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Overwintering populations of *Anabaena*, *Aphanizomenon* and *Microcystis* as potential inocula for summer blooms

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Overwintering cyanobacterial populations of Nostocales and *Microcystis* were investigated in six freshwater reservoirs in Northwestern Spain during two consecutive winters. Surface sediments hosted 10^3–10^5 akinetes mL^{-1} and 10^2–10^4 *Microcystis* colonies mL^{-1}. Sediments from deeper areas close to dam accumulated 2-fold (*Microcystis*) and 11-fold (akinetes) greater concentrations than those at the shallower upstream areas. *Anabaena* spp. and *Microcystis aeruginosa* dominated the sediment pool, with minor amounts of akinetes of *Aphanizomenon* (*Aph. flos-aquae*, *Aph. gracile*) and benthic Nostocales (*Cylindrospermum*, *Nostoc* and *Trichormus*). Our study confirms the dual benthic-pelagic overwintering of *Anabaena, Aphanizomenon* and *Microcystis*, found in the pelagial at 7.5–9.8°C. This study also provides an insight into the little known annual cycle of potential cyanotoxin-producers *Aph. gracile* and *Anabaena circinalis*. Our estimates show that: (i) only a small fraction (<1%) of the sediment pool of akinetes and *Microcystis* was resuspended in the bottom water during winter which, however, may be sufficient inocula to build up the summer maxima under realistic *in situ* growth rates; and (ii) the time required for the development of summer populations is mainly driven by growth rates, and therefore by the environmental conditions faced by the inoculum, with a lower influence (although greater for *Microcystis* than for Nostocales) of the inoculum size.

KEYWORDS: *Anabaena*; *Aphanizomenon*; *Microcystis*; annual cycle; akinetes; sediment

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INTRODUCTION

Planktonic cyanobacteria have achieved notable success in aquatic ecosystems worldwide. Their ability to survive periods of adverse conditions, such as winters in temperate regions or droughts in subtropical or tropical areas, is considered to be one of the main reasons for their ecological success. The survival strategies of cyanobacteria comprise the formation of specialized spore-like cells (akinetes) in the orders Nostocales and Stigonematales and of "dormant" colonies in the order Chroococcales. The planktonic Nostocalean genera *Anabaena* and *Aphanizomenon* and the colonial Chroococcalean *Microcystis* are representative of these two strategies which pose a great concern for water management due to their frequent involvement in bloom formation and cyanotoxin production worldwide (Sivonen and Jones, 1999).

Microcystis and planktonic Nostocales show similar annual life-cycles in temperate freshwaters (Reynolds *et al.*, 1981; Hense and Beckmann, 2006). Summer blooms are followed by autumnal settling of resting stages (akinetes in Nostocales and colonies in *Microcystis*) to the bottom sediments. Here, they overwinter until improved water conditions in spring lead to the reinvasion (recruitment) of the water column supported by increased buoyancy. In Nostocales, the reinvasion of the watercolumn is preceded by the process of akinete germination which generates young buoyant trichomes (Hense and Beckmann, 2006).

Microcystis colonies and Nostocales akinetes overwintering in lake and reservoir sediments have been studied to reconstruct the short-term ecological history of cyanobacteria (Rasanen *et al.*, 2006; Rinta-Kanto *et al.*, 2009; Wood *et al.*, 2009). Furthermore, the role of benthic *Microcystis* and akinetes as potential inocula for subsequent summer blooms has evident implications for the water management strategies conducted to predict (e.g. by mathematical models) (Verspagen *et al.*, 2005; Hense and Beckmann, 2006) and to provide remedial actions (e.g. by dredging or drying the sediments) (Poulickova, 1998) to mitigate the effects of algal blooms.

The presence of large benthic populations of *Microcystis* and Nostocales akinetes has been observed in several studies (Preston *et al.*, 1980; Baker, 1999; Latour *et al.*, 2007). Benthic *Microcystis* and akinetes can remain viable for a long time. This has been demonstrated by the germination of akinetes deposited 120-years b.p. (Wood *et al.*, 2009) and the successful culturing of *Microcystis* colonies buried under 30 cm of sediment (Bostrom *et al.*, 1989). It has been suggested that factors such as light, temperature and oxygen concentration influence the preservation and hence the viability of akinetes and dormant *Microcystis* in sediments (Brunberg and Blomqvist, 2003; Karlsson-Elfgren and Brunberg, 2004; Schöne *et al.*, 2010).

Nostocales and *Microcystis* may overwinter not only in the benthos, but also as small pelagic populations (Verspagen *et al.*, 2005; Suikkanen *et al.*, 2010). For *Microcystis*, most studies consider a predominantly benthic inoculum (Brunberg and Blomqvist, 2003; Ihle *et al.*, 2005; Schöne *et al.*, 2010), whereas others ascribe the main role to the small-size pelagic population considering the sediments to be more of a sink than a source of *Microcystis* (Verspagen *et al.*, 2005).

After overwintering, increased water temperatures and sufficient light intensities facilitated by sediment resuspension, are among the factors which may trigger akinete germination (Kaplan-Levy *et al.*, 2010) and/or the reinvasion of the water column by *Microcystis* colonies (Brunberg and Blomqvist, 2003; Verspagen *et al.*, 2005; Schöne *et al.*, 2010) and young Nostocales filaments (Hense and Beckmann, 2006). The active (by buoyancy regulation) or passive (resuspension driven) nature of reinvasion still remains under discussion hence indicating a combination of both processes. Furthermore, water body morphology (shallow vs. deep areas) may be a key factor influencing the overwintering and reinvasion processes.

The identification of akinetes and *Microcystis* colonies in sediments is traditionally based on their morphology (Ihle *et al.*, 2005; Kravchuk *et al.*, 2006). Therefore, either some knowledge of the cyanobacterial populations in the water during previous years, or the use of germination experiments is recommended (Wood *et al.*, 2009). Recently, less time-consuming molecular tools (like DGGE or Real Time-PCR) have increasingly been used to analyze *Microcystis* (Innok *et al.*, 2005; Rinta-Kanto *et al.*, 2009; Kim *et al.*, 2010) and Nostocales (Wood *et al.*, 2009) in sediments. However, these methods have the drawback of not being able to distinguish between intact colonies/akinetes and cellular debris. In this sense, the CARD-FISH method recently developed by (Ramm *et al.*, 2012) is a promising tool to identify integer Nostocales akinetes in sediments, at a genus level.

Previous research into *Microcystis* and akinetes in sediments has mainly been performed in lakes (Tsujimura *et al.*, 2000; Rasanen *et al.*, 2006; Rinta-Kanto *et al.*, 2009; Rücker *et al.*, 2009; Wood *et al.*, 2009) but less often in reservoirs (Kravchuk *et al.*, 2006; Kim *et al.*, 2010), water bodies with a different hydro physical behaviour (Margalef, 1983). Information about the overwintering of *Microcystis* and Nostocales in reservoirs of Southern Europe is still very scarce despite the fact that cyanobacterial blooms are frequent phenomena in this area (Quesada *et al.*, 2004; Carrasco *et al.*, 2006) and that *Anabaena*, *Aphanizomenon* and *Microcystis* are major components of these blooms (De Hoyos *et al.*, 2004; Carrasco *et al.*, 2006).

The present study aims to provide an insight into cyanobacterial overwintering in freshwaters of Southern Europe by monitoring the sediment and water of six reservoirs in Northwestern Spain during two consecutive winters (2006–2007 and 2007–2008). We determined the size and species composition of the sediment pool of Nostocales akinetes and benthic *Microcystis*, and investigate the spatial and inter-annual differences in the distribution of this sediment pool. Furthermore, we estimated the resuspension of resting stages from sediment to bottom water and their role as potential inocula for summer blooms.

METHOD

Sampling sites

The six reservoirs studied (Table I) are located in Northwestern Spain, within the Cantabrico watershed (La Barca and Trasona) and Miño-Sil watershed (Cachamuiñas, las Conchas, Salas and Prada). Their trophic status varied from mesotrophic to eutrophic. Their mean depths ranged from 6.7 m (Trasona) to 20.2 m (Prada). All the reservoirs were characterized by a marked thermal stratification in summer and winter mixing, except for Trasona and Cachamuiñas which only stratified weakly during short periods in the summer (data not shown). Furthermore, Cachamuiñas showed an unusually low water depth (1–4 m) during the summers of 2006, 2007 and 2008.

Water and sediment sampling

The water and sediments of the six reservoirs were monitored during 2006, 2007 and 2008. Two sampling sites were established within each reservoir, a deeper one close to the dam and a shallower one located upstream. The depth at the sampling sites ranged 3–65 m at the dam sites and 1–30 m at upstream sites (Table I).

On each of the sampling dates in summer and winter, the water column was characterized by vertical profiles of temperature and chlorophyll *a* concentration using a YSI6920 multiparametric probe. Epilimnetic water samples were taken once in the six reservoirs in July–August 2006, July–September 2007 and August–September 2008. An additional water sample was taken in spring (May–June) 2009 in each of the Cachamuiñas, Las Conchas, Prada and Trasona reservoirs. In winter, the epilimnetic samples were only taken when chlorophyll *a* was detected in the vertical profile. All epilimnetic water samples were taken with a 5-L watersampler integrating the water layer from surface to 2.5 times the Secchi depth as a proxy of the euphotic zone (Strickland, 1958).

Bottom sediments were sampled in the six reservoirs in January–February 2007 and January–February 2008. The top 5 cm of the sediments was collected using an Eckmann dredge and stored in black plastic bags. Simultaneously, samples of bottom water were taken with a 5-L watersampler at 1.5 m above the sediment surface. Additional sediment and bottom water samples were taken during late-spring (May–June) 2009 in each of the Cachamuiñas, Las Conchas, Prada and Trasona reservoirs.

All water and sediment samples were kept at 4°C in darkness and transported to the laboratory within 24 h for subsequent analysis. At the laboratory, sediment samples were homogenized by manual kneading and divided into two subsamples. One of the subsamples was fixed with 4% (v/v) formaldehyde and preserved in darkness at 4°C for microscopic analysis. The second subsample was kept at 4°C in darkness for germination experiments, which were carried out within 24 h of arriving at the laboratory.

Identification and quantification of cyanobacteria in epilimnetic water

Total cyanobacterial biomass in epilimnetic water was estimated as chlorophyll *a* concentration (μg Chl*a* L^{-1}) using a benchtop fluorometer (Moldaenke BBE Algae Analyser).

Table I: Characteristics of the water reservoirs studied

Reservoir	Watershed	River	Trophic status	Volume (hm^3)	Depth at sampling points (m)[a]	
					Dam	Upstream
Cachamuiñas	Miño-Sil	Lonia	Eutrophic	0.03	3–11	1–6
La Barca	Cantábrico	Narcea	Meso-Eutrophic	31.1	47–55	25–30
Las Conchas	Miño-Sil	Limia	Meso-Eutrophic	78.3	26–31	6–10
Prada	Miño-Sil	Jares	Mesotrophic	121.1	55–65	13–18
Salas	Miño-Sil	Salas	Mesotrophic	86.9	21–30	6–10
Trasona	Cantábrico	Corbera	Eutrophic	4.1	11–12	4–6

[a]Depth ranges corresponding to the whole study period (2006–2009).

The identification of planktonic cyanobacterial species was performed in a 1-L subsample placed in a wide-mouthed polypropylene bottle and left undisturbed at room temperature overnight. The buoyant cyanobacteria that had accumulated at the surface were collected by a Pasteur pipette, fixed with 4% (v/v) formaldehyde and kept at 4°C until microscopic analysis. Microscopic identification was carried out using an Olympus BH-2 light microscope (Olympus, Germany) equipped with a Leica DC 300F digital camera (Leica Microsystems, Germany). Species identification followed (Komárek and Anagnostidis, 1989, 1999, 2005). Cyanobacterial dominated samples were fixed with acidified Lugol's iodine and kept at 4°C in darkness until sedimented and quantified by Utermöhl's method (Utermöhl, 1958).

Identification and quantification of Microcystis and Nostocales akinetes in sediments and bottom water

The identification of cyanobacterial resting stages in sediments was performed by epifluorescence microscopy on filters containing diluted sediment material. One millilitre of sediment fixed with formaldehyde was diluted 50-fold with GF/F filtered hypolimetic water from the same reservoir. One millilitre of this diluted material was then gently vacuum filtered through a 41-μm-pore nylon mesh to retain most of the inorganic material as well as *Microcystis* colonies and big akinetes. The filtered suspension was recovered and gentle-vacuum filtered through a 25-mm diameter, 0.2-μm pore Anodisc membrane filter (Whatman, England) to retain most of the akinetes and cell debris. The 41-μm mesh and the 0.2-μm Anodisc filter were mounted separately onto microscope slides with a drop of antifading cover-slip medium Aqua-Poly/Mount (Polysciences, Inc., USA). For bottom water, the filtration and mounting procedure was identical to that applied to the sediment, but using 500−1000 mL original water.

Both the 41-μm and 0.2-μm filters were observed under an Olympus BH-2 epifluorescence microscope (Olympus, Germany) equipped with a Leica DC 300F digital camera (Leica Microsystems, Germany). The epifluorescence system BH2-RFCA (Olympus, Germany) consisted of an OSRAM Short Arc HBO UV Hg lamp (OSRAM GmbH, Germany), an excitation filter BP545, a dicroic mirror DM570 and an emission filter O590; the result was green light excitation and visualization of red autofluorescence emitted by cyanobacterial pigments phycocyanins and phycoerythrins.

The entire filter surface was microscopically checked for the presence of akinetes and *Microcystis* colonies. Micrographs of both types of organism were then taken and the cell dimensions (diameter and length) measured using the image analysis software Leica Qwin (Leica Microsystems, Germany). Identification of *Microcystis* species followed (Komárek and Anagnostidis, 1999). Only those *Microcystis* maintaining colony integrity were included in the analyses. The akinetes of Nostocales species were identified based on Komárek and Anagnostidis (Komárek and Anagnostidis, 1989; Li *et al.*, 2000; Rajaniemi *et al.*, 2005; Komárek and Komárkova, 2006). As akinete morphology shows wide intra-species variation and as dimension ranges often overlap in different taxa, we elaborated a list of Nostocales species potentially present in each of the reservoirs based on: (i) the species found in epilimnetic water during the years 2006, 2007 and 2008; (ii) the species resulting from the germination experiments; (iii) some species with very characteristic akinetes, like the long-cylindrical *Aphanizomenon flos-aquae* akinetes, even if they were not found in water or in germination experiments. Only intact fluorescent akinetes were included in the analyses.

Quantification of *Microcystis* cells and akinetes was performed by counting all cells present on the entire surface of each of the two filters (41 and 0.2 μm), and summing both partial counts. The final result was expressed as cells mL^{-1} of fresh sediment after taking into account filtered volumes and the dilution factor applied in each case. Considering microscopic counts as Poisson distributed, and taking into account the dilution factors applied in each of the sediment samples, the detection limit of our technique was 150 akinetes mL^{-1} sediment and 50 *Microcystis* colonies mL^{-1} sediment for each taxon present. Average cell biovolumes (*Microcystis* cells, akinetes) were calculated by assimilating cells to regular geometric bodies and measuring relevant dimensions in at least 200 cells from 20 different colonies or filaments. Cell counts and biovolumes were standardized to wet weight (WW) and dry weight (DW) of each of the sediments. The WW (g mL^{-1}) was determined by weighing 2 mL of fresh sediment placed in a Petri dish. To obtain the DW in g mL^{-1}, the 2 mL were then desiccated at 65°C and weighed periodically until the sample reached a constant weight (typically in 24 h). Organic matter was determined by combustion (500°C, 4 h).

Germination experiments

Sediment samples from the six reservoirs collected in winter 2007−2008 were subjected to two types of germination experiments. The first experiment consisted of diluting 2 g of fresh sediment in 100-mL Erlenmeyer flasks with 50 mL of either $BG11_0$ medium (without combined nitrogen) (Rippka *et al.*, 1979) or modified-BG11 medium diluted 1:4 with deionised water (Sanchis

et al., 2004). The flasks were kept under two intensities of white continuous light (30 and 60 μmol photons m^{-2} s^{-1}) until cyanobacterial biomass was macroscopically visible (normally 1–2 weeks). The second experiment included a pre-concentration step of the cyanobacterial biomass in the sediments by density-gradient centrifugation. Three grams of fresh sediment was added to 30 mL of centrifugation medium containing 90% (v/v) Percoll (Amersham Biosciences, Sweden) and 10% (v/v) sucrose 2.5 M and centrifuged at 4°C (20 000 × *g*; 15 min). Centrifugation facilitated the separation of the inorganic matrix of the sediment (which remained at the bottom of the centrifugation tubes) from the cyanobacterial biomass and cell debris which accumulated in the top centimeters. The top centimeters were recovered by Pasteur pipette and filtered through a 10-μm-pore nylon mesh, which was then rinsed with 2 mL of GF/F-filtered bottom water from the corresponding reservoir. The aliquots were placed in sterile 24-multiwell plates containing 2 mL of each culture medium per well, which were kept at 30 and 60 μmols photons m^{-2} s^{-1} until the cyanobacterial biomass was macroscopically visible (in 1–2 weeks). Cyanobacterial suspensions obtained from both kinds of experiments were checked under the microscope and the Nostocales and/or *Microcystis* species were identified following the criteria described above.

RESULTS

Planktonic cyanobacteria in epilimnetic water

The abundance (as Chl *a* concentration) and species composition of cyanobacteria in epilimnetic water are summarized in Table II. Maximum cyanobacterial Chl *a* concentrations ranged from 2.6 μg Chl *a* L^{-1} in Salas dam to 43.4 μg Chl *a* L^{-1} in La Barca dam. Maximum cyanobacterial abundances in the dam and upstream were similar in Cachamuiñas, Las Conchas and Salas, but not in Trasona, Prada and, especially, La Barca, where maximum Chl *a* was higher in the dam than upstream (1.7-fold in Trasona; 3-fold in Prada and 10-fold in La Barca).

Microcystis spp. were present at both sampling sites of La Barca, Las Conchas and Trasona, and in Prada dam. They were not detected either in Cachamuiñas or in Salas. Planktonic Nostocales were present in all six reservoirs, with *Aphanizomenon* spp. appearing in two (Cachamuiñas and Las Conchas) and *Anabaena* spp. in the other four reservoirs. *Anabaena circinalis* was the only Nostocales identified in both sites of Prada and Salas. Other buoyant cyanobacteria, namely *Woronichinia naegeliana* (Chroococcales) and *Planktothrix agardhii* (Oscillatoriales) were also observed in some epilimnetic water samples from Las Conchas, La Barca and Trasona (Table II).

Nostocales akinetes in reservoir sediments

The overall results for akinete abundance and species composition are summarized in Table III. Akinete quantities were low in La Barca and Trasona, with up to 1900 and 2700 akinetes mL^{-1} sediment, and moderate in Salas, Cachamuiñas and Las Conchas with up to 11 100, 17 000 and 17 900 akinetes mL^{-1} of sediment, respectively. The Prada reservoir showed particularly high akinete concentrations of up to one order of magnitude higher than the other reservoirs, with 186 900 akinetes mL^{-1} found at the dam on 10 January 2008. Dam sites showed the highest akinete abundance in the six

Table II: Cyanobacterial presence in epilimnetic water

					Planktonic cyanobacteria (presence)[b]		
Reservoir	Point	Period	Samples (*n*)	Chl *a*[a] (μg L^{-1})	*Microcystis* spp.	Nostocales	Others
Cachamuiñas	Dam	2006–2008	4	0–11.9	—	6	—
	Upstream	2006–2008	4	0–10.9	—	6	—
La Barca	Dam	2006–2008	3	0–43.2	7	1, 4	10
	Upstream	2006–2008	3	0–4.1	7	1, 4	—
Las Conchas	Dam	2006–2008	4	0.5–4.5	7, 8	—	—
	Upstream	2006–2008	4	0.1–4.6	7, 8	5	10, 11
Prada	Dam	2007–2008	2	5.1–6.2	7	2	11
	Upstream	2007–2008	2	6.6–18.3	—	2	11
Salas	Dam	2007–2008	2	0.6–2.6	—	2	—
	Upstream	2007–2008	2	0.4–2.8	—	2	—
Trasona	Dam	2006–2008	4	5.2–28.4	7, 8, 9	1, 3	10, 11
	Upstream	2006–2008	4	0–16.9	7, 8, 9	1, 3	10, 11

[a]Cyanobacterial Chl *a* concentrations estimated by fluorometry.
[b](i) *Anabaena crassa*; (ii) *Anabaena circinalis*; (iii) *Anabaena flos-aquae*; (iv) *Anabaena planctonica*; (v) *Aphanizomenon flos-aquae*; (vi) *Aphanizomenon gracile*; (vii) *Microcystis aeruginosa*; (viii) *Microcystis flos-aquae*; (ix) *Microcystis wesenbergii*; (x) *Planktothrix agardhii*; (xi) *Woronichinia naegeliana*.

Table III: Akinete abundance in bottom sediments

Reservoir	Point	Total akinete abundance				Dominant species
		10^3 cells mL^{-1}	10^3 cells g^{-1} WW	10^3 cells g^{-1} DW	mm^3 L^{-1}	
Cachamuiñas	Dam	17	15.2	85	67.1	*Anabaena* spp.
	Upstream	0.2–1.2	0.2 – 1.0	0.9–5.2	2.3–3.4	*Anabaena* spp.
La Barca	Dam	0.2–1.9	0.2 – 1.8	1.0–9.0	0.5–5.1	*Anabaena* spp.
	Upstream	0.7–1	0.5–0.7	2.1–2.9	1.6–2.1	*Anabaena* spp.
Las Conchas	Dam	0.3–17.9	0.3–17.5	0.9–55.9	0.5–29.4	*Anabaena* spp.
	Upstream	4.3	3.6	15.9	5.8	*Anabaena* spp.
Prada	Dam	111.1–186.9	113.8–190.7	796.4–1335.0	188.5–327.2	*A. circinalis*
	Upstream	2.9–6.6	2.5–5.8	8.5–19.4	5.9–13.1	*A. circinalis*
Salas	Dam	6.7–11.1	6.0–9.9	25.8–42.7	13.7–23.2	*A. circinalis*
	Upstream	0.7–2.7	0.6–2.3	1.5–5.7	1.4–4.8	*A. circinalis*
Trasona	Dam	0.5–2.7	0.5–2.5	1.9–10.4	2.1–10.8	*Anabaena* spp.
	Upstream	0.9–2.2	0.8–2.0	3.9–9.6	1.5–3.8	*Anabaena* spp.

DW, dry weight; WW, wet weight; nd, not detected.
Numbers refer to the range of all samples analyzed, except for Cachamuiñas dam and Las Conchas upstream where only one sample was available.

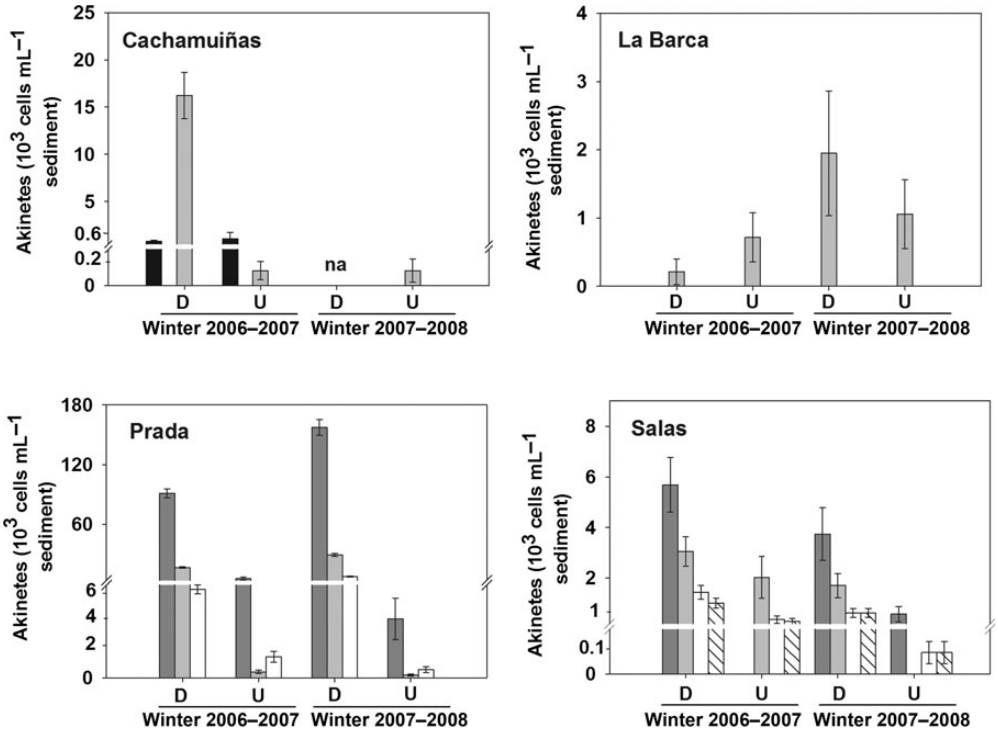

Fig. 1. Species composition of akinetes overwintering in sediments of Nostocales-dominated reservoirs. D: dam, U: upstream. Dark grey bar: *Anabaena circinalis*; Grey bar: Other *Anabaena spp*; White bar: *Aphanizomenon flos-aquae*; Black bar: *Aphanizomenon gracile*; Striped bar: benthic Nostocales; Na: not analyzed. Error bars represent 95% confidence intervals for counts.

reservoirs studied, representing on average 11-fold higher than those at upstream site in the same reservoir. Significant inter-site variations were observed, ranging from 1.2-fold in Trasona to 63-fold in Prada.

Figure 1 shows the species composition of akinetes in the Nostocales-dominated reservoirs. Planktonic members of the genus *Anabaena* dominated the akinete pool in all the reservoirs and in almost all the sediment samples analyzed ($n = 22$) with the only exception of Cachamuiñas upstream in 2006−2007, which was dominated by *Aph. gracile* (Fig. 1). *Anabaena circinalis* was the dominant species in Prada and Salas, representing up to 63 and 89% of the akinetes in each reservoir, respectively. Other *Anabaena* species included in the taxonomic complexes *A. flos-aquae/lemmermannii/mendotae*, *A. planctonica/viguieri/smithii* and *A. crassa/spiroides* dominated the akinete record in

Cachamuiñas, La Barca, Las Conchas and Trasona and were also present in Prada and Salas. The genus *Aphanizomenon* appeared in Cachamuiñas, which was subdominated by *Aph. gracile*, whereas akinetes of *Aph. flos-aquae* were present in Las Conchas, Prada and Salas despite representing <4% of the akinete pool. Interestingly, we also found quantifiable numbers of akinetes of benthic Nostocales *Cylindrospermum* sp., *Nostoc* sp. and *Trichormus variabilis* in Salas (Fig. 1) but these were below our quantification limit (150 akinetes mL^{-1}) in Cachamuiñas, Prada and Trasona. Of the six reservoirs, Prada and Salas showed the highest diversity of akinetes, with low interannual variations in abundance and species composition. Cachamuiñas, La Barca, Las Conchas and Trasona showed lower akinete diversity but more pronounced annual variations.

Microcystis in reservoir sediments

Microcystis colonies were detected in the sediment of four reservoirs (La Barca, Las Conchas, Prada and Trasona) (Table IV) but were not observed either in the water or the sediments of Cachamuiñas and Salas.

La Barca, Trasona and Prada had moderate abundances of *Microcystis* of up to 134 000, 266 000 and 301 000 cells mL^{-1}, respectively. Las Conchas hosted a much more abundant benthic *Microcystis* population with >14 million of cells mL^{-1}, equivalent to ~28 000 colonies mL^{-1}. Similar to the results found for akinetes, dam sites had higher maximum *Microcystis* concentrations, the only exception being Trasona in the winter of 2006–2007. Dam vs. upstream ratios varied from 1.1 (Trasona) to 4 (La Barca). The most extreme case was Prada, with >300 000 cells mL^{-1} in the dam but no *Microcystis* detected upstream.

Microcystis aeruginosa was the dominant species in the four reservoirs (Table IV). *Microcystis flos-aquae* was present in Las Conchas in small amounts, and in Trasona representing up to 48% of the cells (dam, winter 2007–2008) (Fig. 2).

Akinetes and Microcystis in bottom water: an estimation of resuspension events

Akinetes and *Microcystis* colonies were also present in bottom water samples taken at 1.5 m above the sediment

Table IV: Microcystis *abundance in bottom sediments*

		Total *Microcystis* abundance				
Reservoir	Point	10^4 cells mL^{-1}	10^4 cells g^{-1} WW	10^4 cells g^{-1} DW	mm^3 L^{-1}	Dominant species
La Barca	Dam	nd – 13.4	nd – 12.6	nd – 63.7	nd – 3.7	*M. aeruginosa*
	Upstream	nd – 3.4	nd – 2.4	nd – 10.0	nd – 0.9	*M. aeruginosa*
Las Conchas	Dam	426.2–1434.6	417.9–1406.4	1332.0–4483.0	248.4–470.0	*M. aeruginosa*
	Upstream	314.7	262.3	1165.7	109.1	*M. aeruginosa*
Prada	Dam	nd – 30.1	nd – 30.7	nd – 215.2	nd – 28.0	*M. aeruginosa*
	Upstream	nd	nd	nd	nd	—
Trasona	Dam	9.1–26.6	8.4–24.6	35.1–102.2	10.8–13.9	*M. aeruginosa*
	Upstream	18.1–25.8	16.7–23.9	78.5–112.2	11.6–12.7	*M. aeruginosa*

DW, dry weight; WW, wet weight; nd, not detected.
Numbers refer to the range of all samples analyzed, except for Las Conchas upstream where only one sample was available.

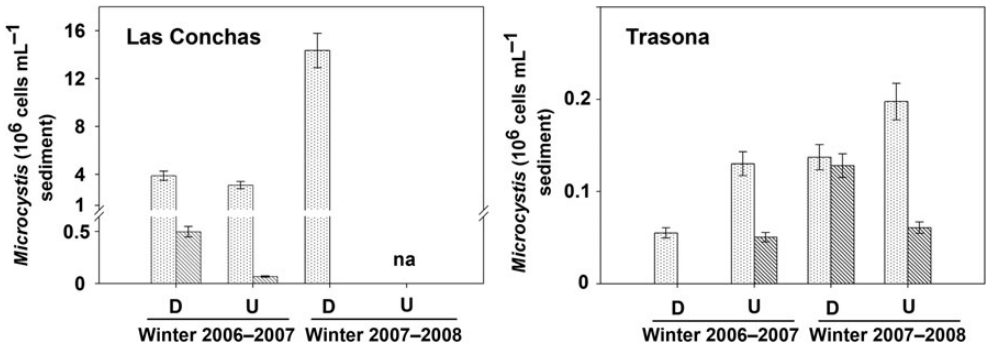

Fig. 2. Species composition of *Microcystis* overwintering in the sediments of *Microcystis*-dominated reservoirs. D: dam, U: upstream. Dotted vertical bar: *Microcystis aeruginosa*; Stripped vertical bar: *Microcystis flos-aquae*; Na: not analyzed. Error bars represent 95% confidence intervals for counts.

surface, although in very low concentrations, <4 akinetes mL^{-1} and 240 *Microcystis* cells mL^{-1} (data not shown).

We estimated the akinete and *Microcystis* concentration per unit area (cells cm^{-2}) in the first 5 cm of sediment by using the sediment cell concentrations (cells mL^{-1}) (Tables III and IV) and assuming a homogeneous distribution through the whole 5-cm layer. Furthermore, we calculated akinete and *Microcystis* concentrations (cells cm^{-2}) in the 1.5-m water layer above the sediment surface by using the cell concentrations found in the bottom water (cells mL^{-1}) and also assuming that this 1.5-m layer was homogeneously distributed. This allowed us to estimate the percentage of the sediment pool of akinetes and *Microcystis* which were suspended in water (Figs 3 and 4).

During winter, resuspended akinetes represented <1% of the total akinete pool in the sediment of all six reservoirs, with a maximum of 0.5% in the water of Cachamuiñas upstream during the winter of 2007–2008. Nevertheless, this value is likely to have been influenced by the *Aph. gracile* population (12 000 cells mL^{-1}) developing in the epilimnion during that period. The maximum resuspension values in the other reservoirs were 0.02% in Las Conchas dam, 0.04% in La Barca upstream, 0.1% in Las Salas upstream and a 0.2% in Trasona dam and Prada upstream. Even with these apparently low sediment resuspension, the total akinete pool estimated for the 1.5-m water layer above the sediment was significant, varying from 15 000 (La Barca upstream) to >6 million akinetes per m^2 (Prada dam).

The late-spring samples ($n = 1$ in each reservoir) taken during May–June of 2009 in Cachamuiñas and Prada were included in the analysis (Fig. 3). The abundance of akinetes at the dam sites of both reservoirs was remarkably lower than the average of the winters 2006–2007 and 2007–2008, with a 10-fold reduction in Cachamuiñas and a 5-fold reduction in Prada, whereas the spring upstream abundances were similar to those observed in winter. The maximum resuspension in spring was found in Cachamuiñas dam (0.2%) and Prada upstream (0.1%), which in both cases was below the winter maxima of the same reservoirs.

As was found with the akinetes, the winter resuspension of *Microcystis* (Fig. 4) was very low reaching a 0.5% maximum (Trasona dam, winter 2007–2008). No *Microcystis* resuspension was observed in La Barca or Prada. The maximum values in Las Conchas were all <0.1%. Nevertheless, taking into account the huge *Microcystis* pool present in Las Conchas sediment, even these low percentages might represent up 360 million cells ($\sim 7 \times 10^5$ colonies) in each m^2 of the 1.5-m water layer above sediment. Additional late-spring samples taken in May 2009 in Las Conchas and Trasona showed that, unlike that observed for akinetes, *Microcystis* abundance in dam water was higher during spring than the average of the winters 2006–2007 to 2007–2008 in both reservoirs (Fig. 4), with a 1.8-fold increase in Las Conchas and a 2.2-fold increase in Trasona. Spring *Microcystis* abundance upstream was different in the two reservoirs, with a striking 56-fold reduction in Las

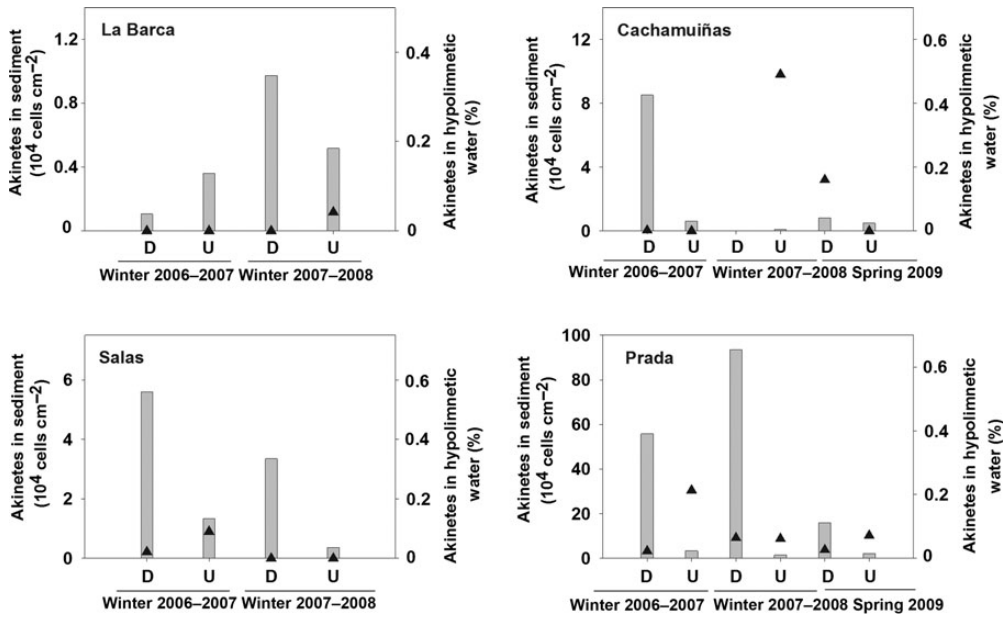

Fig. 3. Akinete pool in sediments and estimated akinete resuspension in Nostocales-dominated reservoirs. D: dam; U: upstream. Grey vertical bars represent the total akinete pool in the top 5 cm of sediment. Black triangles represent resuspended akinetes (%) in the 1.5-m water column above sediment surface. Na: not analyzed.

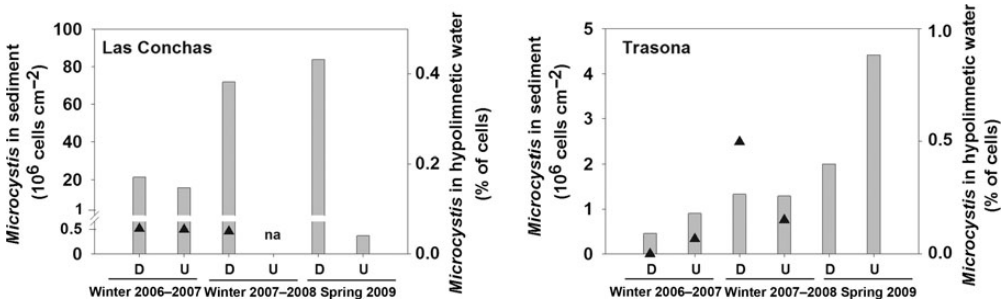

Fig. 4. *Microcystis* pool in sediments and estimated resuspension in *Microcystis*-dominated reservoirs. D: dam; U: upstream. Grey vertical bars represent the total *Microcystis* pool in the top 5 cm of sediment. Black triangles represent resuspended *Microcystis* in the 1.5-m water column above sediment surface. Na: not analyzed. *Microcystis* in the bottom water were not sampled in spring 2009.

Conchas compared with the winter 2006–2007, and a 4-fold increase in Trasona. No resuspended colonies of *Microcystis* were observed in the spring samples from Las Conchas and Trasona.

Germination experiments

Germination experiments yielded 17 cyanobacterial-enriched suspensions dominated by Nostocales and/or *Microcystis* spp. (see Supplementary data, Table SI and Fig. S1) combined with minor amounts of small Oscillatoriales and green algae. Germinated cyanobacteria were obtained from four of the six reservoirs, most of them (59%) from Cachamuiñas. Seventy-six percent of the suspensions belonged to benthic Nostocales genera *Trichormus* (41%), *Nostoc* (23%) and *Cylindrospermum* (12%). Planktonic species *Aph. gracile* and *Microcystis* (*M. aeruginosa, M. flos-aquae*) were also obtained from Cachamuiñas and Trasona. There was no apparent influence of the sampling point, as similar 59 and 41% of suspensions originated from dam and upstream sites, respectively. The pre-concentration step by density-gradient centrifugation appeared to be useful for germination experiments as 76% of the germinations were obtained using this method.

DISCUSSION

Multiple studies have investigated the presence of akinetes and *Microcystis* in lake sediments in Central and Northern Europe. However, data from Southern European countries remain scarce. This study is the first to describe the overwintering sediment-pool of *Microcystis* and Nostocales akinetes in water reservoirs in Spain.

The six reservoirs studied during a 2-year period (2006–2008) represented different ecological scenarios: La Barca, Trasona and Las Conchas were *Microcystis*-dominated water bodies, whereas Cachamuiñas, Prada and Salas were dominated by Nostocales. The abundance of the resting stages in sediments resembled that previously found in water bodies where dense cyanobacterial blooms have occurred. Thus, the 11 000–187 000 akinetes mL^{-1} in the Nostocales-dominated reservoirs are in the range of the 10^4–10^5 akinetes mL^{-1} in *Anabaena*-dominated lakes elsewhere (Tsujimura and Okubo, 2003; Kim *et al.*, 2005; Faithfull and Burns, 2006). Similarly, the 250–600 *Microcystis* colonies mL^{-1} in La Barca and Trasona resemble the value of 100–1000 *Microcystis* colonies mL^{-1} in surface sediment layers of the Grangent reservoir, France (Latour *et al.*, 2007). Prada and Las Conchas hosted huge benthic populations of akinetes and *Microcystis* of up to 187 000 akinetes mL^{-1} and 28 000 *Microcystis* colonies mL^{-1}, respectively. By considering the average densities of akinetes (1.1 g cm^{-3}) (Hori *et al.*, 2002) and *Microcystis* (0.99 g cm^{-3}) (Reynolds *et al.*, 1981), the sediment densities of Prada and Las Conchas (1.14 and 1.02 g cm^{-3}, respectively) and the organic matter content measured in Cachamuiñas (10 mg g^{-1} DW), we calculated that *Microcystis* in Las Conchas and akinetes in Prada might represent up to 0.6 and 1%, respectively, of the organic matter content in the sediments. This highlights the importance of benthic *Microcystis* and akinetes in mass fluxes of lakes, which agrees with their demonstrated influence on the phosphorus (Brunberg and Boström, 1992) and nitrogen cycles (Zhang *et al.*, 2010) in these ecosystems.

This study reinforces previous observations regarding the accumulation of cyanobacterial resting-stages in deeper areas of lakes and reservoirs (Verspagen *et al.*, 2005; Kravchuk *et al.*, 2006). Akinete and *Microcystis* concentrations in dam were on average 11-fold (akinetes) and 2-fold (*Microcystis*) greater than those measured at the shallower upstream sites. These differences did not appear to be directly related to pelagic cell concentrations in previous summers, as shown in Prada sediment (winter 2007–2008). This sample had 28-fold higher akinete concentration in dam vs. upstream contrasting to

the 7-fold lower cell concentration observed in the dam in the only water sample collected in the previous summer (on 21 August 2007). Therefore, the differences in benthic stocks of dam and upstream may be explained not only by the deeper water column at the dam sites (which could provide an increased settling-cyanobacterial population) but also by "sediment focusing" or the natural re-distribution of fine particles from shallow to deep areas observed in other lakes (Evans, 1994; Verspagen *et al.*, 2005; Kravchuk *et al.*, 2006). Hypothetically, this process might be greater in the reservoirs studied here due to the influence of the river flow.

The genus *Anabaena* dominated the akinete pool in all the reservoirs, with the minor presence of *Aphanizomenon* (*Aph. gracile*, *Aph. flos-aquae*). Some authors suggest different annual cycles in *Anabaena* spp., with akinete-based overwintering, and *Aph. flos-aquae*, able to overwinter as vegetative pelagic populations even under the ice (Suikkanen *et al.*, 2010; Üveges *et al.*, 2012). We found a dual strategy in *Anabaena* spp., with major akinete production combined with the existence of pelagic overwintering of *A. flos-aquae* filaments in Trasona water at 9.8°C (28 000 cells mL^{-1} on 7 February 2008); but also in *Aph. gracile* from Cachamuiñas, producing low akinete amounts and appearing in the epilimnion at 7.5°C (12 000 cells mL^{-1} on 29 January 2008). This provides novel data on the poorly known annual life-cycle of *Aph. gracile*, a potential producer of the cyanotoxins cylindrospermopsin (Kokociński *et al.*, 2013) and paralytic shellfish poisoning toxins (PSP) in Europe (Ballot *et al.*, 2010); and *A. circinalis*, a well-known PSP-producer in Australia (Negri *et al.*, 1995) scarcely studied in Europe, here confirmed as a major akinete producer (11 000−187 000 akinetes mL^{-1}) in Prada and Salas reservoirs.

Our results confirm a benthic-pelagic overwintering of *Microcystis* in Las Conchas during both winters of 2006−2007 and 2007−2008, where a large benthic stock (4−14 million cells mL^{-1}) co-occurred with small epilimnetic populations (1150−1290 cells mL^{-1}) at water temperatures of 8−9.2°C, which concurs with the findings in a more northerly lake from The Netherlands (Verspagen *et al.*, 2005).

Most studies on the annual life-cycles of *Microcystis* and planktonic Nostocales consider that small spring inocula, combined with resuspension events, are able to build important pelagic populations during the bloom period (Karlsson-Elfgren and Brunberg, 2004; Verspagen *et al.*, 2005). We estimate that only 0.02−0.5% of *Microcystis* and Nostocales in sediments were mobilized into bottom water by resuspension during winter and spring. Some of these values may be affected by epilimnetic populations co-occurring in winter (e.g. 12 000 *A. gracile* cells mL^{-1} in Cachamuiñas), but unlikely by large pelagic summer populations which, considering average settling velocities of 0.2−0.7 m day^{-1} for resting stages (Cirés *et al.*, 2013) and the depth of the reservoirs studied here, might be completely settled in 2−5 weeks after bloom decay. The apparently low percentages of resuspension might mean up to 6 million akinetes m^{-2} (Prada dam, winter 2007−2008) or 7×10^5 *Microcystis* colonies m^{-2} (Las Conchas dam, winter 2006/07) in the 1.5-m water layer above sediments. Compared with the epilimnetic populations in subsequent summers, these potential inocula represented 0.06% of the 20 000 *Anabaena* cells mL^{-1} (extending into a 0.5-m wide water layer) in Prada dam, and a higher percentage (1.2%) of the *Microcystis* population (10 000 cells mL^{-1} in a 0.5-m layer) observed in Las Conchas dam in summer of 2007, which is in the range of 0.003−8% found for *Anabaena* and *Aphanizomenon* (Barbiero and Welch, 1992; Barbiero and Kann, 1994; Karlsson-Elfgren and Brunberg, 2004) or 0.5−4% for *Microcystis* (Brunberg and Blomqvist, 2003).

To summarize our results on bottom-water populations as potential inocula, we estimated the period required to reach maximum pelagic populations assuming two scenarios of low or high *in situ* growth rates obtained from the literature (Table V). Our estimations do not include any potential reduction in the inocula sizes or the growing population due to flushing, grazing or degradation, as quantitative values for these processes are not available in the literature. According to our calculations, the maximum inocula we measured may need a minimum of 2−5 weeks (high growth rates) or maximum of 9−25 weeks (low growth rates) to build up summer populations. Therefore, the bottom-water population resuspended in winter or early spring may be enough to explain summer populations if optimum growth conditions occur. The estimates in Table V suggest that the growth rate of the initial population has a bigger impact on the time needed to form a bloom than the size of the inoculum, with longer periods in *Microcystis*-dominated reservoirs than in Nostocales-dominated ones. To check this observation, we performed a sensitivity analysis with maximal inocula from Prada and Las Conchas (Table VI), the reservoirs hosting the largest overwintering populations of Nostocales akinetes and *Microcystis*. According to results shown in Table VI, growth rate would be the main factor influencing the time period to reach pelagic populations in both reservoirs, with a 2-fold increase in growth rate meaning a 50% decrease in time. This compares with a minor influence of inoculum size for which a 2-fold increase meant only 9−22% reduction in time. This supports conclusions indicated by models of *C. raciborskii* from German lakes, pointing to a low influence of inoculum size on summer populations (Rücker *et al.*, 2009) and a major impact of water conditions in

Table V: Estimation of the time period (T_{inoc}) required to reach maximum pelagic population of Nostocales and Microcystis *under different growth rate scenarios*

Reservoir	Point	Type of inoculum	Max. hypolimnetic inoculum (cells m^{-2})	Max. pelagic population (cells m^{-2})	Estimated T_{inoc} (weeks)	
					Low μ[a]	High μ[b]
Cachamuiñas	Dam	Akinetes	1.3×10^5	2.5×10^{10}	13.3	3.9
	Upstream	Akinetes	4.1×10^4	1.3×10^{10}	13.9	4.0
Prada	Dam	Akinetes	6.0×10^6	1.9×10^{10}	8.9	2.6
	Upstream	Akinetes	7.1×10^5	1.3×10^{11}	13.4	3.9
Salas	Dam	Akinetes	1.2×10^5	3.1×10^9	11.2	3.2
	Upstream	Akinetes	1.2×10^5	1.8×10^9	10.6	3.1
Conchas	Dam	*Microcystis*	3.6×10^8	2.5×10^{10}	11.9	2.3
	Upstream	*Microcystis*	8.5×10^7	1.8×10^{10}	14.9	2.9
Trasona	Dam	*Microcystis*	6.6×10^7	5.6×10^{10}	18.9	3.6
	Upstream	*Microcystis*	1.9×10^7	1.6×10^{11}	25.1	4.9

Calculations were performed assuming exponential growth.
[a]$\mu = 0.13°$ for Nostocales (Rücker *et al.*, 2009) and $\mu = 0.05$ day^{-1} for *Microcystis* (Verspagen *et al.*, 2005).
[b]$\mu = 0.45$ day^{-1} for Nostocales (Westwood and Ganf, 2004) and $\mu = 0.26$ day^{-1} for *Microcystis* (Reynolds *et al.*, 1981).

Table VI: Sensitivity analyses for the period required to reach maximum population (T_{inoc}) of Nostocales and Microcystis *in Prada and Las Conchas reservoirs*

Sample	Parameter		Expected T_{inoc} (days)	T_{inoc} decrease (%)
Prada (dam)	Inoculum	Initial[a]	62.3	—
		2X	56.9	9
		4X	51.6	17
	Growth rate	Initial[b]	62.3	—
		2X	31.1	50
		4X	15.6	75
Conchas (dam)	Inoculum	Initial[a]	83.0	—
		2X	64.8	22
		4X	51.3	38
	Growth rate	Initial[b]	83.0	—
		2X	41.5	50
		4X	20.8	75

Calculations were performed assuming exponential growth.
[a]Initial inocula of 6.0×10^6 and 3.6×10^8 cells m^{-2} for Prada and Las Conchas dam, respectively (see Table V).
[b]Initial growth rate of 0.05 day^{-1} for Las Conchas dam and 0.13 day^{-1} for Prada dam.

spring, particularly water temperature (Wiedner *et al.*, 2007; Jöhnk *et al.*, 2011). Table VI also suggests a higher impact of inoculum size in *Microcystis* (a 4-fold size increase meant a 38% decrease in time) than in Nostocales (4-fold size increase meant a 17% decrease), which has been previously hypothesized by other authors based on the lower growth rate of *Microcystis* (Reynolds *et al.*, 1981; Verspagen *et al.*, 2005). However, these estimations need to be checked by mathematical models, including the overwintering pelagic population and multi-annual data series, as has been done in more northerly European lakes (Verspagen *et al.*, 2005; Wiedner *et al.*, 2007; Rücker *et al.*, 2009; Jöhnk *et al.*, 2011). In view of our findings, obtaining accurate measurements of the germination ratio of akinetes and the initial *in situ* growth rates are crucial for these models. In summary, the overwintering of cyanobacteria in both the sediments and the water column, the need for very small inocula to build up summer populations, and the evident difficulties of altering water conditions (e.g. temperature) other than nutrient loads, suggest that water management strategies based on the annual life cycles need to be coupled with eutrophication control to effectively cope with cyanobacterial blooms.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

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