 m	10 m	20 m	Pelagial	Littoral	20 m
27 Jul	30	30	27	21	—	58	60	—
11 Aug	30	30	26	27	—	59	60	—
15 Oct	30	30	30	28	30	60	60	—
25 Oct	30	30	30	30	30	60	60	60 (4)
03 Nov	30	30	30	30	30	60	60	60 (5)
11 Nov	30	30	30	30	26	60	60	60 (3)
24 Nov	30	29	27	19	22	60	54	60 (6)

sampling point to study *Microcystis* sedimentation until the end of the study period.

Water sampling—Two types of water samples were collected during the study period (Table 1). First, discrete-depth water samples were collected at the pelagic sampling point at 0.5 m, 3 m, 5 m, 10 m, and 20 m using a 5 liter water sampler (Uwitec). These samples were used for (1) the determination of total Chl *a* and algal group composition, (2) the identification of present cyanobacterial taxa, (3) *Microcystis* cell density calculations and biovolume estimations, (4) quantification of sestonic MCs, and (5) *Microcystis* chemotyping at individual depths (chemotyping was only performed when sufficient colonies were present; *see* Table 2 for dates). At these sampling days, integrated net samples of the first 20 m were also collected with a 20 μm mesh-size plankton net (Uwitec) at both pelagic and littoral sampling points and were used for chemotyping of single *Microcystis* colonies.

After sampling, water samples were transported to the laboratory at 4°C and processed within 2 h. Chl *a* concentrations and algal group compositions were determined using an Algae Analyzer benchtop fluorometer (Moldaenke BBE), allowing the determination of the individual contributions of cyanobacteria and other algal groups to total Chl *a* concentrations in the sample. A 100 mL aliquot of discrete-depth water samples was fixed in acid Lugol's solution for subsequent microscopic identification, cell counts, and biovolume estimations of waterborne *Microcystis*. The remaining water was filtered through GF/F glass fiber filters (Whatman) and stored at −20°C for MC analysis.

Sediment-traps sampling—Sediment traps were designed and constructed by SegaInvex (Universidad Autónoma de Madrid). They consist of three identical black polyvinyl chloride cylinders (4.4 cm inner diameter) that prevent photosynthetic growth. The traps were attached to two buoys fixed by two anchors. A central weight allowed further stabilization. Traps were thereby freely suspended in the water column at a depth of 20 m. After their deployment on 15 October, traps were collected simultaneously with water samples (Table 1). Settled material was collected from the traps by carefully discarding the supernatant of each tube and collecting the 100 mL remaining in the trap. After thorough homogenization of the settled material, a first aliquot was fixed in formaldehyde 4% (v:v) and stored dark at 4°C for microscopic species identification and quantification. A second aliquot was vacuum-filtered through GF/F filters and stored at −20°C for MC extraction and analysis. The remaining volume was used to collect single colonies for chemotyping. All measurements were performed individually for each tube, and results were expressed as average values of three replicates.

Sediment-core sampling and separation of benthic Microcystis—In order to analyze the chemotype composition of the benthic *Microcystis* population, three profundal sediment cores and three littoral sediment cores were collected at each sampling point at the beginning of the study period using a gravity corer (Uwitec). Immediately after sampling, cores were transported undisturbed to the laboratory and sliced. *Microcystis* colonies were successfully separated from the sediment after centrifugation of 2 g of homogenized fresh sediment (0–1 cm slices) with 30% LUDOX® TM-50 silica suspension (Sigma-Aldrich) at 400 × *g* during 20 min. After centrifugation, colonies accumulated in the supernatant and 60 colonies per sediment core were collected for chemotyping analysis.

Identification and quantification of Microcystis *in water and sediment trap samples*—Identification and quantification of cyanobacteria in acid Lugol fixed samples was performed following Utermöhl's technique (Utermohl 1958). Morphospecies identification was performed under a Leica DM IL inverted microscope (Leica Microsystems) following Komárek and Anagnostidis (1999). In the case of sediment traps, formaldehyzed aliquots of the settled material of each trap were diluted 10-fold and 1 mL of the resulting suspension was filtered through 0.2 μm pore-size Anodisc membrane filters (Whatman) under gentle vacuum to avoid colony disruption. The filters were mounted on microscope slides with an added drop of anti-fading mounting oil Aqua PolyMount (Polysciences) and examined under an Olympus BH2 microscope equipped with a BH2-RFCA epifluorescence system (Olympus), using the BP545 excitation filter, the DM570 dicroic mirror, and the O590 emission filter. *Microcystis* colonies and cells were counted on the whole surface of the

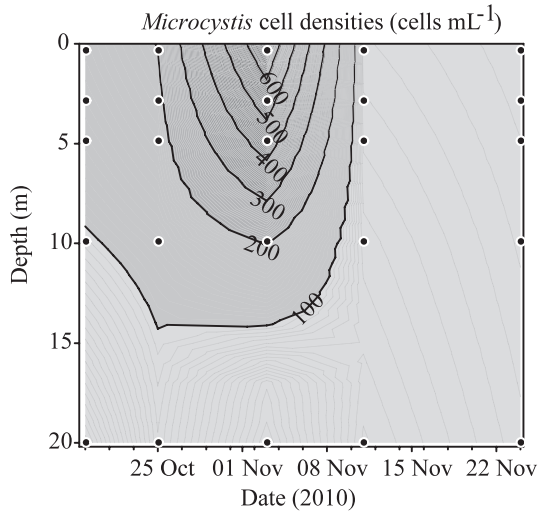

Fig. 1. Estimated depth–time distribution of *Microcystis* cell numbers (cell mL^{-1}) in the first 20 m of Valmayor reservoir. Actual counts are represented by black dots. Data were negative-exponentially smoothed using SigmaPlot version 11.0.

filter. Thereby total settled *Microcystis* colonies and cells could be calculated for each sedimentation period.

Estimation of pelagic net growth and sedimentation rates—Cell counts in individual discrete-depth water samples allowed the estimation of *Microcystis* depth–time distribution along the first 20 m during the sedimentation period (Fig. 1). The vertical distribution of the four dominant chemotypes was estimated analogously, considering their relative abundances in each discrete-depth water sample. The calculated settling rates of the whole *Microcystis* population and individual chemotypes (cells m^{-2} d^{-1}), obtained from cell counts of the settled *Microcystis* and the relative abundances of chemotypes in the traps, could be related to total *Microcystis* (and individual chemotypes) cells in the overlying 20 m water column above the traps. Thereby, settling rates could be assimilated to normalized sedimentation rates (d^{-1}) for both the whole *Microcystis* population and the individual chemotypes.

In order to quantify the contribution of natural sedimentation to the dynamics of chemotypes, a simple conceptual model was employed that can be described with the equation

$$\Delta N = NGR - SR \quad (1)$$

where ΔN (d^{-1}) is the recorded variation in the integrated chemotype cell numbers between sampling dates; SR is the calculated sedimentation rates (d^{-1}); and NGR stands for a net growth rate estimate (d^{-1}), which comprises chemotype intrinsic growth, including overall cellular losses different from sedimentation (grazing, pathogenic cell lysis, programmed cell death, etc.).

Microcystis *chemotyping*—When sufficiently abundant, individual *Microcystis* colonies from individual discrete-depth water samples, integrated net samples, sediment trap samples, and sediment core slices were collected under a dissecting microscope using disposable glass capillaries. The number of colonies collected from each type of sample and date is detailed in Table 2. Once collected, individual colonies were placed in 0.2 mL Eppendorf tubes and allowed to dry at room temperature for 2 h. Ten microliters of an acetonitrile, ethanol, and water (1 : 1 : 1) extractant solution, acidified with 0.03% (v : v) trifluoracetic acid were added to each sample. Samples were introduced in liquid nitrogen to induce cell lysis and enhance oligopeptide extraction. Colonies were then stored at $-80^{\circ}C$ until they were analyzed. The analysis of individual colonies was performed by Matrix Assisted Laser Desorption Ionization—Time of Flight Mass Spectrometry (MALDI-TOF MS) using a Bruker Reflex MALDI mass spectrometer equipped with a TOF (Time of Flight) detector. Subsequent oligopeptide identification and spectral data processing were performed using Bruker FlexAnalysis 3.0 software (Bruker Daltonics). MALDI-TOF MS data acquisition and spectral data processing are described in detail in Agha et al. (2012). Chemotype identification was carried out by cluster analysis of the mass spectral oligopeptide compositions by performing an Ascending Hierarchical Classification, using Euclidean distances, and applying Ward's agglomeration method. Optimal classification was considered when intra-cluster variability was zero for all clusters. Chemotype composition of each sample was expressed as the relative abundance of each chemotype. To analyze the temporal dynamics of chemotypes in the reservoir, a principal component analysis (PCA) was performed. Thereby, dimensionality of the data was reduced. Each set of colonies from each individual sample was plotted in a two-component plain. The diversity of chemotypes in the individual samples was expressed as the Shannon index of diversity.

Microcystin extraction and liquid chromatography MS-MS analysis—Sestonic microcystins retained on GF/F filters from discrete-depth water samples and sediment trap samples were extracted twice by sonication into methanol (90%). Extracts were vacuum-concentrated and prepared for ElectroSpray Ionization LC MS-MS analysis. Microcystin analysis was performed on a Varian 500 Ion Trap Mass Spectrometer equipped with two Varian 212 LC chromatographic pumps and a 410 autosampler. Chromatographic separation of MC-LR, MC-YR, and MC-RR was achieved using a Pursuit C-18 2 × 150 mm column as stationary phase and mobile phases (A) MilliQ water and (B) methanol, both acidified with 0.2% formic acid and buffered with 2 mmol L^{-1} ammonium formate. Gradient (%A : %B) applied 60 : 40 to 0 : 100 in 18 min. Quantification of MC variants was made by injecting commercial standards (Danish Hydraulic Institute) to plot calibration curves.

Results

Water column sampling—Cyanobacterial abundances during the study period were moderate and no massive cyanobacterial proliferation was observed along the season. Valmayor reservoir was thermally stratified at the beginning of the sampling period, with a thermocline located at 6 m,

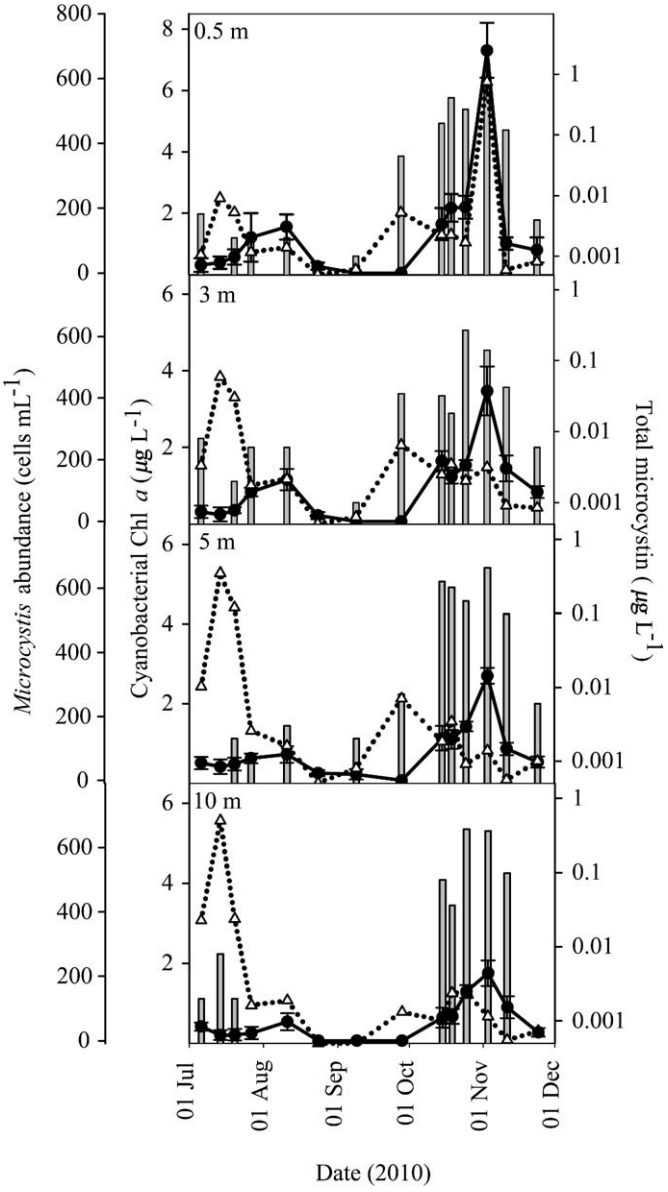

Fig. 2. Temporal evolution of cyanobacterial Chl *a* (dotted lines), *Microcystis* cell densities (bold lines), and sestonic microcystin (bars) concentrations at the pelagic sampling point in Valmayor reservoir.

which migrated deeper in the course of the season; it reached 16 m on 11 November, when autumnal mixing of the water column occurred. During July and the first 2 weeks of August, green algae dominated the planktonic community, although diatoms and cyanobacteria (represented by *Microcystis* spp., *Woronichinia naegeliana*, and filamentous *Anabaena crassa*) were also present at low abundances. Cyanobacterial Chl *a* concentrations, and *Microcystis* cell densities, were accordingly low (Fig. 2). After a prolonged clear phase in late August and September, cyanobacterial presence in the water column increased, reaching cyanobacterial Chl *a* concentration maxima around 6 μg L^{-1} on 03 November. During this period and until the end of the study, *Microcystis aeruginosa, M. flos-aquae,* and *M. novacekii* appeared as the dominant species in the cyanobacterial community, reaching subsurface cell densities of up to 700 cells mL^{-1}. Mixing of the water column occurred on 11 November, and cyanobacterial abundance in the water column rapidly decreased.

Dynamics of Microcystis *chemotypes*—In the analysis of 2175 single *Microcystis* colonies, a wide range of oligopeptides were detected, belonging to different oligopeptide families. MCs were the most abundant oligopeptides (present in 88% of the analyzed colonies), including multiple chemical variants (MC–LR, –RR, –YR, –H4YR, –WR, and other demethylated variants). Aeruginosins (101, 602, and 670a variants), cyanopeptolins (1063b, 1034Ac, 986A, 972A, and 920B variants), and microginins (FR3, FR4, 478, and 755 variants) were also detected. Spectral data processing and subsequent cluster analysis enabled the identification of 53 different chemotypes throughout the study period, characterized by presenting unique combinations of individual oligopeptides. Most chemotypes (41 out of 53; 77%) never reached relative abundances $> 5\%$ in individual samples. In contrast, only six chemotypes (11%) jointly accounted for 40–90% of the *Microcystis* population in the individual water samples. The six remaining chemotypes could only be detected as benthic populations collected from sediment samples, but never as waterborne colonies. The different *Microcystis* morphospecies (*M. aeruginosa, M. flos-aquae,* and *M. novacekii*) were represented by several chemotypes, and no clear relationship could be found between chemotype affiliation and colony size (data not shown).

The comparison of chemotype compositions of each analyzed sampling point and date was expressed as a bi-factorial PCA plot (Fig. 3). The proximity of data points in the PCA space expresses the similarity between chemotype assemblages in each sample (i.e., closely positioned points represent samples that display resembling chemotype compositions). Consequently, the shift of data points across the PCA plane can be interpreted as the dynamics of chemotypical subpopulations in Valmayor reservoir. Factor 1 was significantly and positively correlated with CT11 and negatively correlated with CT17, CT4, and CT22. Factor 2 was positively correlated with CT11 and CT17 and negatively correlated with CT1. Together, both factors accounted for 67.47% of the variance in the data. Chemotype dynamics during the study period displayed a non-cyclic trend, with clearly dissimilar chemotype assemblages at the beginning and end of the study period. Points corresponding to the same sampling date grouped together, independently of their pelagic or littoral origin, indicating that the seasonal succession of chemotypes occurs homogeneously in both habitats. Interestingly, points corresponding to water samples were clearly separated from that of profundal sediment samples, evidencing marked differences between benthic and waterborne chemotype assemblages during the blooming season. Surprisingly, no colonies could be found in the sediment cores collected from the littoral area. The reason for their absence remains unclear and might result from sediment focusing into deeper areas of the reservoir (Hilton 1985), or enhanced

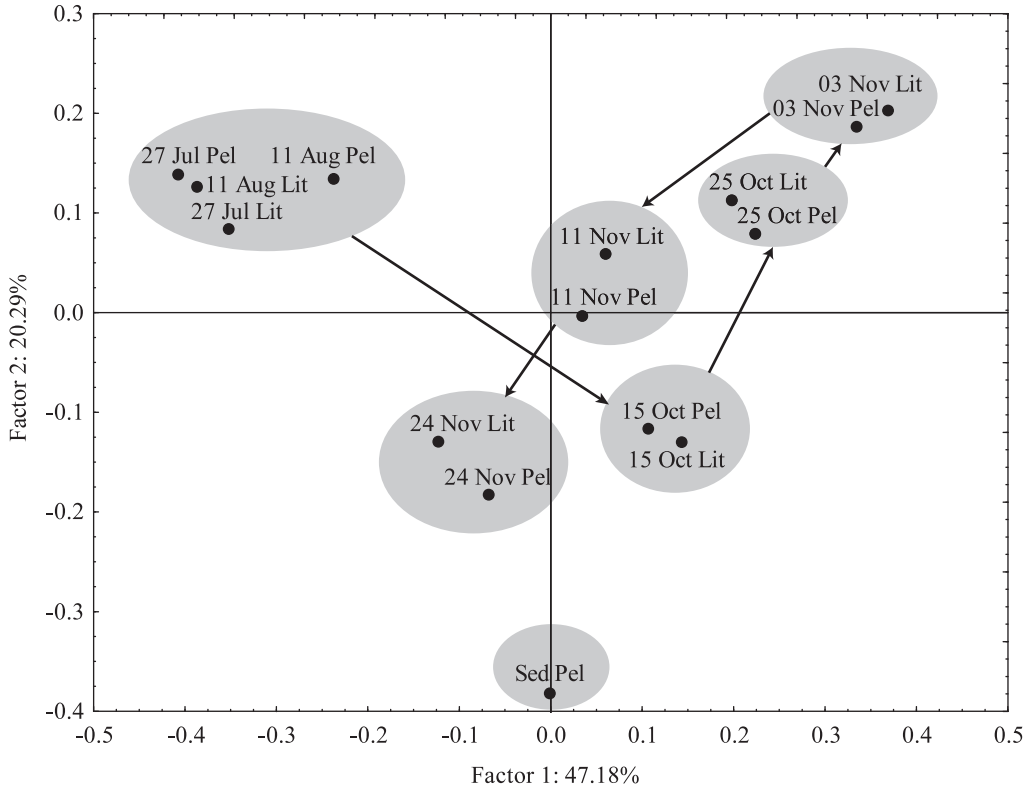

Fig. 3. Bifactorial principal component analysis (PCA) plot illustrating *Microcystis* chemotype compositions in Valmayor reservoir from July until November 2010. Next to each data point, the corresponding sampling date and sampled point (pelagic [Pel]; littoral [Lit]) are specified. Arrows indicate the temporal evolution of the samples. "Sed Pel" stands for chemotype assemblages corresponding to profundal sediment samples.

recruitment of colonies in shallow sediments with increased light availability (Schoene et al. 2010).

Chemotype diversity was high during the whole study period, although significant temporal variations in the Shannon index could be observed (Fig. 4). During the end of July and August (preceding the clear phase) chemotype diversity was lowest (Shannon Diversity Index, H = 1.42–1.56) with chemotypes CT4, CT22, and CT17 dominating the population, jointly accounting for 73–94% of the colonies in the different samples during this period. After a clear phase of several weeks, *Microcystis* colonies reappeared in the water column on 15 October, comprising a significantly more diverse population than during the previous period (one-way ANOVA, $p < 0.01$; H = 2.64–2.69). Chemotype diversity declined then steadily until 03 November (H = 1.69–1.87) coincident with the seasonal cyanobacterial density maxima. In the next week (11 Nov) diversity reached its maximum (H = 2.56–2.90) and remained constant until the end of the study period (no significant differences). Despite the marked succession of chemotypes, Spearman rank correlation tests did not reveal any significant ($p > 0.01$) relationship between depth, light irradiance, macronutrients, or water temperature and the relative abundance of individual chemotypes along the study period (data not shown).

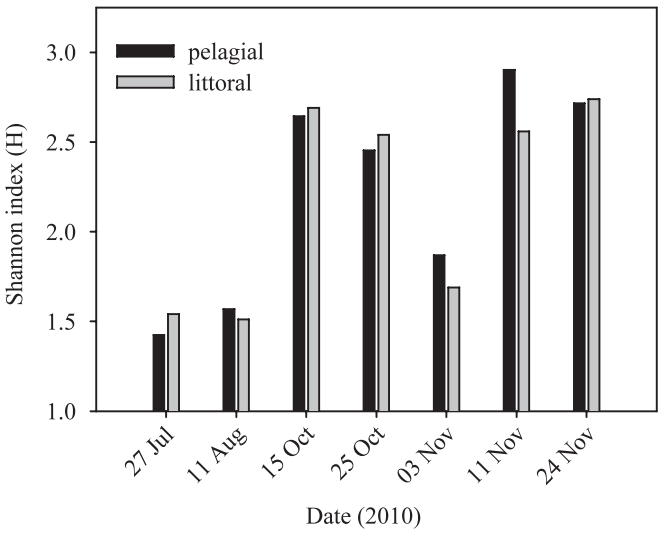

Fig. 4. Shannon index of chemotype diversity at pelagic and littoral sampling points in Valmayor reservoir.

Microcystins and chemotype compositions—In line with cyanobacterial abundances, total microcystin concentrations in Valmayor reservoir were, in general, low, varying from 0.03 μg L^{-1} to 1.49 μg L^{-1}. Temporal fluctuations in sestonic toxin concentrations showed no clear agreement with cyanobacterial Chl *a* concentrations or *Microcystis* abundances in the water column (Fig. 2). Instead, the proportion of toxic chemotypes in the population recorded at each date,

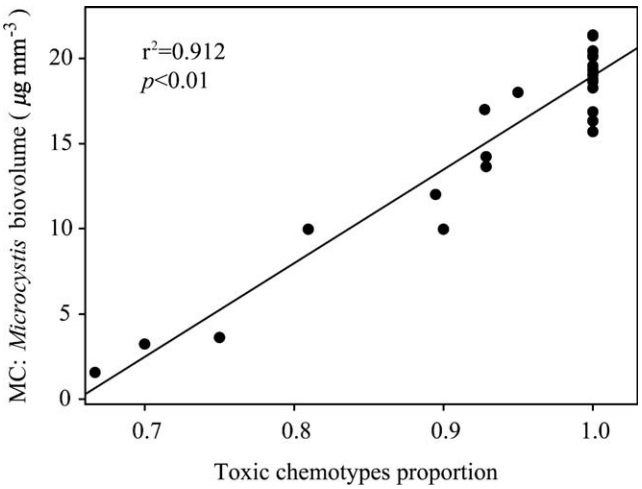

Fig. 5. Correlation between the proportion of toxigenic chemotypes and microcystin : *Microcystis* biovolume ratios in Valmayor reservoir. Points correspond to discrete-depth water samples.

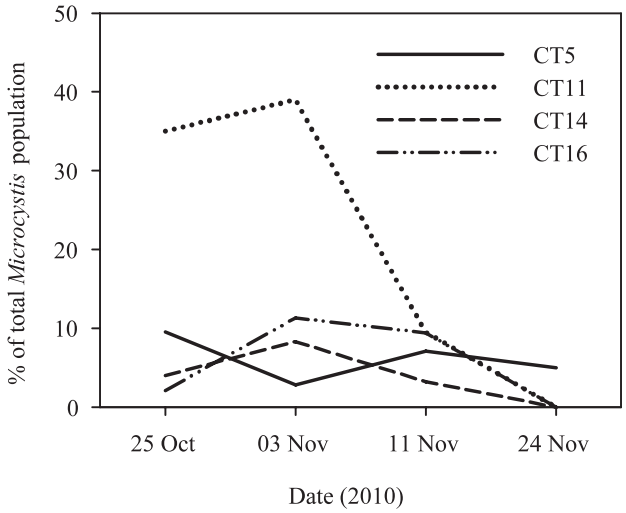

Fig. 6. Relative abundances of the four major *Microcystis* chemotypes (CT5, CT11, CT14, and CT16) in the whole water column during the sedimentation study period.

sampling point, and depth showed a highly significant linear correlation with total microcystins : *Microcystis* biovolume ratios ($r^2 = 0.912$; $p < 0.01$; Fig. 5), evidencing that sestonic microcystin contents in Valmayor reservoir are effectively modulated by shifts in the composition of chemotypes.

Sedimentation study—Together with the reappearance of *Microcystis* colonies after the clear phase, sedimentation traps were installed at the pelagic sampling point on 15 October in order to track and quantify sedimentation losses of individual *Microcystis* chemotypes. Colonies collected from the sedimentation traps showed, in general, intense red autofluorescence when microscopically inspected and generally provided satisfactory mass spectra in the chemotyping analysis. A few colonies, however ($n = 18$; Table 2), provided putatively incomplete mass spectra, showing no coincidence with any of the peptide patterns observed in waterborne or benthic subpopulations.

For the sedimentation study, we focused on the most abundant chemotypes during the last month before mixing, namely chemotypes CT5, CT11, CT14, and CT16. The phenology of these chemotypes (Fig. 6) was characterized by a marked dominance of CT11 during the first 2 weeks, accounting for up to 39% of the population, followed by a later dramatic decline and disappearance from the water column. The other chemotypes presented relative abundances between 3% and 10% that fluctuated without clear trends during this period until 24 November, when cyanobacterial densities dramatically decreased and only CT5 could be found in the water column.

Although the *Microcystis* whole-population showed moderate sedimentation rates (0.01–0.12 d^{-1}), the dominant chemotypes displayed significantly higher sedimentation and net growth rates ($p < 0.01$; one-way ANOVA; Fig. 7). During the first sedimentation period, all major chemotypes displayed a rather similar behavior with marginal sedimentation rates and high net growth rates. In later sedimentation periods, CT5, CT14, and CT16 exhibited lower pelagic net growth rates and higher sedimentation rates (although significantly different among chemotypes; Fig. 7), denoting that sedimentation was the main loss process affecting these subpopulations. Interestingly, CT11 (the dominant chemotype at the beginning of the sedimentation study) showed a completely different behavior. After a period of 2 weeks dominating the community (accounting for 35% and 39% of the colonies on 25 Oct and 03 Nov respectively; Fig. 6), a sharp decrease in the abundances of this chemotype was evident. Strikingly, no colonies with the CT11-characteristic oligopeptide signature could be found in the sediment traps. To evaluate whether this observation was an analytical artifact, we explored the possibility of distortion of the oligopeptide pattern caused by suboptimal physiological conditions during sedimentation, which would mask the settling fluxes of CT11 (Agha et al. 2013). However, we ruled out this possibility because colonies providing unknown peptide patterns in the traps (i.e., not consistent with any observed benthic or pelagic chemotype [*see* above]) presented not only a lack of CT11-specific signals, but also one or more additional signals corresponding to oligopeptides not synthesized by CT11. As a result of the absence of sedimentation, net growth estimates corresponding to CT11 showed negative values, indicating that pelagic loss processes affecting this chemotype were of a different nature, but not due to sedimentation.

Discussion

The coexistence of conspecific lineages within a population is a common feature in many microorganisms. In cyanobacteria, population subdivision has been observed in picocyanobacteria *Prochlorococcus* and *Synechococcus,* which show niche partitioning among ecotypes (Johnson et al. 2006; Becker et al. 2007). In more complex cyanobacteria, coexisting subpopulations have been recognized with regard to the production of non-ribosomal

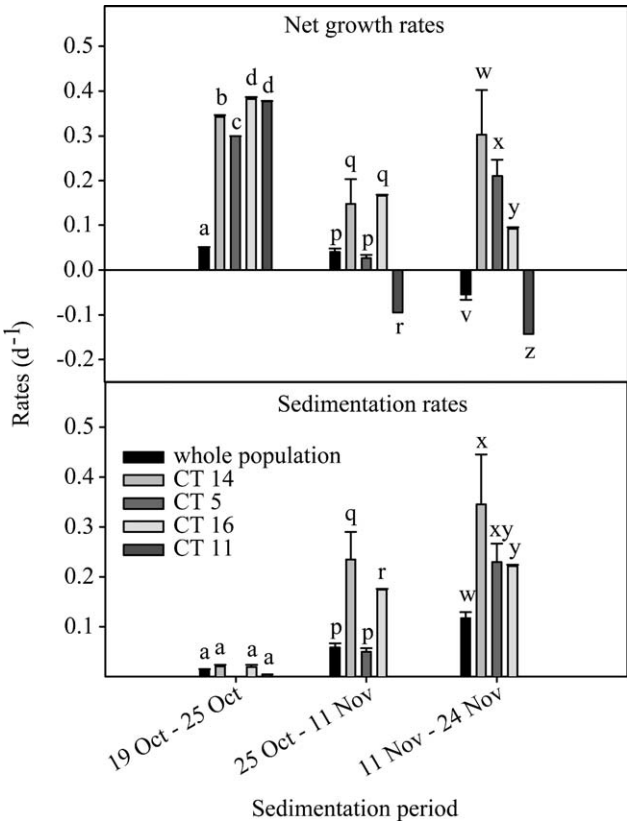

Fig. 7. Calculated net growth rates and sedimentation rates of the whole *Microcystis* population and the four major *Microcystis* chemotypes during the last weeks before mixing in Valmayor reservoir. Sedimentation rates were calculated based on the analysis of sedimentation traps. Net growth rates were estimated considering variations in chemotypes' waterborne cell numbers among sampling dates and their calculated sedimentation rates during each period. Letters represent significant differences among chemotype-specific rates at the same date (one-way ANOVA, post hoc Holm–Sidak test; $p < 0.05$).

peptides in genera *Microcystis* (Welker et al. 2007), *Planktothrix* (Rohrlack et al. 2008), and *Nodularia* (Fewer et al. 2009). The use of oligopeptides as markers of subpopulations is an emerging approach to undertake studies on the ecological significance of intraspecific chemical polymorphisms in cyanobacteria and thereby grasp insights into the unknown biological role of oligopeptides. Classic botanical criteria used for the classification of cyanobacterial species are unsuitable to discriminate among chemically dissimilar strains within a population, as supported in this study by the lack of correspondence between chemotype affiliation and *Microcystis* morphotypes. Similarly, phylogenetic classifications show little or no agreement with the widely variable oligopeptide compositions (Neilan et al. 1997; Rounge et al. 2010). Despite these incongruences, our study shows that chemical conspecific polymorphisms in genus *Microcystis* delineate subpopulations that interact differently with their environment in terms of sedimentation losses, pelagic growth, and habitat distribution. Therefore, chemical diversification represents a novel dimension in cyanobacterial biodiversity, which encapsulates functional units with a potentially greater ecological significance than that of the morphology-based concept of species alone.

The present study reveals the existence of a highly diverse community of *Microcystis* chemotypes in Valmayor reservoir, which are subject to marked dynamic changes in their composition, leading to a continuous succession of chemotypes along the season. Coincident with previous studies in nutrient-rich waterbodies (Welker et al. 2007), the high diversity of chemotypes in Valmayor reservoir at a given time point was characterized by the presence of a few abundant dominating subpopulations and a remarkable number of chemotypes coexisting at low cell densities. The disappearance of abundant chemotypes throughout the season gave rise to a sharp increase in other subpopulations. The resulting succession of chemotypes during the season was shown to critically control average microcystin cell quotas in the population as a result of fluctuations in the proportion of toxigenic and non-toxigenic subpopulations. This is of special relevance when addressing uncertainties derived from the wide variations in toxin concentrations typically observed during bloom events. The inclusion of chemotypical approaches using MALDI-TOF MS in the development of monitoring strategies of cyanobacterial blooms has, in fact, been recently proposed (Agha et al. 2012) and constitutes a rapid and simple alternative to quantify the proportion of toxigenic clones within a population, compared with genetic approaches based on the amplification of genes responsible for toxin production.

The temporal dynamics of *Microcystis* chemotypes were shown to be synchronic in both horizontal and vertical gradients. No clear differences in chemotype assemblages among pelagic and littoral areas were evident. Similarly, no correlation between depth and the distribution of individual *Microcystis* chemotypes in the water column could be found. The uniform vertical distribution of chemotypes is attributable to buoyancy control and consequent daily vertical migration of *Microcystis* colonies, but contrasts with the depth-dependent distribution of *Planktothrix* chemotypes observed by Rohrlack et al. (2008). In that case, however, *Planktothrix* oligopeptide chemotypes and gas vesicle genotypes (*sensu* Beard et al. 2000) showed a full agreement, and hence it seems difficult to ascertain whether oligopeptides are effectively involved in the vertical distribution of chemotypes. Despite the homogenous distribution of *Microcystis* chemotypes in both vertical and horizontal gradients, chemotype compositions in the water column and sediments were markedly different. Although studies on *Microcystis* benthic recruitment indicate that pelagic re-invasion from the sediment is triggered by physical and rather unspecific factors such as temperature (Reynolds et al. 1981; Latour et al. 2004), light (Rengefors et al. 2004; Schoene et al. 2010), and sediment re-suspension and bioturbation (Stahl-Delbanco and Hansson 2002; Verspagen et al. 2005), positive selection of *mcy+ Microcystis* genotypes during recruitment has also been evidenced (Schoene et al. 2010; Misson et al. 2011). The latter indicates that benthic recruitment in genus *Microcystis,* instead of being an unspecific process solely mediated by physical factors, might act as a selective process influencing the composition of strains in pelagic summer populations.

Although a putative involvement of oligopeptides (not only MCs) in the successful re-invasion of the water column remains to be examined, the segregation of chemotypical subpopulations among benthic and pelagic habitats indicates that individual chemotypes go through shifts in their annual life-cycle with different outcomes and strongly suggest differences in their interaction with the environment.

The succession of chemotypes in Valmayor reservoir was generally reflected well by differential participation in sedimentation losses among chemotypes at each trap-sampling date (Fig. 7). Recorded whole-population sedimentation rates are in the range of other studies (Reynolds and Rogers 1976; Fallon and Brock 1980; Verspagen et al. 2005), displaying sedimentation maxima toward the end of the season. However, sedimentation rates of the dominant chemotypes greatly exceeded whole-population rates, especially during the last 2 weeks before mixing. Strikingly, sedimentation rates of CT14 showed maximum values of $0.34 \pm 0.1\ d^{-1}$, corresponding to sinking velocities of $6.8 \pm 2\ m\ d^{-1}$, which are, to our knowledge, the highest settling rates reported for *Microcystis*. However, although sedimentation rates reported for *Microcystis* typically refer to whole-population rates, no studies specifically addressed the settling patterns of subspecific populations. The moderate rates recorded at the whole-population level and the conspicuously higher settling rates of some dominant chemotypes indicate that these subpopulations are the ones primarily contributing to *Microcystis* losses at a given time point and suggest that chemotypes represent subpopulations that undergo sedimentation losses with dissimilar severity. Carbohydrate accumulation and reduced respiration under low temperatures (Visser et al. 1995), as well as the attachment of sediment particles to the mucilage (Verspagen et al. 2006), have been proposed as possible mechanisms resulting in loss of buoyancy and responsible for *Microcystis* sedimentation. Although such physical and rather unspecific processes may result in unspecific settling (e.g., during autumnal temperature decrease), these mechanisms alone seem insufficient to explain the differences in sedimentation patterns among chemotypes. Instead, the significant differences in net growth and sedimentation rates among subpopulations suggest that individual chemotypes present dissimilar physiological conditions at a given time point that result in asynchronous proliferation of chemotypes along the season, leading to a continuous succession of subpopulations. In addition, whereas *Microcystis* losses were mainly caused by sedimentation, our data also indicate that individual chemotypes (in this study, CT11) may undergo massive chemotype-specific loss processes unrelated to sedimentation. Although the nonappearance of CT11 colonies in the traps evidences the absence of substantial sedimentation losses affecting this chemotype, the elucidation of such alternative loss process (es) falls outside the scope of this study and hence remains unclear. However, the existence of chemotype-specific loss processes further supports the idea that *Microcystis* chemotypes represent bona fide ecologically distinct subpopulations that interact differently with their environment.

Individual chemotypes in their environment can be controlled either by bottom-up or top-down mechanisms. Bottom-up control processes are mediated by resource competition among chemotypes (e.g., light, nutrients). However, no significant correlation between temperature, light availability, or macronutrients and the prevalence of particular chemotypes could be found. Similar results were obtained elsewhere for genus *Planktothrix* (Rohrlack et al. 2008), raising questions about the existence of bottom-up mechanisms effectively controlling oligopeptide chemotypes. Conversely, top-down mechanisms (e.g., grazing, viral lysis, parasitism) shaping the dynamics of chemotypes constitute a more plausible explanation: Sonstebø and Rohrlack (2011) could demonstrate the existence of chemotype-selective chytrid infection in genus *Planktothrix* and proposed an evolutionary scenario in which *Planktothrix* chemotypes constitute functional evolutionary units that co-evolve with parasites by reciprocal adaptations. Because of their shorter generation times, parasites typically display higher evolutionary rates. Under these circumstances, hosts would be strongly selected toward diversification (De Bruin et al. 2008), resulting in the subdivision of *Planktothrix* into different chemotypes with different susceptibility to parasites. In fact, recently, Rohrlack et al. (2013) convincingly showed by knockout mutagenesis that oligopeptides microcystins, anabaenopeptins, and microviridins could play a major role as antichytrid compounds in genus *Planktothrix*. Although formulated for genus *Planktothrix,* this hypothesis could in principle explain the metabolic diversification of *Microcystis* into oligopeptide-based subpopulations. Rather than maintaining an ideal genotype, *Microcystis* may profit from preserving a wide array of chemotypes and thereby hamper the ability of parasites to optimally exploit the whole population. In our study, the dramatic decline of the most abundant chemotype toward the end of the season (CT11, accounting for 35–39% of the total *Microcystis* population) and its subsequent absence in the sediment traps is compatible with the idea of chemotype-selective epidemics; according to the kill-the-winner concept (Thingstad and Lignell 1997), the most abundant host population (i.e., superior competitor) is also the one most affected by pathogenic top-down control, resulting in the maintenance of a highly diverse community in spite of competitive differences among coexisting chemotypes. Furthermore, chytrid infection has been observed to induce rapid changes in the buoyancy of the host (T. Rohrlack pers. comm.), which might explain the chemotype-specific sedimentation patterns recorded in this study.

Existing research on naturally occurring cyanobacterial oligopeptide chemotypes shows wide differences in terms of diversity. Welker et al. (2007) found 37 distinct *Microcystis* chemotypes in hypereutrophic Brno reservoir (Czech Republic) in a period of 5 months, while we identified 53 *Microcystis* chemotypes in eutrophic Valmayor reservoir in the same period. In another study, 15 different *Planktothrix* chemotypes could be isolated from a single plankton net sample taken from lake Maxsee, Germany (Welker et al. 2004). This contrasts with the remarkably lower diversity of *Planktothrix* chemotypes in the oligotrophic lake Steinsfjorden (Norway), where only four chemotypes were observed over more than three decades (Rohrlack et al. 2008). In the frame of the proposed evolutionary scenario, these marked differences in chemotype diversity, although observed for

different genera, could be related to the trophic state of the different ecosystems. In contrast to oligotrophic waterbodies, where species co-existence and plankton diversity is promoted (i.e., wide host ranges), highly eutrophic systems typically display lower specific diversity and are usually dominated by oligo- or monospecific cyanobacterial communities, represented by one or a few dominant taxa (Rasconi et al. 2012). These communities constitute rather narrow host ranges that can be expected to be more readily exploited by the pool of co-existing parasites. We therefore hypothesize that monospecific cyanobacterial communities, typically occurring in eutrophic systems, undergo intense selective pressure by coexisting parasites and pathogens and therefore are more strongly selected toward diversification (De Bruin et al. 2008), resulting in populations characterized by a more diverse community of chemotypes. Although this hypothesis remains to be tested, it provides a plausible explanation for the marked differences in chemotype diversity reported elsewhere.

In summary, this study revealed the co-existence of highly diverse *Microcystis* oligopeptide-based subpopulations in Valmayor reservoir, whose dynamics proved to be responsible for fluctuations in the average toxicity of the population. We demonstrate that the succession of chemotypes results from temporal differences in chemotype-specific net growth and losses. Although such losses generally lead to colony sedimentation, we provide evidence for alternative loss process other than sedimentation selectively affecting individual chemotypes. Although the nature of such processes remains to be clarified, the existence of extensive chemotype-selective loss processes, the temporal differences in terms of sedimentation during pelagic growth, as well as the partitioning of subpopulations among benthic and pelagic habitats, clearly demonstrate that *Microcystis* chemotypes interact differently with their environment and display dissimilar ecological traits. Recognizing the subpopulation level as a relevant basis for biological process thus seems needed to increase our understanding of the dynamics and ecology of cyanobacterial populations.

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