

Cyanobacterial dynamics and toxins concentrations in Lake Alto Flumendosa, Sardinia, Italy

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ABSTRACT

Seasonal blooms of cyanobacteria (CB) are a typical feature of Lake Alto Flumendosa (Sardinia, Italy). The waters of this lake are used for drinking water supply, for agricultural and industrial uses, and fish farming activities. Since cyanotoxins are not monitored in edible organisms, diet could be a relevant route of human exposure. CB also represent a threat for the health of wild and domestic animals that use lake water for beverage. Therefore, to characterize the CB community and assess the risk for human and animal population, CB dynamic, *mcyB*⁺ fraction, and microcystins (MCs) concentration have been followed monthly for 18 months, in three stations. Results confirmed the presence of several toxigenic species. *Planktothrix rubescens* dominated between August 2011 and April 2012 (3.5×10^6 cells L⁻¹), alternating with *Woronichinia naegeliana* (8×10^6 cells L⁻¹) and *Microcystis botrys* (9×10^5 cells L⁻¹). *Dolichospermum planctonicum* was always present at low densities (10^4 cells L⁻¹). MCs were detected, at values well below the 1 µg L⁻¹ threshold of WHO for drinking water. The molecular analysis of *mcyB* gene for *P. rubescens* indicated the presence of a persistent toxic population (average 0.45 *mcyB*/16S rDNA). Highly significant linear regressions were found between *P. rubescens* and the sum of the demethylated MC variants, and between *M. botrys* and the sum of MC-LR and MC-LA, also when co-occurring, suggesting that these two species were responsible for different MC patterns production. The regression lines indicated a quite stable MC cell quota. However, in some spotted samples very different values were obtained for both MC concentrations and cell quota (from 10-fold lower to 30-40-fold higher than the 'average') showing an unexpected significant variability in the rate of toxin production. The relatively low cell densities during the monitoring period is consistent with the low-to absent MC contamination level found in trout muscle; however, the analytical method was affected by low recovery, probably due to MC-protein binding. Our results show that, during the study period, no risk of exposure for the human and animal population occurred. However, the persistence of a complex CB community characterised by a significant toxic fraction suggests the need for periodic monitoring activity. Particularly, the hidden deep summer *P. rubescens* blooms, located where water is taken for drinking water supply, and *M. botrys*, able to produce the most toxic MC variants with high cell quota, should be kept under control. The documentation and interpretation of sudden changes in toxins concentrations deserve special attention. This is particularly relevant in proximity of fish farming plants and water catchment sites.

Key words: Toxic cyanobacteria; cyanotoxins; microcystins; human and animal exposure; health risk.

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INTRODUCTION

Cyanobacteria are a common and naturally occurring component of freshwater environments, although they can be found in all terrestrial and aquatic ecosystems (Whitton, 2012). They are important primary producers and play a key role in ecosystem functioning and biodiversity. However, they can pose risks to environment and aquatic consumers through the production of cyanotoxins (CTX), a large group of toxins that comprise microcystins (MCs), which are the most numerous and studied chemical variants (Buratti *et al.*, 2017). During dense blooms, which are increasingly occurring due to eutrophication and climate changes (Paerl and Paul, 2012; Planas and Paquet, 2016), CTX can reach very high concentrations, affecting

both humans and aquatic animals, as well as wild and livestock animals (Funari and Testai, 2008; Moreira *et al.*, 2013; Hilborn and Beasley, 2015; Testai *et al.*, 2016b; Wood, 2016; Buratti *et al.*, 2017). Reports of animal poisonings attributable to CTX have been documented worldwide for more than a century. A diverse range of animals has been affected - from dogs, cattle and fish, to flamingos, bats and bees (Carbis *et al.*, 1995; Frazier *et al.*, 1998; Briand *et al.*, 2003; Stewart *et al.*, 2008). Some animals appear to be attracted by cyanobacteria in water and dried crusts on top of the water, even when clean water was plainly accessible (Codd *et al.*, 1992; Lopez Rodas and Costas, 1999). Oral human exposure to CTX can occur through the ingestion of drinking water from a contaminated source or through the ingestion of inadver-

tently swallowed contaminated water during recreational activities (Funari and Testai, 2008; Testai *et al.*, 2016a). Furthermore, humans can be orally exposed through the ingestion of cyanobacteria-based food supplements (Saker *et al.*, 2005; Vichi *et al.*, 2012) or food items such as fish and shellfish with bioaccumulated toxins (*e.g.*, through filtration of contaminated water) (Ibelings and Chorus, 2007; Berry, 2013). Additional routes of exposure are dermal contact and accidental inhalation during recreational activities in waters subjected to a toxic bloom.

In Sardinia, more than 90% of the drinking water originates from artificial lakes which are generally eutrophic (Lugliè *et al.*, 2013), the ideal environment for cyanobacterial blooms. Indeed, cyanobacteria have become dominant in many reservoirs over time (Sechi and Lugliè, 1992, 1996; Aktan *et al.*, 2009; Pulina *et al.*, 2011). However, only a few studies have assessed the presence of CTX, namely MCs (Messineo *et al.*, 2009; Sulis *et al.*, 2014; Mariani *et al.*, 2015). Furthermore, a toxic strain of *Microcystis aeruginosa* (Kützing) Kützing, associated with a fish kill event, was isolated in Lake Liscia (Pellegrini *et al.*, 1995), and a toxic bloom of *Planktothrix rubescens* (formerly *Oscillatoria rubescens*) (de Candolle ex Gomont) Anagnostidis and Komárek was documented in 1986 in the Flumendosa reservoir (Loizzo *et al.*, 1988). In the last 30 years, the Flumendosa reservoir has been used as the main water supply for civilian, agricultural and industrial uses in Southern Sardinia (Botti *et al.*, 2001). In addition, a trout farming plant is located nearby, whereas water is utilised also by wild animal and livestock for beverage. In the 1990s, Cyanophyceae, together with Bacillariophyceae and Chlorophyceae, were the dominant classes in the Flumendosa reservoir system (Lugliè *et al.*, 1997), with CB representing 90-100% of phytoplankton since 2002 (Sulis *et al.*, 2014). The CB with the highest density described so far are the *Planktothrix* and *Microcystis* genera, which have been responsible for the most frequent onset of blooms, while the *Dolichospermum* genus, except for a bloom in 2003, appeared sporadically (Sulis *et al.*, 2014). To our knowledge, data on MCs in this lake have been assessed only in two occasions and were associated with the presence of *P. rubescens* (Messineo *et al.*, 2009; Sulis *et al.*, 2014). A recent 18-month study on four reservoirs in Northern Sardinia showed significantly different seasonal variations in CB community composition, with several toxigenic species, and large variability in MCs concentrations (Mariani *et al.*, 2015). The results highlighted the need to increase the knowledge of these aspects in each reservoir, whose use could affect human and animal health. In June 2010, a mass mortality of fish occurred in the Flumendosa reservoir, concurrently with an extensive surface bloom of *P. rubescens*. The cause for fish mortality was potentially attributed to a combination of oxygen deficiency and CB toxins. Following this event, our study was aimed: i) to characterize the

CB community of the reservoir; ii) to verify the applicability of a linear model used in single species blooms to predict their toxicity (Salmaso *et al.*, 2014); and iii) to assess the potential risk for humans and animals. We followed the dynamics of cyanobacteria for 18 months and determined MCs concentrations, toxicity profile (MC variants concentration), frequency of toxic genotype and accumulation of MCs in farmed trout.

METHODS

Description of the site and sampling strategy

The reservoir of the Alto Flumendosa, located in centre-eastern Sardinia (Italy) (latitude: 39°42'38" N; longitude: 9°17'25" E) (Fig. 1), is an artificial lake (volume 64×10⁶ m³) with a surface area of 9 km² (Begliutti *et al.*, 2007) and a maximal depth of around 50 m. It is a complex and multiuse water system, which interconnects with other reservoirs (Flumendosa-Campidano). The reservoirs are linked in a cascading sequence and from each one pressure pipelines and open channels guarantee water for residential use and irrigation of the Campidano plain (Sechi and Sulis, 2009). Once used for the production of electrical energy in three underground stations, the water is conveyed through a system of tunnels in the Lake Teaula, about 286 m asl, east of Villagrande. At this point, waters are directed through the Tortoli flat for irrigation and for civilian uses (*e.g.*, drinking water) (Cabras *et al.*, 2013).

Water samples were collected in three sites: (1) Zattere (maximum depth 50 m) at about 10 m from a trout's floating cages farming plant; (2) Middle site in the centre of the lake (maximum depth 25 m), in front of the water off-take site (collecting water also for drinking purposes); and (3) Rio Osiana (maximum depth 7 m) close to the inflow of a small temporary tributary stream (Fig. 1). During our sampling period (October 2011 - May 2013), the stream periodically underwent dry phases. After Oct 2012, the site 3 was no longer sampled, since the values of the studied parameters were not different from the other sites. Since in some periods some CB species density was very low, two different strategies of sampling were used to monitor their temporal dynamics: i) discrete samples were collected monthly in the whole sampling period (18 months) in the three stations at the surface (S1, S2, S3) and in the stations 1 and 2 at 20 m depths (P1, P2; depth of the water off-take site, which is important to check when *P. rubescens* occurs) (Manganelli *et al.*, 2016) using a Van Dorn bottle; ii) periodically, integrated samples were collected with a 20 µm phytoplankton net on a water column of 30 m (R1), 25 m (R2) and 7 m (R3). Samples were collected and stored in acid clean polycarbonate bottles, in cold and dark conditions until the arrival in the laboratory where they were aliquoted for the different analysis.

Five fish samples were collected from November 2011 to September 2012, every two-three months, from the trout farming plant (2-3 animals for sampling).

Physical measurements and chlorophyll-a analysis

Chlorophyll-a (Chl-a) was extracted according to Jespersen and Christoffersen (1987). Samples were filtered onto GF/F filters (Whatman) and stored in 96% ethanol in the dark at room temperature over-night. The fluorescence of the extract was measured with a TURNER 10-AU-005 fluorimeter (Holm-Hansen *et al.*, 1965). Temperature was read from a thermometer inside the sampling bottle, as soon as the sample arrived at the surface, and pH was measured in the field with a pH meter probe.

Cyanobacteria abundance and isolation

Cyanobacteria taxonomic determination was carried out according to Komárek and Anagnostidis (1999, 2005) and Komárek and Zapomělová (2007). Cyanobacteria abundance was determined by epifluorescence microscopy, since phycoerythrin and phycocyanin contained in CB,

when examined under blue light excitation, fluoresce orange and red, respectively (Walsby and Avery, 1996; Ernst *et al.*, 2006). This technique, based on sample concentration on filters, allows the concentration of large volumes in few minutes and the counts of species present within a large range of densities (Hallegraeff *et al.*, 2004). Fifty mL of formaldehyde-fixed samples (final concentration 4%) were filtered onto 5 μm black membrane filters (25 mm diameter) (Whatman). The number of filaments and colonies was counted by autofluorescence under blue light with an upright microscope (Olympus BX51) at 100x. Two replicate filters per sample were counted, with a variation between filters generally well within 20%. The number of cells per filament/colony was determined by average on 50 filaments/colony on both filters, using an image analysis software (ImageJ, <http://rsbweb.nih.gov/ij/>).

Monoclonal strains of *P. rubescens* were isolated as follows. The filaments were plated on BG-11 medium 1% agar (DIFCO) plates. The plates were then incubated at 14°C, at a photosynthetic photon flux density of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in a light:dark cycle (16:8h). Filaments were isolated by micromanipulation according to Rippka (1988)

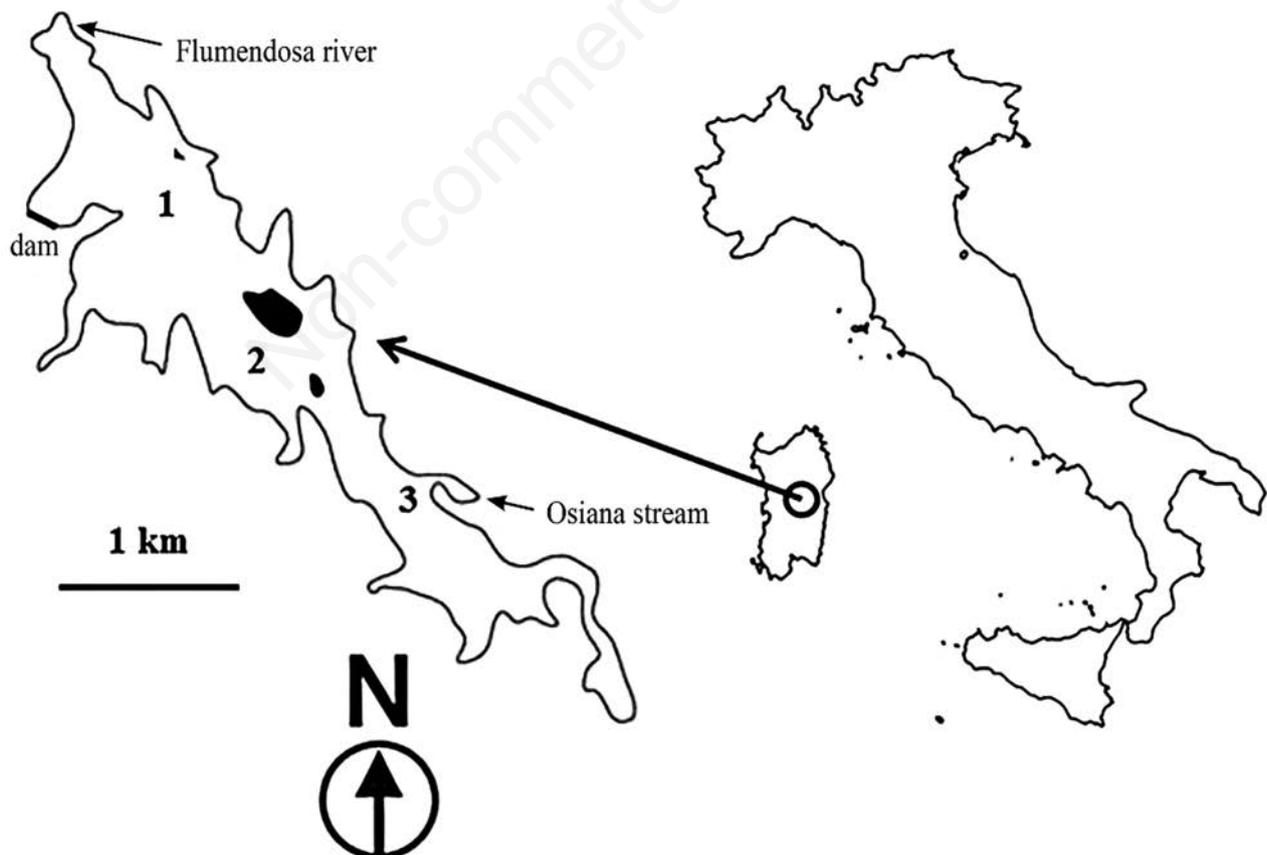


Fig. 1. The Flumendosa reservoir and sampling sites.

and incubated at the same conditions described above, in liquid BG-11 medium. We also tried to isolate colonial cyanobacteria, like *Woronichinia naegeliiana* (Unger) Elenkin, as described by Sedmak *et al.* (2008). The samples were concentrated in test tubes under natural light. Colonies floated towards the surface due to their buoyancy mechanism. The remaining phytoplankton together with zooplankton sank to the bottom. The surface layer with cyanobacterial material was collected and re-examined by microscopy resulting monospecific (over 99%). The colonies were then incubated in liquid BG-11 at the same conditions as above. However, all isolation attempts failed. *W. naegeliiana* did not persist in culture more than one month and no further analysis was carried out.

Phytoplankton abundance

Samples for the identification and counting of phytoplankton other than cyanobacteria were fixed with formaldehyde (final concentration 4%) and/or Lugol's solution (final concentration 1%). Ten mL samples were sedimented according to the Utermöhl method (1958) and counted under an inverted microscope (Olympus IX50) at 40x and 400x magnification, in bright-dark field.

Detection of microcystins

ELISA

Total (intra- and extra-cellular content) MCs were analysed in untreated water samples by an enzyme-linked immunosorbent assay (ELISA). After three freeze/thaw cycles, the samples were analysed using an ELISA kit (Microcystins ADDA-ELISA Microtiter Plate, ENVIRONMENTAL LOGIX, Portland, ME, USA) according to the manufacturer's protocol. Microscope observation of freeze thawed samples confirmed the complete lysis of cells. The results were expressed as MC-LR equivalents (MC-eq). Methanol extracts from fish samples were also tested, after being dried and re-dissolved in water, with minor modification from Papadimitriou *et al.* (2012). Briefly, 5 g of fish muscle from each animal were added with 10 mL methanol, homogenized, centrifuged at 4000 rpm for 10 min and the supernatant collected. The extraction step was repeated twice and the supernatant fractions were pooled. Three aliquots of 5 mL each were gently evaporated to dryness at 45°C in rotavapor. The residue was reconstituted in 500 µL of distilled water. Samples were considered positive when the MC concentration was higher than the detection limit of the method (0.10 µg L⁻¹).

LC-MS/MS analysis

To discriminate the different MC variants, which have a different toxicity (Funari and Testai, 2008; Buratti *et al.*, 2017), samples have also been analysed by liquid chro-

matography tandem mass spectrometry (LC-MS/MS). The extraction procedure was different for water samples and complex matrix as tissues.

Water samples

Each sample was divided into two 500 mL aliquots: one was used to determine dissolved MCs and one to determine total MCs. For dissolved fraction detection, samples were filtered onto GF/C discs (Whatman) in order to eliminate cells. The filtrate, acidified, was cleaned-up by solid-phase extraction (SPE), as previously described (Buratti *et al.*, 2011). Briefly, samples were purified and concentrated through ODS SPE cartridge, rinsed with 20% MeOH (4 mL), then eluted with 4 mL MeOH. The eluate was dried under a gentle nitrogen stream, and re-dissolved in 0.5 mL of acetonitrile:water (30:70) containing 0.1% (v/v) formic acid. To determine total MCs (dissolved plus intracellular MCs), the aliquot was freeze-thawed three times, to cause cell lysis with the consequent release of intracellular CTX and then treated as described above for the dissolved fraction. The intracellular content of MCs was calculated as the difference between total and dissolved concentration.

Fish

Fish tissues have been extracted as reported above for ELISA assay.

Separation, identification and quantification of the MC variants (MC-LR, -LA, -YR, -RR, -LF, -LW, -dem-LR, -dem-RR, for which standards were available) were performed with two MicroPump and an autosampler PE series 200 (Perkin Elmer Inc., Waltham, MA, USA), coupled to a triple-quadrupole mass spectrometer equipped with a TurboIonSpray source (MDS Sciex, Concord, Canada). MS tuning and optimization were achieved by infusing MCs standards (Alexis, San Diego, CA, USA); detection was carried out using Multiple Reaction Monitoring (MRM) mode. MCs separation, in MRM analysis, was conducted as previously described (Buratti *et al.*, 2011). All samples were run in MRM mode, utilizing the specific fragmentation reaction of single or doubly protonated MCs to m/z 135, the specific fragment associated to the Adda fragment. The amount of MCs was quantified by the external standard quantification procedure, referring to a calibration straight line with 4–6 known amounts of the analytical standards in solvent (coefficient of determination, R² ≥ 0.98), for water samples, and in the blank fish matrix, for fish samples, in order to exclude any interference due to matrix effect. Indeed, salts that are hardly removed during extraction procedures and sample preparation may influence samples ionization, causing a quenching of the signal for some congeners whereas for others the signal can be overcompensated (Vichi *et al.*, 2012). In fish samples, recovery was also investigated

spiking pre- and post-extraction with known amounts of standard MCs. We found around 80% recovery for the different MC variants tested, except for MC-RR, MC-dem-RR and MC-LA, for which recovery was never higher than 50%. For the applied method, the LOQ ranged between 2 ng L⁻¹ (MC-RR) and 9 ng L⁻¹ (MC-LW) for water samples, and between 5 ng g⁻¹ (MC-LR) and 15 ng g⁻¹ (MC-LF) for fish samples.

Molecular investigation

Each field sample (250-500 mL) was filtered on 0.2 µm Supor Membrane Filters to harvest the cells; filters were stored at -20°C. Total genomic DNA was extracted using the commercial DNeasy plant mini kit (Qiagen), following the manufacturer's instructions. Genomic DNA concentration was measured by a biophotometer (Eppendorf), and purity was assessed by calculating the ratio of the absorbance at 260 nm and 280 nm.

Molecular analyses were performed exclusively on 83 samples, where the *P. rubescens* cells abundance, estimated by counting before storage and DNA extraction, exceeded or was equal to the threshold of 10⁴ cells L⁻¹, in order to obtain reliable and consistent results.

A preliminary qualitative PCR was carried out to identify the MC-producing genera *Planktothrix* and *Microcystis*, following the methods of Rantala *et al.* (2006) and Vaitomaa *et al.* (2003), based on the detection of the genus-specific MC synthetase gene E (*mcyE*). The quantitative estimation of *Planktothrix* cells and the fraction of individuals carrying MCs biosynthesis genes were further investigated by qPCR employing a StepOne™ Real-Time PCR System (Applied Biosystems), according to Ostermaier and Kurmayer (2009). Two independent Taq Nuclease Assays (TNAs) were used, one to quantify the general population of *Planktothrix* using the 16S rDNA region as specific genus marker, and the other one to quantify the potential MC-producing genotypes within the community by checking the amplification of the first adenylation domain of *mcyB* (*mcyBA1*).

Genotypes of the unknown samples were quantified relating their threshold cycle (Ct) to the starting cell concentration, by applying the standard curve methodology. For both 16S rDNA and *mcyB* genes the standard curves were established using serial dilutions of DNA extracted from known cell concentrations of *P. rubescens* strains CCAP1460/3 and CCAP1460/10. The serial dilutions and the samples were amplified simultaneously to determine their Ct values under the same experimental conditions. Each measurement was performed in triplicate.

A similar qPCR approach was applied to quantify the toxic fraction of *Microcystis* (Vichi *et al.*, 2012); however, some cross reactivity was observed when primers were tested in the presence of *W. naegeliana* cells, which often co-occurred with *Microcystis* in the environmental sam-

ples, thus making this method unreliable for the quantitative estimation of *Microcystis mcy+* cells.

Statistics

Differences between set of data were tested with ANOVA, with the software STATISTICA 6 (StatSoft, Inc. OK, USA), after testing for normality. Since data from surface sampling sites were not statistically different they have been averaged and average ± SD has been considered. Correlation analysis between different variables has been calculated with the software SigmaPlot 9.01 (Systat) on all raw data.

RESULTS

Temperature

Data of temperature are shown in Fig. 2. The lake was generally stratified from March-April until December.

Cyanobacteria abundance and isolation

Several potentially toxic species were detected during the monitoring period, with a variable relative abundance over the year: *Planktothrix rubescens*, *Woronichinia naegeliana*, *Microcystis botrys* Teiling and *Dolichospermum planctonicum* (Brunnthal) Wacklin, L. Hoffmann & Komárek (Fig. 3).

P. rubescens was always present (Fig. 3a); it dominated in both surface and 20 m depth samples, from November 2011 to summer months in 2012, being significantly lower in the remaining months. During the stratification period, with surface temperatures reaching a maximum of 22.5°C, the highest densities were at 20 m depth, with values oscillating between 1.5 to a maximum of 5 × 10⁶ cells L⁻¹, according to the typical seasonal dy-

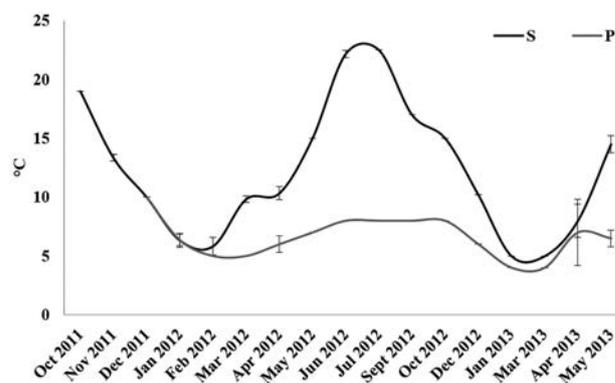


Fig. 2. Temperature variation at the surface (S, average of the 3 surface sites ± SD) and 20 m depth (P, average of the 2 depth sites ± SD). Aug 2012, Nov 2012, Feb 2013 no sampling.

dynamic of this species. After July 2012, *P. rubescens* density was about one order of magnitude lower than in the previous year (Fig. 3a). During the stratified period, sporadic samples in S1 were collected every 5 m from surface to -20 m, confirming that *P. rubescens* was homogeneously distributed between -10 and -20 m with higher density than surface (*data not shown*).

W. naegeliana was numerically dominant from September to December 2012 when it reached high values on the surface ($4.3\text{--}7.5 \times 10^6$ cells L^{-1}) and at 20 m depth ($6\text{--}8 \times 10^6$ cells L^{-1}), that is about one order of magnitude higher than in 2011 (Fig. 3b). In the remaining months, it was totally absent or present at much lower densities.

M. botrys was found from the beginning of the monitoring activity until January 2012 and from May to October 2012 in surface samples and sporadically in depths samples (Fig. 3c). A peak of density (up to 8×10^5 cells L^{-1} , sites S1 and S2) was observed in October 2011 at the surface, when it represented the dominant species among CB (45%); it sharply decreased to less than 5% of total CB community in the following months.

D. planctonicum was always present at low densities (Fig. 3d). In surface samples, it never exceeded 4×10^4 cells L^{-1} ; densities at depths were similar or lower. The lowest values were measured in samples collected in 2013.

When integrated samples were considered, *M. botrys*

was the only species virtually absent from the lake between December 2012 and May 2013, when it was again detectable at very low density (1.7×10^4 cells L^{-1}) although only in R2 (Tab. 1). *P. rubescens* and *W. naegeliana* were always present at very high density (up to 1.5×10^9 cells L^{-1} and 2.4×10^9 cells L^{-1} respectively). *D. planctonicum* was characterized by low densities (maximum 7×10^6 cells L^{-1}).

Phytoplankton abundance

The phytoplankton community showed a high variability in both cell abundance and species composition. It consisted of green algae like *Chlorella* sp., *Scenedesmus* sp. and *Chlorococcum* sp., and diatoms like *Aulacoseira* sp., *Fragilaria* sp. and *Asterionella* sp. In terms of cell number, they represented on average only $\sim 30\%$ of the community (median = 15%). However, their biomass was more relevant. Indeed, two significant correlations, one for summer and one for winter ($r=0.73$ $P<0.001$ and $r=0.90$ $P<0.001$, respectively), identified by two ranges of temperature, were found between the density of the whole phytoplankton community (CB and algae) and Chl-a, used as a proxy for biomass (Fig. 4). Excluding non-CB algae from the number of cell data, correlations were no longer significant, implying that in this lake, even if

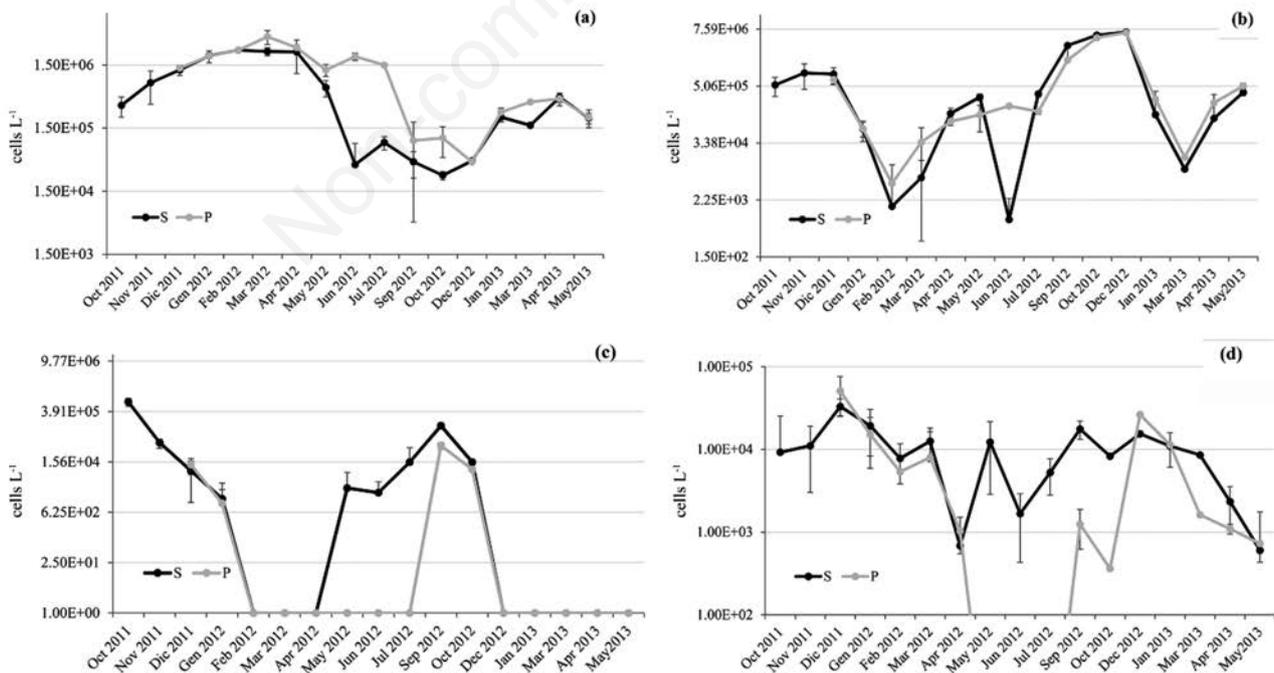


Fig. 3. Seasonal variations in the densities of (a) *P. rubescens*, (b) *W. naegeliana*, (c) *M. botrys* and (d) *Dolichospermum* sp. at the surface (S, average of the 3 surface sites \pm SD and at 20 m depth (P, average of the 2 depth sites \pm SD) (Y axis log scale). Aug 2012, Nov 2012, Feb 2013 no sampling.

CB were numerically more abundant than algae, Chl-a could not be used as an index of CB abundance.

Detection of microcystins

ELISA

The MCs concentrations in water were always detected in the range 0.1-1 $\mu\text{g L}^{-1}$ (*data not shown*). The highest values were found in October 2011 in a surface sample, when the dominant species was *M. botrys*.

LC-MS/MS

MC-LA, -LR, -dem-LR, -RR, -dem-RR were detected in all samples in different relative proportions, whereas the other tested congeners MC-LF, -LY and -LW were always below the detection limit (9 ng L^{-1}). Consistently with the ELISA data, in all discrete samples MCs concentrations never exceeded 1 $\mu\text{g L}^{-1}$ (Supplementary Tab. 1). The levels of MCs in integrated samples ranged between 0.4 and 100 $\mu\text{g L}^{-1}$ (Tab. 2). The highest value was measured in May 2012 when a 10-fold difference was observed between R1 and R3, although the relative proportion of the different variants was the same.

The pattern of congeners in both discrete and integrated samples revealed that generally, but not always, MC-dem-RR accounted for ~92% (up to 100%) and MC-dem-LR up to 11%, with the sum of the other variants <1% (Tabs. 2 and 3). Indeed, in some samples, the pattern of congeners was totally different, with MC-LR and MC-LA accounting for ~60% and 30%, respectively.

The fit of linear models between single congeners and cyanobacterial species allowed distinguishing the potential producers of different MC variants. In discrete samples, highly significant correlations were observed

between *P. rubescens* and MC-dem-RR and MC-dem-LR concentration ($R^2=0.81$) (Fig. 5a) and between *M. botrys* and MC-LR, -RR, -LA ($R^2=0.86$) (Fig. 5b). In the integrated samples, no correlation between *P. rubescens* and dem-MC was observed when all data were included in the analysis. Excluding the values measured in May 2012, which deviated from the main pattern, the relationship between MC concentrations and *P. rubescens* densities was significant ($R^2=0.67$) (Fig. 6a), with a slope not different from that obtained from the discrete samples (t-test $t=0.34$, $P>0.5$) (Fig. 5a). The correlation between MC-LR and MC-LA and *M. botrys* was still highly significant ($R^2=0.94$) (Fig. 6b). Also in this case, the slopes of linear models computed on data measured in discrete and inte-

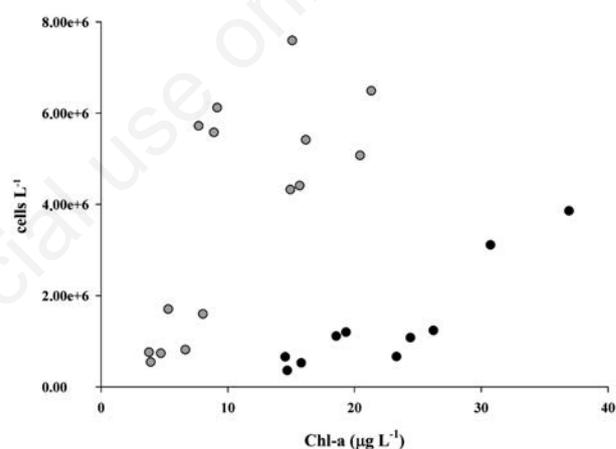


Fig. 4. Variation of number of cells (phytoplankton including cyanobacteria) vs Chl-a in surface sites (Grey, warm season; black, cold season).

Tab. 1. Cyanobacteria density (cells $\text{L}^{-1} \times 10^6$) in integrated samples in the three sites.

Site	Date	<i>M. botrys</i>	<i>P. rubescens</i>	<i>W. naegeliana</i>	<i>D. planctonicum</i>
R1	Oct 2011	52.6	7.00	36.2	2.02
	May 2012	nd	181	158	7.41
	Sept 2012	30.8	7.40	2390	0.68
	Oct 2012	7.04	9.87	755	0.77
	Dec 2012	nd	3.29	2150	2.51
	Jan 2013	nd	50.4	118	1.55
	Mar 2013	nd	94.1	13.3	0.001
	Apr 2013	nd	252	355	1.31
	May 2013	nd	21.8	127	0.12
R2	Oct 2011	71.9	13.1	100	1.63
	May 2012	17.3	1510	486	5.27
	Apr 2013	nd	75.8	67.3	0.12
	May 2013	0.02	14.8	93.3	0.07
R3	Oct 2011	58.6	6.76	131	2.53
	May 2012	3.52	1350	82.9	1.50

nd, not detected.

grated samples were not statistically different ($t=0.25$, $P>0.5$).

During the study, 10 strains of *P. rubescens* were isolated and grown in laboratory conditions. The strains were all toxic, producing only MC-dem-RR (94%±2%) and -dem-LR (6%±2%).

Detection of MC in fish

The application of ELISA tests to fish muscle tissues showed a weak contamination of the examined samples (Tab. 4). These results were not confirmed by the LC-MS/MS analyses that revealed at most not quantifiable traces of MC-LR and MC-dem-LR.

Molecular investigation

The potential toxigenicity of the two genera *Microcystis* and *Planktothrix* was qualitatively confirmed by PCR in some randomly selected integrated samples with high CB cell densities. The environmental samples were amplified together with the reference strains of *M. aeruginosa* and *P. rubescens*, used as positive controls for the presence of the *mcyB* gene. The absence of any cross-reactivity between DNA from *Planktothrix* and *Microcystis* and the selected primers was verified and excluded with preliminary PCR assays on each isolated environmental culture; in addition, no PCR product was obtained when the same primers were tested in samples dominated by *W. naegeliana*. No conclusions could be drawn on the potential toxigenicity of the *W. naegeliana* strains present in the lake, since no isolated reference strains (neither discriminating primers) were available.

The quantitative estimate of the toxic genotypes performed on the *Planktothrix* population by qPCR revealed

a permanent and fairly constant fraction (about 40-50%) of *mcyB*⁺ cells throughout the monitoring period, with

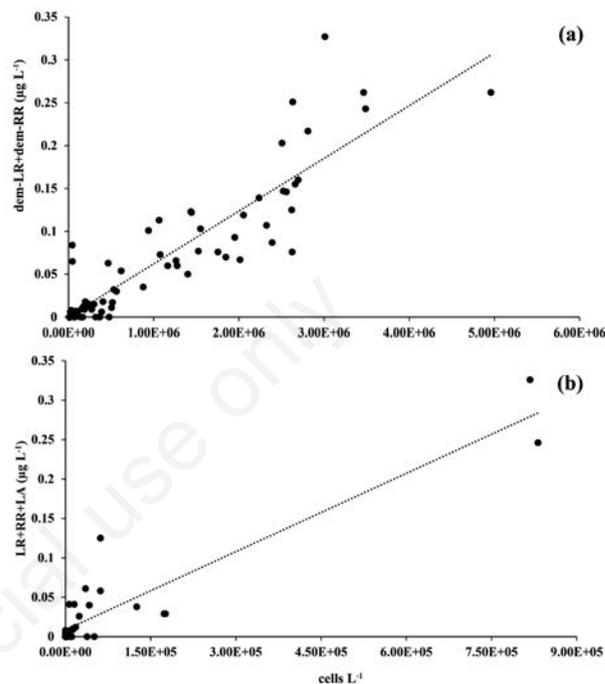


Fig. 5. a) Correlation between *P. rubescens* and MC-dem-LR + MC-dem-RR in discrete samples; the regression line is $Y=6.17 \times 10^{-8}(\text{SE } 3.60 \times 10^{-9}) \cdot X + 8.61 \times 10^{-5}(\text{SE } 5.65 \times 10^{-3})$; $R^2=0.81$, $P<0.001$. b) Correlation between *M. botrys* and MC-LR + MC-RR + MC-LA in discrete samples; the regression line is $Y=3.96 \times 10^{-7}(\text{SE } 2.18 \times 10^{-8}) \cdot X + 3.68 \times 10^{-3}(\text{SE } 3.38 \times 10^{-3})$; $R^2=0.86$, $P<0.001$.

Tab. 2. Microcystins concentrations ($\mu\text{g L}^{-1}$) in integrated samples in the three sites.

Site	Date	Microcystins						TOT
		RR	dem-RR	LR	dem-LR	LA		
R1	Oct 2011	nd	0.000	38.720	nd	9.180	47.900	
	May 2012	nd	92.097	nd	8.061	0.000	100.158	
	Sept 2012	nd	0.865	10.381	0.266	2.573	14.085	
	Oct 2012	0.447	nd	4.036	nd	1.345	5.829	
	Dec 2012	nd	nd	0.523	nd	nd	0.523	
	Jan 2013	nd	1.449	0.043	0.102	0.044	1.638	
	Mar 2013	nd	4.476	nd	nd	nd	4.476	
	Apr 2013	nd	6.673	0.194	1.490	nd	8.356	
	May 2013	nd	0.369	0.006	0.038	nd	0.413	
R2	Oct 2011	nd	nd	39.940	nd	15.000	54.940	
	May 2012	nd	69.520	0.196	5.810	nd	75.526	
	Apr 2013	nd	7.383	nd	1.372	nd	8.755	
	May 2013	0.012	0.692	0.015	0.057	nd	0.775	
R3	Oct 2011	nd	nd	36.070	nd	11.880	47.950	
	May 2012	nd	8.338	0.221	1.181	nd	9.740	

nd, not detected.

similar proportions in the surface and deep sampling sites ($48\pm 19\%$ vs $40\pm 15\%$), and in integrated samples ($56\pm 0.18\%$).

Cell quota

Based on the correlations between MC congeners and CB, the toxins cell quota were calculated for *P. rubescens* and *M. botrys*. The cell quota of the dem-MC produced by *P. rubescens* in the discrete samples averaged 0.104 ± 0.274 pg MC cell⁻¹ (Tab. 5). The high average and s.d. were due to two values measured in December 2012, S1 and P1, in which the cell quota was 30–40 fold higher than the rest of the values which were quite homogeneous (1.44 and 1.91 vs 0.059 ± 0.04 pg MC cell⁻¹ in the remaining samples). When the cell quota was calculated considering only the potentially toxic fraction (*mcyB*⁺/16S

rDNA), the values increased, as expected (0.130 ± 0.076 pg MC cell⁻¹, respectively) (Tab. 5). Due to a snowfall during sampling, in December 2012 no sample was collected for molecular biology analysis and therefore it was not possible to verify whether the abnormally high cell quota was related to a population 100% composed of *mcyB*⁺ cells, or to other reasons. However, even in case of a 100% toxic population the cell quota would still be ~10 times higher than usual. Only assuming a possible contribution by *W. naegeliana*, the cell quota would be in the range of the other values, 0.010 and 0.013 pg MC cell⁻¹; however, this possibility is unlikely, based on what is known on *Woronichinia* toxicity. To see if the toxic fraction of *P. rubescens* population was a better predictor of MC concentration than the whole population, the fit of linear model between MC variants and the potentially toxic cells (*mcyB*⁺ cells) was used, but the variability ex-

Tab. 3. Percentage of the different microcystins variants in discrete samples.

Site	Date	Microcystins				
		RR	dem-RR	LR	dem-LR	LA
S	Oct 2011	0	0	60	0	40
	Nov 2011	4	39	44	4	9
	Dec 2011	1	79	9	9	1
	Jan 2012	0	90	0	10	0
	Feb 2012	0	90	0	10	0
	Mar 2012	0	91	0	9	0
	Apr 2012	0	94	0	6	0
	May 2012	0	100	0	0	0
	Jun 2012	0	100	0	0	0
	Jul 2012	0	27	9	0	64
	Sep 2012	0	16	59	0	25
	Oct 2012	0	9	62	0	29
	Dec 2012	0	68	24	0	7
	Jan 2013	0	100	0	0	0
	Mar 2013	na	na	na	na	na
	Apr 2013	0	100	0	0	0
	May 2013	0	100	0	0	0
P	Oct 2011	na	na	na	na	na
	Nov 2011	na	na	na	na	na
	Dec 2011	2	75	12	9	2
	Jan 2012	0	89	0	11	0
	Feb 2012	0	92	0	8	0
	Mar 2012	0	93	0	7	0
	Apr 2012	0	95	0	5	0
	May 2012	0	92	0	8	0
	Jun 2012	0	92	0	8	0
	Jul 2012	0	92	0	8	0
	Sep 2012	na	na	na	na	na
	Oct 2012	0	100	0	0	0
	Dec 2012	na	na	na	na	na
	Jan 2013	na	na	na	na	na
	Mar 2013	0	97	3	0	0
	Apr 2013	0	100	0	0	0
	May 2013	na	na	na	na	na

S, average of the 3 surface sites; P, average of the 2 depth sites; na, not available.

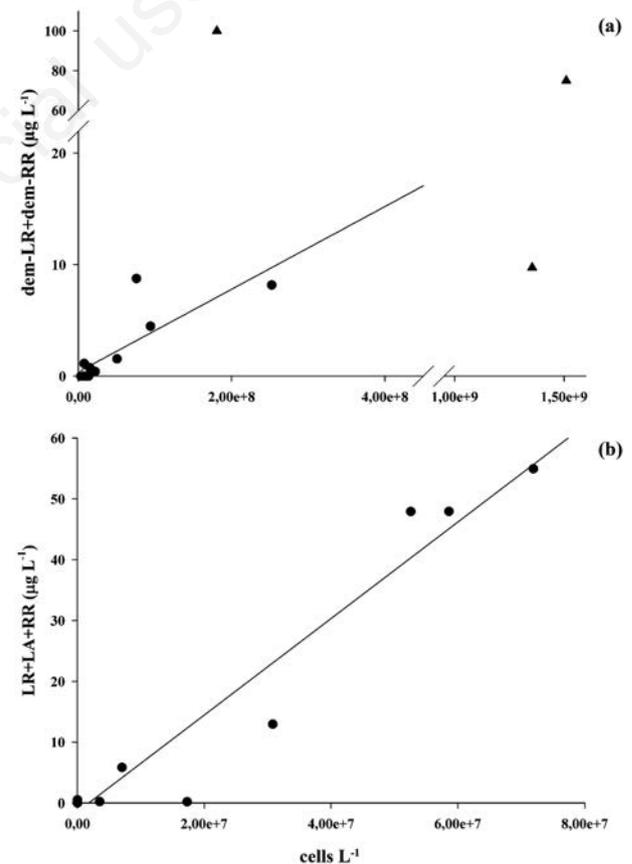


Fig. 6. a) Correlation between *P. rubescens* and MC-dem-LR + MC-dem-RR in integrated samples; the regression line is $Y = 3.71 \times 10^{-8} (\text{SE } 8.18 \times 10^{-9}) \cdot X + 0.38 (\text{SE } 0.68)$; $R^2 = 0.67$, $P < 0.001$; data from May 2012 have been excluded from the regression. b) Correlation between *M. botrys* and MC-LR + MC-RR + MC-LA in integrated samples; the regression line is $Y = 7.94 \times 10^{-7} (\text{SE } 5.44 \times 10^{-8}) \cdot X + 1.40 (\text{SE } 1.58)$ $R^2 = 0.94$ $P < 0.001$.

plained by the regression was much lower (54% vs 81%) (*data not shown*).

The cell quota calculated on the integrated samples were generally consistent with the average values determined in the discrete ones, except for the values obtained in May 2012 (Tab. 6), which were out of range. Indeed, the cell quota from the discrete sample was 0.069 pg MC cell⁻¹, whereas the one in R1 was almost 10 times higher (0.554 pg MC cell⁻¹) and in R3 10 times lower (0.007 pg MC cell⁻¹).

The cell quota of *M. botrys* in discrete samples averaged 0.771±1.280 pg MC cell⁻¹ (median 0.303 pg MC cell⁻¹) (Tab. 5); in the integrated samples cell quota averaged 0.668±0.495 pg MC cell⁻¹, very similar to the discrete sample values (Tab. 6).

DISCUSSION

Sardinia has a typical semiarid mediterranean climate, and relies for a 57% on a complex water distribution network of surface artificial reservoirs exploited for civilian, agricultural, and industrial purposes (ISRI, 2006), and accounting for about 90% when only drinking water is concerned (Lugliè *et al.*, 2013). Therefore a good water management plan is essential. Most of the Sardinian reservoirs were eutrophic (Total Nitrogen, 594-2057 µg L⁻¹; Total Phosphorus, 23-180 µg L⁻¹), with phytoplankton

communities dominated by CB (Sechi and Lugliè, 1996; Begliutti *et al.*, 2007; Marchetto *et al.*, 2009). The Alto Flumendosa reservoir maintains an intermediate trophic level between oligotrophy and mesotrophy, as shown by the nutrient concentrations reported by Cabras *et al.* (2013) (Supplementary Tab. 2), which refer to the period of the monitoring activities described in this paper. The results reported in this latter work are consistent with other previous studies (Begliutti *et al.*, 2007; Sulis *et al.*, 2014).

Sulis *et al.* (2014), who used a very large chemical and phyecological dataset for the Flumendosa-Campidano system to calibrate a model of water quality, described a variable phytoplankton community over the years (annual averages from 1996 to 2012), with a dominance of CB (>90%) from 2002 onward. Our study, which was carried out between the end of 2011 and mid 2013 fully confirmed the dominance by CB, although in terms of biomass other phytoplankton groups were also relevant. The species composition of the CB community in the Flumendosa reservoir has been previously described as dynamic, with alternating dominating species (Lugliè *et al.*, 1997; Meregalli *et al.*, 2002; Begliutti *et al.*, 2007). As reported by Sulis *et al.* (2014), *P. rubescens-agardhii* has been continuously dominant (>90%) between 2002 and 2009 (peak density 35×10⁶ cells L⁻¹), then decreased to ~20% of the whole phytoplankton community in 2010. *Microcystis* spp. has also been always present, generally at the onset

Tab. 4. ELISA and LC-MS/MS results for microcystins in fish tissues.

Date		ELISA			LC-MS/MS (ng g ⁻¹)			
		(ng g ⁻¹)	RR	dem-RR	LR	dem-LR	LA	TOT
08/11/11	Trout1	0.054	nd	nd	nd	nd	nd	nd
	Trout2	0.144	nd	nd	Traces	Traces	nd	nd
	Trout3	0.936	nd	nd	Traces	Traces	nd	nd
28/02/12	Trout1	0.200	nd	nd	nd	nd	nd	nd
	Trout2	0.132	nd	nd	Traces	Traces	nd	nd
24/04/12	Trout1	0.092	nd	nd	nd	nd	nd	nd
	Trout2	*	nd	nd	Traces	Traces	nd	nd
6/06/12	Juveniles	0.048	nd	nd	nd	nd	nd	nd
25/09/12	Trout1	0.046	nd	nd	Traces	Traces	nd	nd
	Trout2	0.152	nd	nd	Traces	nd	nd	nd

*Lost sample; nd, not detected.

Tab. 5. Average of cell quota computed for different microcystins variants (pg cell⁻¹) in *P. rubescens* and *M. botrys* in discrete samples (see text). Computations refer to the whole sampling period.

	Species		
	<i>P. rubescens</i>	<i>P. rubescens</i> mcyB ⁺	<i>M. botrys</i>
Discrete samples	0.104 (0.055) 0.274 n=72	0.130 (0.128) 0.076 n=30	0.771 (0.303) 1.281 n=21

Mean (median) SD.

of the blooms, while *Dolichospermum* bloomed in 2003, but after that low densities were reported sporadically over the years (Sulis *et al.*, 2014). The overall density of CB community during our study was about 10 times lower than what was observed before (Messineo *et al.*, 2009; Sulis *et al.*, 2014), but an even more complex alternation of species was observed. *P. rubescens* was always present and generally dominant until summer 2012, with the typical trend of depth blooms in summer and more homogeneous distribution along the water column during the other seasons (Kurmayer *et al.*, 2016). These results confirmed the need to sample the metalimnetic layers, especially when they are the source of drinking water supply (Manganelli *et al.*, 2010, 2016). The dominating species alternating with *P. rubescens* was *W. naegeliana*, previously described in the lake only in 1995 and 1996 (Meregalli *et al.*, 2002). Blooms of *W. naegeliana*, particularly in late summer and autumn, are not infrequent in temperate lakes, and have been reported in Northern Europe (including Poland, the Czech Republic, Sweden, Finland, Belgium, Russia), North America (United States) and Australia (Komárek and Anagnostidis, 1999; Bucka and Wilk-Woźniak, 2002; Annadottér *et al.*, 2005; Rajaniemi-Wacklin *et al.*, 2005). Furthermore, at the beginning of our study, *M. botrys* was the dominating species, then disappearing in the following months. In addition, *D. planctonicum* was never dominant but always present as supported by data from integrated samples on the 20 m water column, useful to detect the presence of CB species at low density. Since during the winter months *M. botrys* was undetectable also in the integrated samples, its re-appearance during the summer months can be explained considering that colonies overwintered on the bottom sediments (Reynolds *et al.*, 1981).

We tested whether CB abundance could be a good proxy for MC concentration in the lake, as it has been shown by many studies on *Planktothrix* (Briand *et al.*, 2005; Catherine *et al.*, 2008; Dolman *et al.*, 2012; Salmaso *et al.*, 2014). A prerequisite for this relationship to be significant is the presence of a single dominant species (Salmaso *et al.*, 2014). In the present study, the toxic species occurring simultaneously were compared with the different MC-variants, analysed separately. The pattern of MC variants was demonstrated to be highly dependent on the potentially producing species: highly significant linear regressions were obtained between *P. rubescens* and the demethylated form of MC-RR and MC-LR, and between *M. botrys* and MC-LA and MC-LR, in both discrete and integrated samples. Each species was characterized by a different slope (*t*-test, $t=2.79$, $P<0.01$). The determination of linear regressions with the same slope in both type of samples, for each species, reinforces the validity of our results, since in the integrated samples values of cell density and MC concentration were well above the detection limits. In the elaboration of *P. rubescens* integrated data, results from May 2012 were not considered, being totally out of range. These results allowed to associate the potential producing species with the different MC variants and furthermore to obtain a model which explained more than 80% of the variation in MC concentration in this system. The association between *P. rubescens* and MC-dem-RR and -dem-LR was also confirmed by the 10 monoclonal isolated strains producing only the two variants, and is in line with data from other Italian lakes (Messineo *et al.*, 2006; Manganelli *et al.*, 2010, 2016).

Even if no correlation between MCs and *W. naegeliana* was found, we cannot definitely rule out its ability to pro-

Tab. 6. Cell quota of microcystins variants (pg cell⁻¹) in *P. rubescens* and *M. botrys* in integrated samples in the three sites.

Site	Date	Species		
		<i>P. rubescens</i>	<i>P. rubescens</i> mcyB ⁺	<i>M. botrys</i>
R1	Oct 2011	0.000	0.000	0.911
	May 2012	0.554	1.152	nd
	Sept 2012	0.153	na	0.421
	Oct 2012	0.000	0.000	0.828
	Dec 2012	0.000	0.000	nd
	Jan 2013	0.031	na	nd
	Mar 2013	0.048	0.075	nd
	Apr 2013	0.032	0.057	nd
	May 2013	0.019	0.031	nd
R2	Oct 2011	0.000	0.000	0.764
	May 2012	0.050	0.075	0.011
	Apr 2013	0.116	0.195	nd
	May 2013	0.050	0.070	1.526
R3	Oct 2011	0.000	0.000	0.818
	May 2012	0.007	0.012	0.063

na, not available; nd, not detected.

duce some MC congener or other CTX. Since *W. naegeliana* was simultaneously detected with other toxic species, no conclusive considerations on its toxicity have been reported so far. Sivonen *et al.* (1990) reported that the neurotoxicity of bloom samples collected from Finnish lakes was associated statistically with *Anabaena (Dolichospermum) lemmermannii*, *Anabaena (Dolichospermum) flos-aquae* and *W. naegeliana*. In other studies, various microcystins were found during blooms in which *W. naegeliana* was dominant (Willame *et al.*, 2005; Baudin *et al.*, 2006), and the presence of *mcy* genes in environmental samples characterized by the presence of this species was also reported (Oberholster *et al.*, 2006). However, up to now, there are no pure isolated cultures of *W. naegeliana* in the world cyanobacterial collection, and we also failed to maintain any culture after isolation.

With respect to *Microcystis*, and confirming our results on *M. botrys*, other studies in the field have observed significant correlations between the population abundances and MC concentrations (Wang *et al.*, 2010; Horst *et al.*, 2014, among others).

The strong relationship between CB and MC in *P. rubescens* implies a high stability of population genetic structure over time (Salmaso *et al.*, 2014; Kurmayer *et al.*, 2016), which is indeed what we found in the Flumendosa reservoir. But this is not always the case: in Lake Vico (Central Italy), where *P. rubescens* was dominant, the fraction of the *mcyB*⁺ cells was highly variable over time, especially during the blooms. In those conditions, the correlation between cell density and MC could explain only about 50% of MC variation (Manganelli *et al.*, 2016). The quantification of *mcy* genes of *P. rubescens* has been indicated as a better predictor of MC concentrations (Ostermaier and Kurmayer, 2010; Hautala *et al.*, 2013). However, in this study the application of the linear regression to the *mcyB*⁺ cells and MC reduced the percentage of MC variation explained, from >80% to ~50%, *i.e.*, a value very close to what was observed by Briand *et al.* (2008) in a population of *P. agardhii* and consistent with what we previously described in Lake Vico (Manganelli *et al.*, 2016). There are several possible explanations for this outcome, as summarized in Kurmayer *et al.* (2016), such as other coexisting non identified toxic species, problems in the amplification of *mcy* genes or mutation inactivating *mcy* genes. Or it could be also hypothesized that other factors, in addition to the genetic make up, influence the relationship between density and toxicity, and the relation between the whole population and MC, representing the environmental cell quota (ECQ, *sensu* Salmaso *et al.*, 2014), includes those factors.

Since the relationship between MC content and the dynamics of the relative producer was quite strong, the slope of the linear regression could be a good estimate of the cell quota (Salmaso *et al.*, 2014), reducing the variability

due to extreme values. A reliable estimate of the content of MC per cell can be very important when the possible risk for the population has to be assessed when only the density of CB is known.

The 'average' density-based *P. rubescens* cell quota determined in this study were in the range of other values reported in literature (Naselli-Flores *et al.*, 2007; Briand *et al.*, 2008; Kosol *et al.*, 2009; Manganelli *et al.*, 2010, 2016). Interestingly, the cell quota determined for *M. botrys* were constantly about 10 times higher than *P. rubescens* and in the range of *Microcystis* cell quota in the field estimated by Yu *et al.* (2014) (0.001 and 1.326 pg cell⁻¹ on the whole population and 0.012 and 1.876 pg cell⁻¹ on the toxic cells). The high cell quota and the production of the more acutely toxic variants (MC-LA and MC-LR, showing the lowest LD₅₀ values) make *M. botrys* a highly toxic species, whose dynamic should be carefully monitored from spring to fall, for possible surface blooms.

The levels of MC content during the study never exceeded 1 µg L⁻¹ in all raw water discrete samples, excluding risk of acute and subchronic intoxication by drinking water for the population (WHO, 2004) and the domestic and wild animals (considering the action levels established by the California State EPA, for dairy cows, beef, cattles and dogs; Butler and Linville, 2012). Also the average cell quota was in the range of what is known for the CB species present. However, in the field some unpredictable events are possible (see May and December 2012) for which an exaggerated production of toxin per cell occurs (cell quota values up to 40 fold the average one in December) due to still unknown phenomena. The data used to compute these high values coincided with periods of high MC concentrations and *P. rubescens* density, making highly improbable the influence of measurement errors on the toxin quota estimates. A sudden increase in toxin concentration, in a time-frame of a few hours, has been observed in *Microcystis* in the field and in laboratory experiment, and it has been associated to the fast increase in cell density and to faster MC production rate (Wood *et al.*, 2011, 2012). We speculate that the integrated sample could partly reproduce such a situation, since the ambient conditions of the cells change completely within few minutes, including a rapid increase of cell density. However, this explanation does not fit with the discrete December samples, as well as with the integrated R3 sample in May. This is definitely a field to explore, to define adequate strategies of management, since these situations are of particular concern when the risk for the exposed consumers has to be estimated.

The relatively low cell densities found in the monitoring period is consistent with the low MC contamination level found in trout muscle. The discrepancy between the ELISA and LC-MS/MS methods (estimated concentrations up to 0.9 ng g⁻¹ and trace values, respectively) may

be explained by multiple reasons: i) the matrix effects affecting the ELISA detection, interfering with the optical density detection; ii) the ELISA kit can reveal the presence of those MC congeners for which analytical standard are not available for LC-MS/MS; and iii) the presence of conjugation products in fish tissue, which can cross-react with anti-MC antibodies. The trace levels detected by LC-MS/MS might be due to better compensation of matrix effects by the use of an appropriate calibration curve. However, low recovery of some MC variants could result to underestimation of MC concentrations in the muscle samples. In this respect, as pointed out by a review on the occurrence of cyanotoxins in food items (Testai *et al.*, 2016a), the availability of reliable, validated methods for detecting MC in complex matrices is a priority to collect good quality data to be used in the estimation of the possible risk due to human consumption. In addition, in view of the variability in the rate of toxins production by the CB communities, and in order to have a reliable estimate of the average ingestion of potentially contaminated fish and shell-fish grown in a specific water body, it is necessary to measure cyanotoxin content in the edible parts of representative species. Furthermore, the sampling should be carried out over an adequate period of time to take into account seasonal variations in cyanotoxins production and possible differences among fish species depending on their diet, as well as on their capacity of retention/detoxication and elimination of cyanotoxin residues. It has indeed been shown that the bioaccumulation of MCs was species specific, with a ten-fold higher bioaccumulation in catfish rather than in carp (Singh and Asthana, 2014).

CONCLUSIONS

Toxic cyanobacteria blooms can be a serious problem for the surface water distribution system in Sardinia. In the oligo-mesotrophic Flumendosa reservoir, toxic cyanobacteria were continuously present, although with relatively low densities and variations over time. Three different species were alternatively dominating, namely *P. rubescens* (producing the demethylated form of MC-RR and MC-LR), *M. botrys* (producing MC-LR and -LA) and *W. naegeliana* (whose toxicity is still unknown). Particular attention should be paid to the not visible deep *P. rubescens* summer blooms, since they are located where water is taken for drinking water supply. *M. botrys*, although not detectable during winter, was abundant in summer, meaning that inocula of the population are always present and should be carefully monitored since it produces the most toxic variants with a high cell quota.

Our results show that due to the relatively low density of CB and limited MC concentration found in the lake during the study period, there is no immediate risk of ex-

posure to MC for the human and animal population. However, a variable CB community, characterised by the persistence of a significant toxic fraction, and the evidence of spotted variable toxin production rates suggest the need to take the system under control to manage possible sudden changes in toxins concentrations. The validity of the linear model applied to separate MC variants, to be further verified, show the possibility to expand its use in more complex CB communities, to obtain slopes representative of the studied systems.

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