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ARTICLE

Vertical and horizontal distribution of the microcystin producer *Planktothrix rubescens* (Cyanobacteria) in a small perialpine reservoir

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ABSTRACT

Among cyanobacteria, *Planktothrix rubescens* (De Candolle ex Gomont) Anagnostidis & Komárek is a species that is well adapted to develop in moderately nutrient rich and deep lakes. In this typology of waterbodies, the competitive abilities of this species rely in its capacity to stand and growth in the dimly illuminated metalimnetic layer during the warmer months. In this work, we have studied the seasonal development and distribution of this species in Lake Ledro, a meso-oligotrophic reservoir located in the Eastern Alps. During the last decade, this species has given rise to numerous and extended surface bloom episodes, causing the reddening of vast areas of the lake. In summer, the light intensities in the zone of greater development of this cyanobacterium (the metalimnion, between the euphotic depth and the layer of maximum development of the species) were between 2 and 20 µmol m^{-2} s^{-1}, *i.e.* values that were well within the light intensities required to sustain the optimal growth of filaments. The formation of the autumn and winter blooms was triggered by the cooling of surface waters and the deepening of the mixed layer, which, eroding the metalimnion, entrained the filaments of *P. rubescens* in the surface mixed layers. The formation of the surface blooms was associated with the presence of high amounts of microcystins, which in a few occasions reached concentrations between 10 and 22 µg L^{-1}, posing potential problems for the exploitation of water resources.

Key words: Cyanobacterial blooms; cyanotoxins; *Planktothrix rubescens*; FluoroProbe.

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INTRODUCTION

Inhomogeneous vertical and horizontal distribution is a well know characteristic of phytoplankton populations (George and Edwards, 1976; George and Heaney, 1978; George, 1981; Salonen *et al.,* 1999; Klausmeier and Litchman, 2001; Oliver and Ganf, 2002; Clegg *et al.,* 2007; Hamilton *et al.,* 2010). The formation of patches, thickening at selected water layers and blooms is driven by interactions between physical and biological processes (Fietz *et al.,* 2005; Hillmer *et al.,* 2008). Some species can change their position in the water column by actively swimming like flagellates (Clegg *et al.,* 2007; Simmonds *et al.,* 2015), or by regulating their buoyancy, like cyanobacteria (Walsby *et al.,* 2004). Their distribution is conditioned by a large number of driving factors, which include the allocation of resources (Klausmeier and Litchman, 2001), the temperature gradients (Clegg *et al.,* 2003, 2007), the light (photo response) (Rhiel *et al.,* 1988), and the chemical gradients (Clegg *et al.,* 2004). Vertical migrations finalized at finding optimal conditions for photosynthesis are often observed in Dinoflagellates, such as *Ceratium hirudinella* (O.F. Müller) Dujardin (Alexander and Imberger, 2009; Whittington *et al.,* 2000; Whitton and Potts, 2000) and *Peridinium cinctum* (O.F. Müller) Ehrenberg (Regel *et al.,* 2004). Cyanobacteria are able to regulate buoyancy through the biosynthesis of specialized gas vesicles, which enable populations to actively move to water depths with optimal growth conditions. Vertical movement rates in cyanobacteria range from a few centimetres per hour to a few meters per hour (Salmaso *et al.,* 2014b). As expected, the highest speeds (between 0.5 and 2 m h^{-1}) are reached by the large colonies of *Microcystis* and the filamentous aggregates of *Dolichospermum* and *Aphanizomenon*. Other data quoted in Paerl (1988) report speeds of up to 10 m h^{-1}, whereas Oliver *et al.* (2012) indicated maximum velocities achieved by large aggregates of *Dolichospermum circinalis* (Rabenhorst ex Bornet & Flahault) P. Wacklin, L. Hoffmann & J. Komárek of more than 200 m h^{-1}. The speeds achieved by individual thin filaments of cyanobacteria are much lower and limited; in the case of *Planktothrix rubescens* (DeCandolle ex Gomont) Anagnostidis & Komárek, to a few centimetres per hour, typically 3-5 cm h^{-1}, though higher speeds can be reached by the aggregation of filaments in larger units (Walsby *et al.,* 2006).

P. rubescens is a typical cyanobacterium that is commonly found in deep oligo-mesotrophic waterbodies. The formation of layers of variable thickness in the metalimnetic zone is a peculiar characteristic of this species. This

behaviour is regulated by the vertical light field and the tight coupling between carbohydrate accumulation and gas vesicle buoyancy (Walsby *et al.,* 2004). *P. rubescens* gas vesicles of different strains are characterised by different strength to hydrostatic pressure in function of lake morphometry and depth (D'Alelio *et al.,* 2011). This species is adapted to grow at low light irradiance (up to ~1 µmol m^{-2} s^{-1}) and this characteristic is advantageous not only in summer (filaments located in the metalimnetic layers), but also for populations developing in autumn or dragged down at greater depths during winter mixing (Walsby and Schanz, 2002). The tolerance to low light conditions is due to the presence of phycobiliproteins like phycocyanin and phycoerythrin, which are antenna pigments able to capture the whole spectrum (400-700 nm) of the Photosynthetic Active Radiation (PAR), providing a strong competitive advantage over other algae (Bright and Walsby, 2000; Oberhaus *et al.,* 2007). Furthermore, when inorganic phosphorus is depleted, *P. rubescens* is able to use dissolved organic phosphorus by excretion of alkaline phosphatase (Feuillade *et al.,* 1990).

Similarly to other toxic cyanobacteria, *P. rubescens* produces several microcystin (MC) congeners, most of them highly toxic for animals and humans (Meriluoto *et al.,* 2017; Metcalf and Codd, 2012). The formation of cyanobacterial blooms in waters used for drinking purposes has been associated to the occurrence of human cancer in a number of countries (Fleming *et al.,* 2002; Svircev *et al.,* 2009; Ueno *et al.,* 1996; Zhou *et al.,* 2002). Therefore, the occurrence of toxic algal blooms in reservoirs used for drinking water production and bathing activities is of critical importance for human health (Bogialli *et al.,* 2013; Hitzfeld *et al.,* 2000; Hoeger *et al.,* 2005). When lake water is derived from metalimnetic layers, particular attention must be devoted to the potential risk of contamination (Leboulanger *et al.,* 2002; Cuypers *et al.,* 2011). MC produce adverse effects also on zooplankton and fish (Shams *et al.,* 2014; Sotton *et al.,* 2014; Sukenik *et al.,* 2015).

The methods used for the determination of phytoplankton based on the collection of discrete samples in the euphotic zone lack the necessary resolution to obtain accurate knowledge of the species distribution (Beutler *et al.,* 2002). Determination of the fine vertical distribution of an organism with standard discrete sampling methods is difficult if not unfeasible, in particular when populations are concentrated in thin metalimnetic layers. Conversely, submersible devices measuring in continuous mode and real-time can accurately identify even the tiniest changes of biomass along the water column (Gregor *et al.,* 2005; Humbert and Törökné, 2017). A widely used approach is based on the detection of the fluorometric signal produced by the phytoplankton photosynthetic pigments (Salmaso *et al.,* 2017). Based on this approach, the use of the fluorometric probe has allowed the description of the spatial heterogeneity of phytoplankton along the water column (Alexander and Imberger, 2009; Longhi and Beisner, 2009; Moreno-Ostos *et al.,* 2006; Selmeczy *et al.,* 2016; Serra *et al.,* 2007) and along horizontal gradients (Carraro *et al.,* 2012; Moreno-Ostos *et al.,* 2009; Salcher *et al.,* 2011). The use of these probes is particularly useful to catch short-term variations in the vertical distribution of metalimnetic species like *P. rubescens* due to displacement caused by seiches. These transient changes are poorly described because of their rapid and occasional nature that are difficult to detect with the traditional sampling methodologies (Garneau *et al.,* 2013).

Compared to the large and deep lakes, knowledge of accurate distribution of *P. rubescens* in reservoirs is less known. In this typology of waterbodies, the relationships between the vertical distribution of *P. rubescens*, the thermal structure and the light regime have been poorly investigated. With the aim to fill this gap, we performed an investigation on Lake Ledro, which is a meso-oligotrophic reservoir that has attracted the interest of scientists for paleolimnological and hydrodynamic investigations (Joannin *et al.,* 2013; Magny *et al.,* 2009; Milan *et al.,* 2016; Santo *et al.,* 2017; Simonneau *et al.,* 2013; Vanniere *et al.,* 2013) and that is characterized by periodic *P. rubescens* blooms (Salmaso *et al.,* 2013). The main objective of this study is to describe the vertical distribution of *P. rubescens* based on high resolution profiling and to interpret the distribution patterns as a function of the main environmental drivers. A second objective is to evaluate the toxigenic potential of the species, in relation to the seasonality of its biomass and toxin production, as well as the main use of the lake.

METHODS

Study site

Lake Ledro is located south of the Italian Alps, 6 km north of Lake Garda, at 650 m asl. The catchment area covers ca. 131 km^2. The lake is feed by two tributaries, the Massangla and the Pur rivers that are often dry. The largest amount of water originates from sub-lacustrine inlets. The outlet is the Ponale River, draining into Lake Garda at 65 m asl. Since 1928 the level of Lake Ledro is regulated for hydroelectric exploitation, with seasonal water level fluctuations of several meters. The lake is connected by a penstock to a pumped-storage plant on the shores of Lake Garda, from which water may be again pumped to Lake Ledro. The lake is extended in the NW-SE axis, and delimited by steep slopes surrounding a relatively wide and flat central basin. Prevailing winds, in particular in the summer season, blow from SE in the morning and from NW in the afternoon (Fig. 1).

Between the beginning of the 1970's and the end of the

1990's, Lake Ledro underwent a phase of eutrophication due to wastewater discharges, massive use of phosphate detergents and livestock. Water quality programs aimed at improving waste water treatment plants and reduction of nutrient loading into the lake begun in the 1980s.

Sampling, laboratory analyses and field measurements

Sampling and field measurements were carried out monthly in the deepest zone of the lake from June 2011 to May 2012.

At the time of sampling, profile measurements of temperature, pH, dissolved oxygen and conductivity were made using a multiprobe CTD, Idronaut Ocean seven 316. Light (PAR) intensity was measured with a submersible irradiance sensor (LiCor 192SA). The euphotic depth (z_{eu}) was defined as the depth at which the PAR irradiance drops to 1% of its sub-surface value, *i.e.* $z_{eu} = \ln(100)\, k_d^{-1}$, where k_d is the vertical light attenuation coefficient (m^{-1}) (Kirk, 2011).

Phytoplankton biomass, expressed in µg L^{-1} of chlorophyll-*a* (Chl-*a*), was measured at each sampling date by the submersible fluorometric probe FluoroProbe II (bbe-Moldaenke, Kiel, Germany) (Kring *et al.,* 2014). The device is based on the measurement of chlorophyll fluorescence emission after being excited at five different wavelengths (450, 525, 570, 590, and 610 nm) employing pulsed light-emitting diodes for the excitation of pigments present in the phytoplankton. The FluoroProbe II device allows the discrimination of four algal groups including 'green' algae (Chlorophyta and Euglenophyta), 'blue' algae containing phycocyanin (Cyanophyta), 'brown' algae (Bacillariophyta, Chrysophyta and Dinophyta), and 'red' algae containing phycoerythrin (Cryptophyta, *Planktothrix*). To discriminate Cryptophyta and *P. rubescens* signals, an additional fingerprint must be specifically assigned to the red, phycoerythrin containing *P. rubescens* (Leboulanger *et al.,* 2002). To do this, a strain of *P. rubescens,* isolated from Lake Ledro was used to calibrate the probe, which was first immersed in GF/F pre-filtered lake water to obtain a natural lake blank, and then in lake water dominated by *P. rubescens* with known Chl-*a* concentration. After calibration, we noted a very good correlation of the measured fluorescence with Chl-*a* (Beutler *et al.,* 2002). Moreover, the fluorometer records were checked against cell counting of discrete field samples. In particular, the differences between the slopes obtained by the regressions between the biovolumes of *Planktothrix* and the abundances obtained by the FluoroProbe at different depths were tested by computing ANCOVA statistics (Crawley, 2005).

The fine vertical and horizontal distribution of *P. rubescens* by high resolution spectrofluorometric profiles was further analyzed in one additional one-day sampling campaign carried out on 26 September 2011. The horizontal distribution was determined by 5 vertical spectrofluorometric profiles along a longitudinal transect in the main axis of the lake. Due to the small size of the lake, the time required to complete a whole horizontal survey was very short, and the collected data could be considered representative of the horizontal gradient.

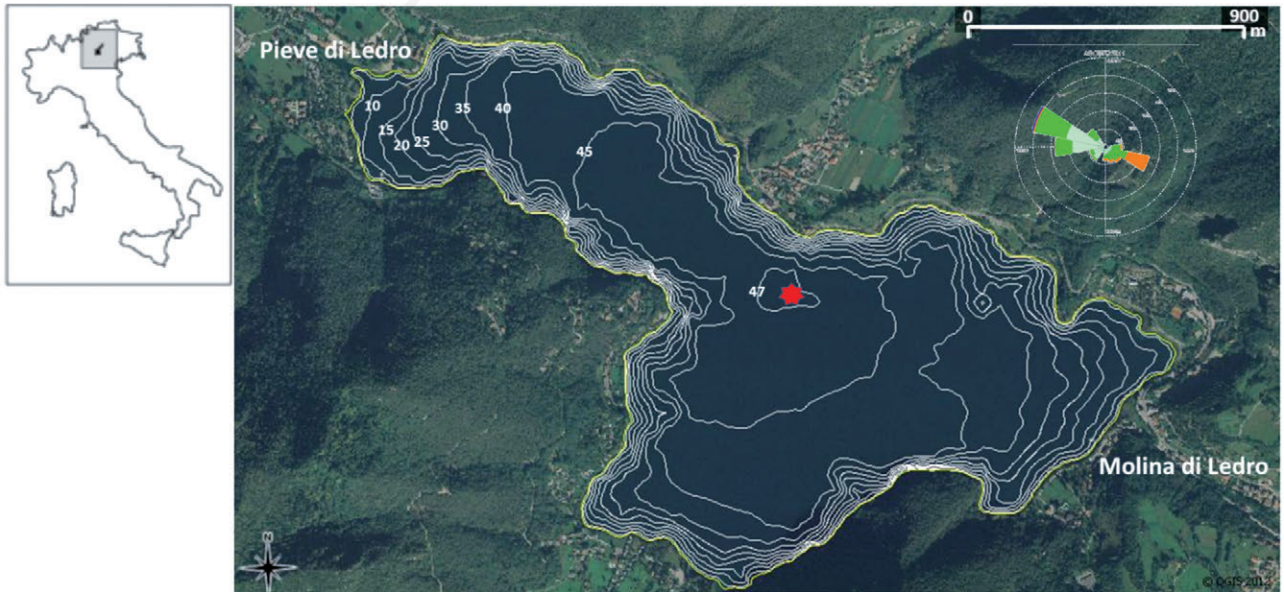

Fig. 1. Location of Lake Ledro and bathymetric map indicating the position of the sampling station (red star). The wind rose shows the wind direction and velocity (m s^{-1}) in August 2012.

Water samples for the chemical analyses were collected using a 5 litres Niskin bottle at 0, 5, 10, 15, 20, 25, 30, 35, 40 m and at 1 m from the bottom. In laboratory, total phosphorus (TP) and nitrate nitrogen (NO_3-N) were measured following standard methods (APHA, 2010).

Phytoplankton samples were collected at 1, 5, 10, 15 and 20 m; after fixation in Lugol's solution, subsamples of 10 mL were analyzed using inverted microscope. Biovolumes were calculated from abundances and specific biovolumes approximated to simple geometric shapes (Rott *et al.,* 2007). In November 2011, during the onset of the bloom, two additional samples were collected.

Samples for the determination of algal toxins were collected at the surface, 10 and 20 m depth between June 2010 and October 2012; a few other occasional samples were collected at 5 and 15 m. The concentration of MC and anatoxin-a (ATX) were determined by UHPLC-MS/MS (Waters Acquity LC coupled to AB Sciex 4000 QTRAP mass spectrometer). Toxin extraction from algal biomass was performed after filtration of the water samples on GF/C filters. The analytical procedures were described in detail in Cerasino *et al.,* (2016) and Cerasino and Salmaso (2012).

RESULTS

Water temperature and nutrients

Since March/April, water temperature slowly increased from 5°C rising to the maxima of 23.5°C in August. The lake showed a stable thermal stratification from April to November, with a stable metalimnion developing between 8 and 20-25 m. Full mixing occurred in winter, with water temperatures reaching 5°C in early January 2011 (Fig. 2A).

During the summer months, epilimnetic and metalimnetic TP concentrations were around 2-11 µg L^{-1} and 9-35 µg L^{-1}, respectively (Fig. 2B), whereas corresponding concentrations of NO_3-N were around 0.65-0.72 mg L^{-1} and 0.52-0.72 mg L^{-1}, respectively. During the winter full mixing, TP and NO_3-N average concentrations were 15 µg L^{-1} and 0.74 mg L^{-1}, respectively. Overall, the annual (2012) average values (±SD) of TP and NO_3-N in the epilimnetic layer (0-20 m) were 14±6.4 µg L^{-1} and 0.83±0.16 mg L^{-1}, respectively.

Transparency and Chl-*a*

Water transparency showed lower values between November and April (2.3-5.8 m). The highest transparencies of the lake were detected between May and October (5.6-11.3 m). The annual (2012) average value (±SD) of transparency was 6.7±2.3 m.

Excluding a peak detected on October 2012 (18.7 µg L^{-1}), between 0 and 10 m, Chl-*a* concentrations were between 1 and 11 µg L^{-1}. In the layer 15-20 m, concentrations were much higher, ranging between 1.5 and 34 µg L^{-1}. The annual (2012) average value (±SD) of Chl-*a* was 6.6±6.6 µg L^{-1}.

Phytoplankton groups

Cyanobacteria were the main phytoplankton group developing in Lake Ledro (Fig. 3). Excluding the first sampling date, their contribution varied between 30% and 93% of the total determined fraction. The main species within this group was represented by *P. rubescens*, whose fraction represented between 98% and 100% on the total cyanobacteria biovolume. The other abundant groups were represented by Bacillariophyta (1-65%; mostly *Fragilaria crotonensis* Kitton, *Asterionella formosa* Hassal, *Cyclotella* spp., *Diatoma elongata* (Lyngbye) C. Agardh and *Fragilaria* spp.) and Chrysophyta (<1-40%; mostly *Dinobryon* spp. and *Mallomonas* spp.).

The biovolumes of *P. rubescens* estimated by the microscopic countings were closely correlated with the abundances estimated by the FluoroProbe. Excluding two outliers detected in June (15 m) and October 2012 (10 m), the relationships between these two estimates were always highly significant (Tab. 1). The slope of the relationship between the two variables was slightly higher at the surface (0-2 m) compared to the deeper layers, particularly at 20 m (ANCOVA, $P<0.01$), indicating a greater fluorometric response of *P. rubescens* in the layer around the metalimnion.

Time and depth distribution of *P. rubescens*

In the whole study period, the temporal development of *P. rubescens* as estimated by the FluoroProbe showed a clear seasonal pattern (Fig. 2C), which was confirmed, in the first 20 m, by the biovolume estimates obtained by microscopic countings (Fig. 2D). During the maximum thermal stratification (June-October) *P. rubescens* was always located in the metalimnetic layer, at the highest density gradient, with maximum biovolumes ranging between 7000 $mm^3\ m^{-3}$ in 2011 and 10700 $mm^3\ m^{-3}$ in 2012. High oxygen saturation was measured immediately above the peak of *P. rubescens* with values up to 190% (*Figure not shown*).

In October, with the decreasing of air temperature, epilimnetic water body cooled and progressively mixed down to the depth of the *P. rubescens* layer, causing the entrainment of filaments in the mixolimnion. Eventually, with the lake fully mixed, the filaments were homogeneously distributed down to the lake bottom (Fig. 2C). In spring, at the onset of thermal stratification, a small subsurface maximum in the *P. rubescens* populations began to develop. During the maximum thermal stratification (June-October), the euphotic depth ranged between 10 and 19 m and the maximum *P. rubescens* density ($peak_{max}$)

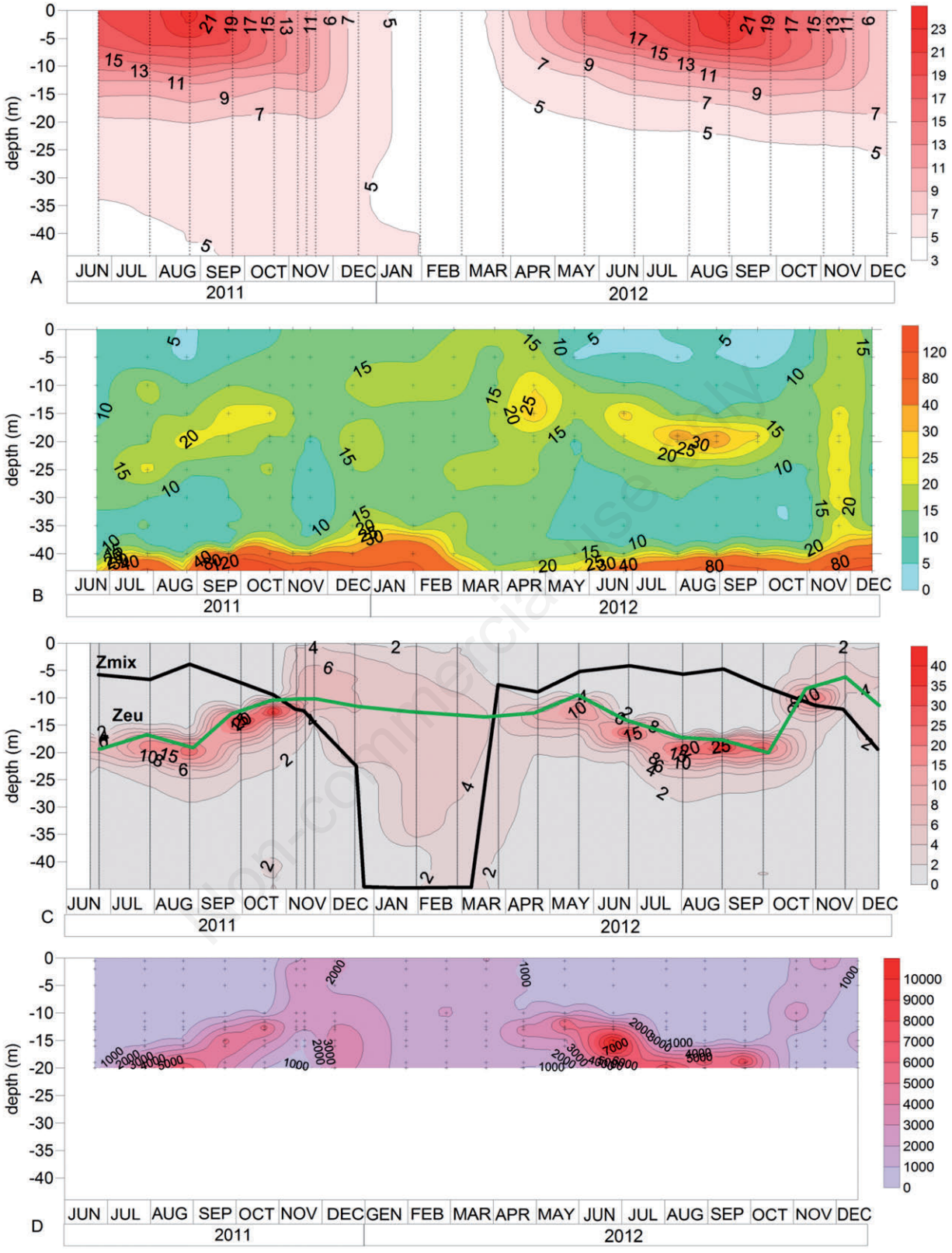

Fig. 2. Temporal development of (A) water temperature (°C); (B) total phosphorus (µg L^{-1}); (C) biomass of *P. rubescens* as determined by the FluoroProbe (Chl-*a* eq, µg L^{-1}); and (D) biomass of *P. rubescens* estimated by microscopic countings (mm^3 m^{-3}) between the surface and 20 m in Lake Ledro. In (C) the black and green continuous lines indicate the mixing layer and the euphotic depths, respectively. Data refer to the period between June 2011 and December 2012.

varied between 12 and 19 m (Fig. 2C). The maximum development of *P. rubescens* was located at about 1.5 m below the depth of the euphotic zone. Moreover, 1% of the subsurface light intensity (z_{eu}) ranged between 6 and 20 µmol m^{-2} s^{-1}; and light intensities at the depth of the maximum peak of *Planktothrix* ranged between 2 and 11 µmol m^{-2} s^{-1}(average 6±2.7 µmol m^{-2} s^{-1}). Overall, in the stratification period (April-November), the depths corresponding to the maximum densities of *P. rubescens* were strongly and positively correlated with the variations in the euphotic depth values (r^2= 0.94; P<0.001) (Fig. 4).

The abundances of *Planktothrix* obtained by the FluoroProbe (expressed as µg Chl-*a* eq L^{-1}) were integrated from the water surface to 40-meter depth to calculate the mean total biomass of *P. rubescens* per surface unit (m^2) (Fig. 5). Values increased from 0.03 g Chl-*a* eq m^{-2} in June 2011 to over 0.13 g Chl-*a* eq m^{-2} in September 2011; values fluctuated between 0.05 and 0.13 g Chl-*a* eq m^{-2} in the following months.

The one-day survey carried out on September 2011 along the main axis of the lake allowed the identification of consistent variations in the vertical distributions of the *P. rubescens* biomass persisting along the major axis of the lake. The depths of the *P. rubescens* peaks were a little bit shallower in the NW zone of the lake compared to the SE zone, with a difference of up to 4 m between the two extremities (Fig. 6). Heterogeneity was documented not only in the vertical distribution of *P. rubescens*, but also in the horizontal, with lower biomasses at the NW end of the lake and higher biomasses at the SE end (Fig. 6).

During the investigation, *P. rubescens* occasionally formed extended surface blooms, which were particularly apparent along the shores, as in the episode occurred between the end of October and the beginning of November 2011 (Fig. 7A). This bloom was strictly localized at the surface and thicket along the shores and bays, and therefore no relevant signals were recorded by the FluoroProbe (Fig. 2C). In other occasions, however, occurred before and after the activities carried out in this research, surface blooms were particularly apparent and extended, as in the events of autumn 2009, spring 2010, and December 2013 (Fig. 7B, and Wilmotte *et al.,* 2017: Fig. 4.1 C,D).

Total MC concentrations varied between 1 ng L^{-1} and 4.4 µg L^{-1} (August 2011, 20 m). Generally, the highest concentrations (>0.2 µg L^{-1}) were detected at 20 m, *i.e.* the maximum depth at which sampling for cyanotoxins analysis was performed; during maximum stratification, between July and September, MC concentrations at 20 m were always higher than 1 µg L^{-1}. Overall, the mean concentrations at 20 m were significantly higher (1.3 µg L^{-1}) than those measured at the surface (0.35 µg L^{-1}) and 10 m depth (0.52 µg L^{-1}) (ANOVA and Tukey's test, P<0.001) (Fig. 8). The more abundant MC congeners were represented by MC-RRdm and MC-LRdm. Other minor MC congeners

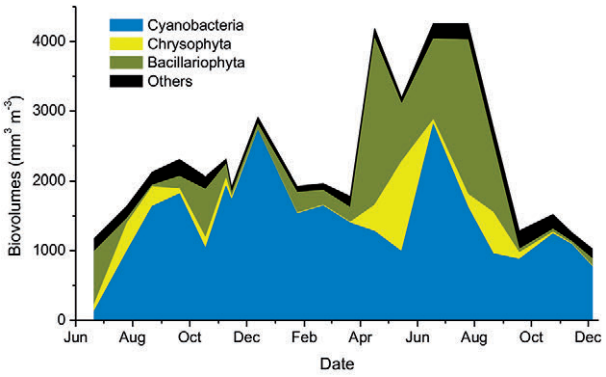

Fig. 3. Temporal development of the dominant phytoplankton phyla between June 2011 and December 2012.

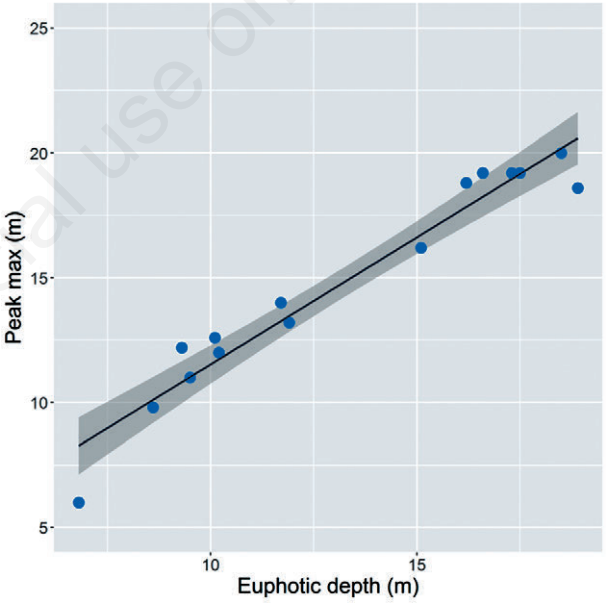

Fig. 4. Relationship between the depth of *P. rubescens* maximum densities ($peak_{max}$) and the euphotic depth (z_{eu}); $peak_{max}$ = 1.35 + 1.02×z_{eu}, r^2 = 0.94, P<0.001. The shaded area indicates the 95% confidence levels.

Tab. 1. Regression between the biovolume values of *P. rubescens* estimated by microscopic countings (mm^3 L^{-1}) and abundances estimated by the FluoroProbe (equivalents of Chl-*a* µg L^{-1}). The parameters a (intercept) and b (slope) were obtained from the regression y = a + b × x, where y is the biovolumes and x the abundances estimated by the FluoroProbe.

	a	b	r2	P
0-2 m	0.14	0.56	0.79	<0.001
5 m	0.15	0.34	0.79	<0.001
10 m	-0.02	0.36	0.88	<0.001
15 m	-0.15	0.42	0.83	<0.001
20 m	0.37	0.27	0.86	<0.001

were MC-HtyrRdm, and MC-RR and MC-LR. Conversely, the neurotoxic alkaloid ATX was always detected with very low concentrations (<0.05 µg L^{-1}). Overall, the total MC concentrations showed a strong relationship with the biovolumes of *P. rubescens* (Fig. 9).

During the blooms, the concentrations of MC at the surface reached quite high values. In a few samples collected at the surface during one of these events (3 November 2011), the total concentrations of MC were between 8.4 and 10.0 µg L^{-1}. An occasional and isolated high value was recorded also at the very surface in March 2012 (9 µg L^{-1}). Before this investigation, total MC concentration measured on a surface sample collected during a bloom on 3 March 2010 was 21.8 µg L^{-1}. In both cases, the dominant congeners were MC-RRdm and MC-LRdm.

DISCUSSION

Ecological and trophic characterization

Populations of *P. rubescens* have been widely studied all over Europe. Traditionally, the first and more numer-

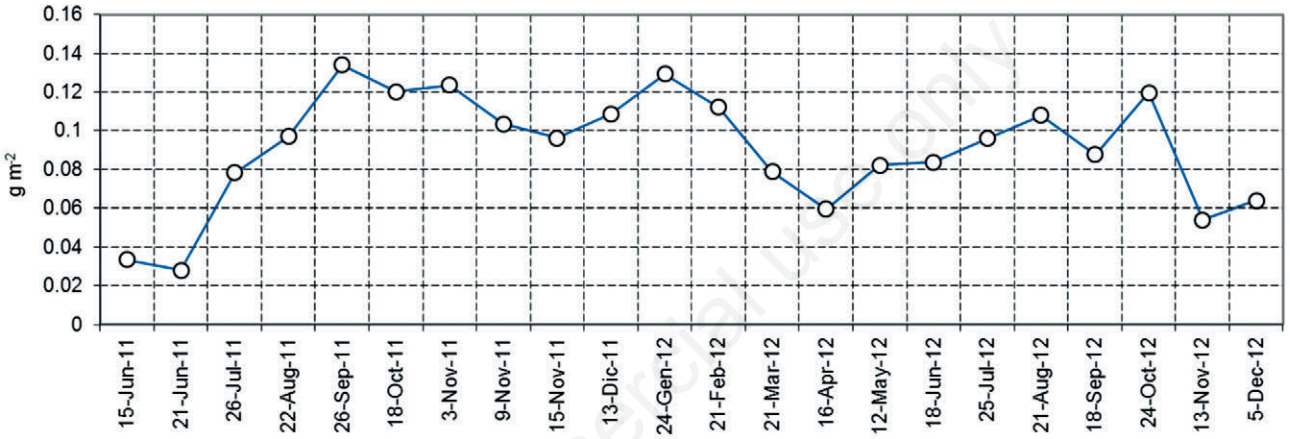

Fig. 5. Integrals of the *P. rubescens* biovolume (g Chl-*a* eq m^{-2}) in Lake Ledro computed from the surface to 40 m depth.

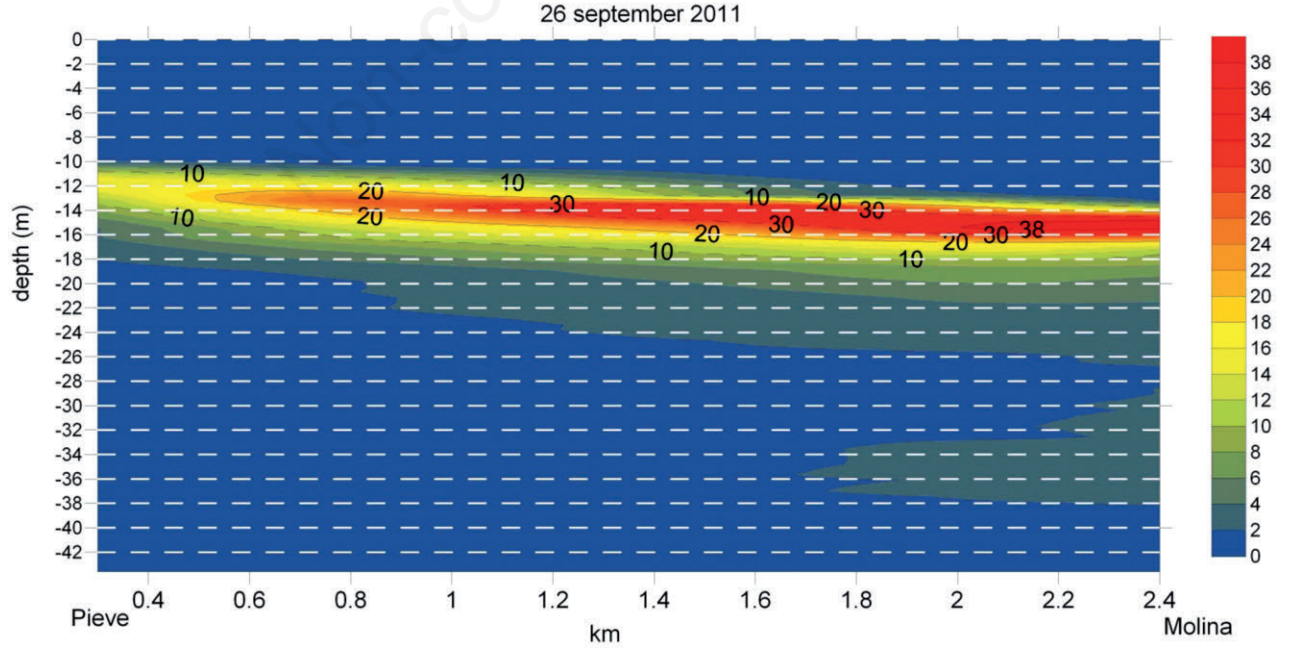

Fig. 6. Two dimensional transects of the distribution of *P. rubescens* biomass obtained by the FluoroProbe as Chl-*a* eq (µg L^{-1}) along the main axis of the lake carried out on 26 September 2011.

ous studies were carried out in the subalpine and perialpine regions (Jacquet *et al.,* 2005; Legnani *et al.,* 2005; Kurmayer and Gumpenberger, 2006; Salmaso *et al.,* 2006; Walsby *et al.,* 2006), Central (Salmaso and Padisák, 2007) and Northern Europe (Halstvedt *et al.,* 2007). For this reason, traditionally this species has been considered a cold-water stenotherm cyanobacterium. Nevertheless, besides the more recent discovery of populations living in Eastern Europe (Vasas *et al.,* 2013), records of this species were documented in many Mediterranean regions, including Central and Southern Italy (Messineo *et al.,* 2006; Assennato *et al.,* 2010), Sicily (Naselli-Flores and Barone, 2000), Spain (Almodóvar *et al.*, 2004), Greece (Vareli *et al.,* 2009) and Turkey (Akçaalan *et al.,* 2014; Köker *et al.,* 2017). More recently, this species has also been identified in Northern Africa (Guellati *et al.,* 2017). The taxonomic position of this species has been widely investigated based both on phenotypic characters (Komárek and Anagnostidis, 2005) and genetic markers (D'Alelio *et al.,* 2013; Gaget *et al.,* 2015; Kurmayer *et al.,* 2015; Salmaso *et al.,* 2016; Suda *et al.,* 2002). Similarly, owing the impact on water quality of the lakes affected by its development, the ecology and trophic preferences of *P. rubescens* have been widely studied in several lakes (Becker *et al.,* 2005; Walsby, 2005; Reynolds, 2006).

Unlike many other cyanobacterial species, *P. rubescens* is usually found in lakes characterized by (oligo-) mesotrophic conditions (Reynolds *et al.,* 2002). In this regard, Lake Ledro confirms the oligo-mesotrophic character of this species; based on the epilimnetic (0-20) annual (2012) values of TP, Chl-*a* and transparency, it can be classified as meso-oligotrophic. *P. rubescens* has the tendency to disappear or develop with rare filaments in oligotrophic and high eutrophic lakes (Jacquet *et al.,* 2014). It has been described as a species that develops during the recovery and oligotrophication processes (Ernst *et al.,* 2001). For example, in Lake Pusiano this species was identified in the early 2000s after the implementation of measures that reduced the wastewater and nutrient loads into the waterbody (Vuillermoz *et al.,* 2006). Similarly, *P. rubescens* gave rise to blooming episodes in Lake Zürich during the early stages of eutrophication, then it disappeared during the eutrophic period, and finally reappeared after reduction of nutrient loads following improvement of sewage treatment plants (Lampert and Sommer, 2007). While the low development of *P. rubescens* in oligotrophic environments is due to deficient concentrations of nutrients or organic matter (Zotina *et al.,* 2003) not sufficient to sustain high growth rates, its decrease in eutrophic environments is due to an excessive reduction of light intensity along the water column and complete metalimnetic darkening due to epilimnetic shading by other algal groups outcompeting cyanobacteria (*e.g.*, see case studies in Jeppesen *et al.,* 2005). Conversely, during summer, in medium enriched lakes, *P. rubescens* is strictly localized in the illuminated metalimnetic layers. The metalimnion is characterized by a strong density gradient with a strong buoyancy force that prevents mixing avoiding entrainment in surface strata (Walsby *et al.,* 2004; next section). With an intensity of light saturation I_k (which indicates the transition from a light-limited to a light-saturated condition) of around 10 µmol m^2 s^{-1} and light inhibition at around 130 µmol m^2 s^{-1}, *P. rubescens* can be considered a true shade phytoplankter, able to grow in low light conditions (Lampert and

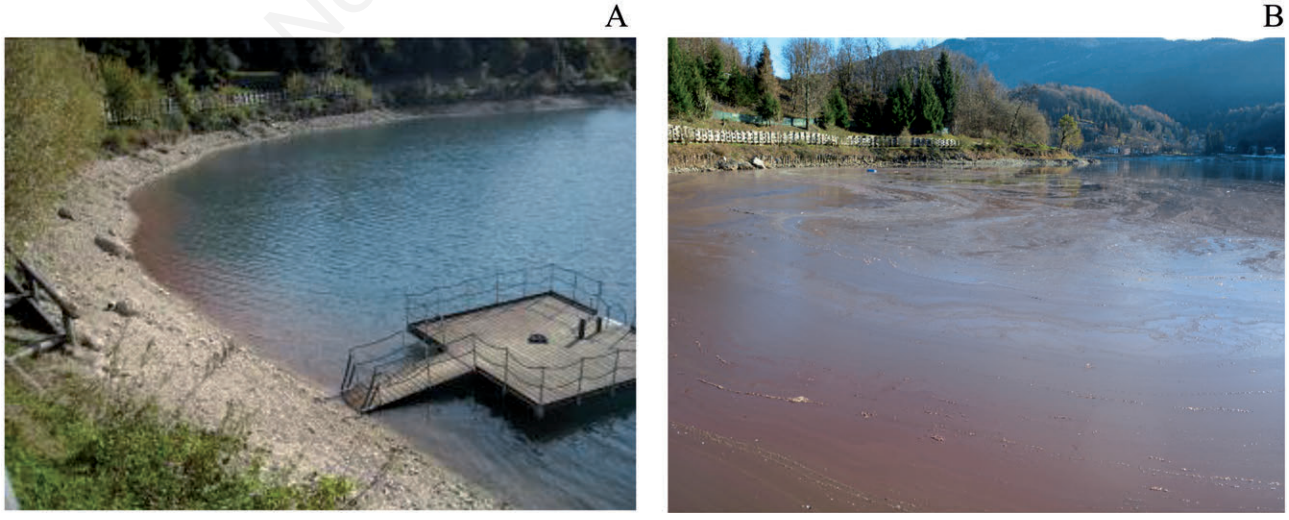

Fig. 7. Surface blooms of *P. rubescens* observed on (A) 28 October 2011 and (B) December 2013 on the northern shore of Lake Ledro.

Sommer, 2007). Photosynthesis in this species is still possible with dim illumination intensities of up to 3-4 µmol m^{-2} s^{-1}. In Lake Ledro, the light intensities in the zone of the greater development of this cyanobacterium (between the euphotic depth and the layer of maximum development of the species) were between 2 and 20 µmol m^{-2} s^{-1}, *i.e.* values within the light intensities required to sustain growth. Therefore, in general, the establishment of conditions suitable for the growth of *Planktothrix* requires a balance between the vertical formation of the metalimnetic layer (where species can concentrate) and its illumination, which, in turn, is controlled by the algal production and shading by other competing species in the epilimnetic layers and, ultimately, trophic state. In Lake Ledro, these conditions appeared fully met.

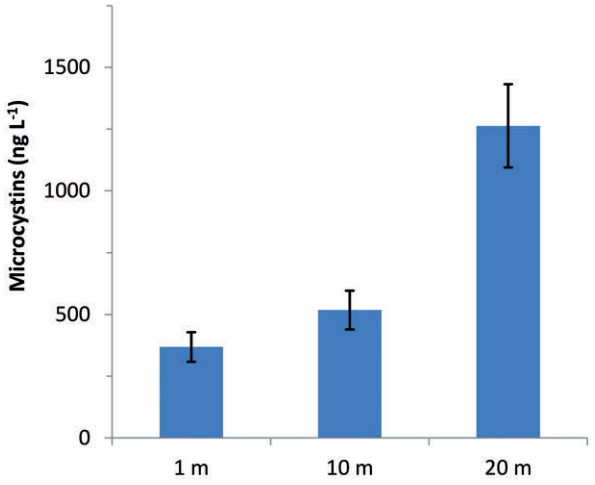

Fig. 8. Mean values of total MC measured at the surface, 10 m and 20 m; data refer to samples collected between June 2011 and December 2012. The vertical bars indicate the standard errors of the means.

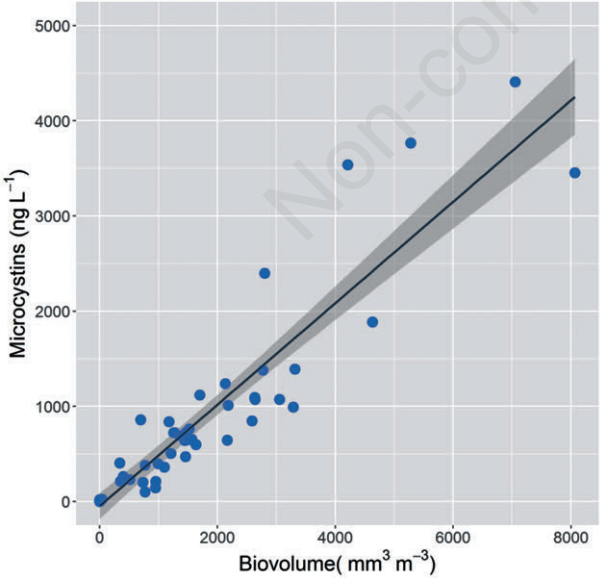

Fig. 9. Relationships between MC and biovolumes of *P. rubescens* (B_{Pr}) in Lake Ledro; MC = -46.6 + 0.53×B_{Pr}, r^2 = 0.86, P<0.001. Data refer to samples collected in the period between June 2010 and October 2012 at the surface and at 10 m and 20 m; a few occasional samples (8) were collected at 5 m and 15 m. The shaded area indicates the 95% confidence levels.

In the metalimnion, cyanobacteria can exploit the nutrients available at the interface between the hypolimnion and the surface depleted layers (Dokulil and Teubner, 2012). In Lake Ledro, during the stratification period, SRP and TP concentrations at the surface were quite low, below 5 µg L^{-1}. The higher concentrations of TP detected around the metalimnion were due to the phosphorus accumulated in the organic matter produced by phytoplankton (mostly by *P. rubescens*) and to the higher availability of nutrients at the interface between epi- and hypolimnion. Overall, this indicated both the ability of cyanobacteria located in the metalimnion to take advantage of nutrients available in the deeper layers and the general availability of a source of nutrients stored in the metalimnion potentially available through bacterial recycling and mineralization processes.

Metalimnetic positioning and bloom formation

In *P. rubescens*, the ability to control the vertical movement and positioning is mostly controlled by the biosynthesis of gas vesicles and by the fraction of carbohydrates (ballast) produced by photosynthesis within the cell (reviewed in Salmaso *et al.,* 2014b). The genes encoding the proteins that constitute the gas vesicles in *P. rubescens* populations isolated from several southern subalpine lakes have been studied by D'Alelio *et al.* (2011). These authors found that the populations of *P. rubescens* living in Lake Ledro were represented by specific genotypes possessing gas vesicles less resistant to the hydrostatic pressure compared to the populations living in the larger and deeper lakes characterized by higher mixing depths, such as, among the others, lakes Garda, Como and Maggiore. Moreover, the limited maximum depth of Lake Ledro (43 m) could assure the survival of the population of *P. rubescens* during winter mixing, giving a competitive advantage to the filaments, which could employ the resources available at the beginning of the vegetative period. In the well illuminated surface layers, the floating caused by the gas vesicles is counterbalanced by higher carbohydrate production, and cells, becoming denser than water, sink. At higher depths and lower light intensity, the production of carbohydrates is low and the cells decrease their ballast and density, positioning themselves in the

metalimnetic layer at a depth (defined neutral buoyancy depth, z_n) (Walsby and Schanz, 2002; Walsby *et al.,* 2006). In Lake Zurich, filaments of *P. rubescens* showed neutral buoyancy after being exposed to PAR illumination of 6.5 µmol m^{-2} s^{-1} and photoperiod of 12:12 hours, corresponding to a daily insolation, Q_n, of 0.28 mol m^{-2} d^{-1} (Walsby *et al.,* 2004; Walsby, 2005). Interestingly, in Lake Ledro the above PAR illumination value is equivalent to the mean light intensity that has been measured in correspondence of the maximum metalimnetic development of *P. rubescens* in the stratified period (*i.e*., 6 µmol m^{-2} s^{-1}). The z_n values increases or decreases in response to high or low brightness conditions, respectively. During the warmer months, when z_n exceeds the mixed depth z_m, *P. rubescens* will be stratified in the metalimnion (Fig. 2 C,D). In autumn, with the concurrent decrease of the depth of neutral buoyancy z_n, which is caused by the decrease in light intensity and day length, and the deepening of the mixed layer, z_{mix}, the metalimnetic thickening of *P. rubescens* begins to erode. When $z_{mix} > z_n$, the filaments are mixed and transported up to the surface, experimenting higher daily average insolation and decreasing buoyancy (Micheletti *et al.,* 1998; Walsby and Schanz, 2002). With further water cooling and increase in z_m, the average daily insolation Q_v experienced by the filaments of *P. rubescens* decreases to values equivalent or below to Q_n, making filaments able to float again and forming, during calm windy conditions, surface blooms (Walsby *et al.,* 2006). Once at the surface, and in the presence of light breezes, the filaments can rapidly move from the pelagic zone to the lake shores and bays (Fig. 7A-B). Conversely, as summarized by Cuypers *et al.,*(2011) and Kurmayer *et al.,* (2015), internal waves can induce pronounced vertical displacements: in a seasonal study these authors described vertical shifts up to 10 m for *P. rubescens*.

The increase in buoyancy and the entrainment of the filaments of *P. rubescens* during the autumn and winter months are the mechanisms explaining the spectacular blooms that have been described in many lakes. These events often influenced the historical folklores and gave rise, for example, to the legend of the Red Cock in Lake Stechlin (Padisák *et al.,* 2010) and to the myth associated with the red discoloration of Lake Murten in Switzerland. The latter one was interpreted as the blood of Burgundian soldiers thrown in the lake in 1476, after the siege of Murten (Walsby *et al.,* 2006). Besides Lake Ledro, in the Italian peninsula intense red autumn and winter blooms were documented in lakes Iseo, Pusiano, Occhito and Vico (Manganelli *et al.,* 2014; Salmaso *et al.,* 2014b).

Biovolume levels and cyanotoxins

The maximum biovolumes attained by *P. rubescens* in the metalimnetic layer during the warmest months (generally between 3000 and 8000 mm^3 m^{-3}) were one order of magnitude higher than those usually recorded in oligotrophic environments, such as the largest and oligotrophic subalpine lakes; conversely, these values were of the same order of magnitude of the biovolumes recorded in smaller waterbodies, such as lakes Occhito and Pusiano (Salmaso *et al.,* 2014a, 2014b). Similarly to Lake Ledro, these two lakes showed the formation of huge surface water blooms. In Lake Occhito, the blooms raised serious concerns due to the use of the waters contaminated by MC for drinking purposes (Di Gregorio *et al.,* 2017).

The concentrations of MC recorded in Lake Ledro showed a clear relationship with the biovolumes of *P. rubescens*. The slope of the regression (Fig. 9) gives an estimate of the increase of MC per unit increase of biovolume (fg μm^{-3}), therefore providing an estimate of the cell quota, CQ, the quantity of toxins per unit biovolume. Though based on a more extended dataset of Lake Ledro, including also data collected between 5 m and 15 m, the slope relating MC and the biovolumes of *P. rubescens* obtained in this work (0.53) was coincident with the slopes computed by Salmaso *et al.,* (2013, 2014a) using both ordinary least square linear regressions (0.51) and Bayesian analyses (0.51). These CQ were the same as those estimated from the samples collected in Lake Garda, suggesting the presence of similar chemotype populations in the two lakes. Overall, the concentrations recorded in Lake Ledro occasionally presented values above 1 µg L^{-1}, which is the limit set by the recent Italian regulatory level for MC in drinking water. However, it is worth to highlight that the highest concentrations were recorded during the summer months in the metalimnetic layers, whereas at the surface occasional values >1 µg L^{-1} were found only during the winter months. The few measurements made during the surface blooms confirmed the presence of high (even >20 µg L^{-1}) concentrations of MC. Nevertheless, as highlighted in previous investigations, a more realistic assessment of toxicity and potential adverse health effects should take into account the actual toxicity of the more abundant congeners (Cerasino and Salmaso, 2012; Salmaso *et al.,* 2014a). Assuming MC-LR as the reference MC with a Toxic Equivalent Factor, TEF = 1, the most abundant MC congeners in the populations of *P. rubescens* living in Lake Ledro, *i.e.* MC-RRdm and MC-LRdm, have TEFs around 4 and 3 times lower than MC-LR, respectively (Wolf and Frank, 2002).

CONCLUSIONS

Among cyanobacteria, *P. rubescens* is a species that is well adapted to develop in moderately nutrient rich and deep lakes, such as Lake Ledro. In this typology of waterbodies, the competitive abilities of this species rely in its capacity to stand and growth in the dimly illuminated metalimnetic layer during the warmer months. As a matter

of fact, during summer (when the touristic presence around the lake is at the top) the lake water is characterized by 'clean' conditions and high transparency, with values generally higher than 8 m. Paradoxically, the lowest transparency values in this lake are measured during the winter months, after the entraining of the cyanobacterium in the mixed layer. The formation of extended red water blooms in this deep meso-oligotrophic lake characterized by the formation of a deep metalimnion is a phenomenon common to other lakes with same characteristics.

P. rubescens populations in Lake Ledro were always toxic in the considered period. Hepatotoxic MCs were constantly associated to the cyanobacterium presence, while the presence of other toxins (namely, the neurotoxic ATX) was not significant. The total MC distribution in the water column was related to *P. rubescens* distribution showing maximum values in the metalimnetic layer (highest value: 4.4 $\mu g\ L^{-1}$ recorded in August 2011). The MC profile resulted to be constant, with the two variants MC-RRdm and MC-LRdm always dominating. The calculated CQ (0.53 $fg\ \mu m^{-3}$) was in agreement with previous observations (Salmaso *et al.,* 2013, 2014a). All these evidences suggest that the chemotype of *P. rubescens* is constant in Lake Ledro.

The development of very dense *P. rubescens* blooms associated to consistent production of MC deserve further investigation of the ecological and toxicological aspects aimed, for instance, at assessing the potential impact of such high toxin levels on the aquatic flora and fauna (fish community), and on the complete genetic and genomic characterization of toxigenic planktic species, including *P. rubescens* and also other cyanobacteria living in the lake.

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