DOTTORATO DI RICERCA IN BIOLOGIA

XXIX CICLO

Toxin-producing cyanobacteria in the large lakes south of the Alps: detection of new producers and molecular identification methods

Tesi di

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2017

UNIVERSITÀ DEGLI STUDI FIRENZE

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Settore Scientifico Disciplinare BIO/01

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Anni 2013/2016

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ABSTRACT

Cyanobacteria represent a serious health hazard for humans and animals, due to their ability to produce a wide range of secondary metabolites including powerful toxins, among which hepatotoxins (e.g. microcystins, MCs) and neurotoxins (e.g. anatoxin-a, ATX). They are able to develop with higher biomasses in nutrient enriched and thermally stable lakes, and the occurrence of cyanobacterial blooms is increasing worldwide.

The deep lakes south of the Alps (i.e. Garda, Iseo, Como, Maggiore, and Lugano) represent the most important lake district in Italy, and have a strong economic and natural value. One of the dominant cyanobacteria in these lakes was represented by *Planktothrix rubescens*, which includes toxic strains responsible of the production of different types of MCs. Nevertheless, in the last decades the deep subalpine lakes went through a change in trophic status and suffered the effects of global warming. At the same time, they experienced the rapid spread of cyanobacterial blooms due to an invasive species, *Dolichospermum lemmermannii*, a potential producers of MCs and anatoxin-a(s). This species appeared for the first time in Lake Garda at the beginning of the 1990s and progressively, summer blooms were observed in lakes Iseo, Como and Maggiore. The exact causes which have determined these huge changes were poorly known. Moreover, besides the widespread of a large number of MCs congeners in the deep subalpine lakes, the presence of ATX was reported in all the lakes, except Lugano. In this lake district, *P. rubescens* was considered the main responsible for MCs production, while the ATX producers resulted unknown, which pointed out the importance of properly identify the toxin producers. Besides, the appearance of new species, the development of blooms, and the consequent potential increase of cyanotoxins can impact the safety of water resources for human use.

The general aim of the PhD project was to identify, at species and strain level, cyanobacteria producing toxins in the deep lakes south of the Alps. A multidisciplinary approach, including taxonomic, phylogenetic and metabolomics analyses, was undertaken on environmental samples and isolated strains, to fill in the picture about the distribution of the dominant toxigenic species in one of the most important lake district in Europe. The project was developed on selected target species, resulted the most nuisance taxa in the deep subalpine lakes in this decade, *D. lemmermannii* and *Tychonema bourrellyi*.

In this study, the pelagic Oscillatoriales *T. bourrellyi* was identified in Lake Garda for the first time and afterwards, in the other deep subalpine lakes, except Lake Lugano. In all these lakes were identified toxic genotypes, able to produce both ATX and homoanatoxin-a, which were probably the responsible of ATX levels in these basins. In Lake Garda, the quantitative determinations on environmental samples, showed the greater importance of this species. Besides, the analysis demonstrated the increase of ATX and the opposite trend of MCs.

The study of *D. lemmermannii* revealed its ability to develop with high biomass in the warmest months (>15°C), although with a low contribution to the total epilimnetic cyanobacterial biovolume. The analysis of the distribution of sub-fossile akinetes preserved in deep sediment showed the establishment of this species in Lake Garda around the middle of the 1960s, which was concurrent to the rapid increase in total phosphorus and water temperature. The spreading of *D. lemmermannii* from northern regions towards southern Europe, raised concerns due to its ability to produce cyanotoxins. Nevertheless, this study demonstrated the inability of populations settled in the deep subalpine lakes to produce a wide range of toxins. On the contrary, MCs production was observed in North European strains. Differences in *D. lemmermannii* genotypes resulted highly influenced by geographic distance, and the establishment of phylogenetic lineages adapted to specific habitats was likely favoured by the contribution of geographic isolation, physical barriers and environmental factors.

1. INTRODUCTION

1.1 Cyanobacteria

Cyanobacteria (blue-green algae) are ancient photosynthetic prokaryotes globally distributed. Their ability to withstand adverse and extreme environmental conditions (high salinity, high and low temperatures, intermittent desiccation, high solar irradiation) has made them excellent colonizers and among the most present organisms on Earth (Whitton, 2012). The advantage over competitors is also due to special adaptions, like nitrogen fixation, buoyancy regulation, light harvesting pigments, and differentiated cell types for resting (Kaebernick and Neilan, 2001).

Cyanobacteria live in a diverse range of environments, including fresh-, brackish- and marine waters, and the illuminated surfaces of rocks and soils. In aquatic environments they commonly exist as planktonic members of the water column, in dispersed form or as aggregates, but can be even found attached to shoreline rocks and sediments. In nutrient enrichment conditions cyanobacterial mass populations can for blooms, scums and biofilms or mats. Nowadays, the anthropogenic pressures forces eutrophication process by the increase of nutrient loading in freshwater and coastal environments. In concurrence with proper conditions of temperature, light penetration, pH, and inlake water residence time, the massive growth of cyanobacteria can be favoured (Schindler 2006; Meriluoto et al., 2016). Hence, the occurrence of cyanobacterial blooms is increasing worldwide. Besides the negative effects on ecosystem biodiversity, the appearance of blooms and the consequent release of odour and taste factors can impact the aesthetic quality and safety of water resources for human use (e.g. drinking water supplies, irrigation, industrial processing, recreation and tourism) (Meriluoto et al., 2016).

Cyanobacteria synthesise clorophyll-a and most of them are able to produce phycobilin proteins and phycocyanin, which give the cells the common bluish colour, while some groups produce the red accessory pigment phycoerythrin (Whitton, 2012). Among the wide range of secondary metabolites synthesised, the release of cyanotoxins into recreational and drinking water raises concern about the negative implications for public health and the environment (Kaebernick and Neilan, 2001; Pearson and Neilan, 2008).

1.2 Cyanotoxins

The exposure to cyanobacterial toxins is associated with adverse health effects on human and animals. Toxicoses can be attributed to cyanobacterial cells or directly to toxins after the extracellular release in the water phase. Different cases were reported, involving wild and domestic mammals, birds, amphibians, fish, and human poisoning (Falconer, 1996; Onodera et al., 1997; Chorus et al., 2000; Carmichael, 2001; Codd et al., 2005; Hoff et al., 2007). Health risks derived by swimming and other recreational activities in water bodies, by drinking water or otherwise consuming tissues or dietary

supplements that have accumulated the toxins (Dittmann et al., 2013). On human, the effects of cyanotoxins exposure can manifest themselves as gastroenteritis, nausea, vomiting, fevers, flu-like symptoms, sore throat, blistered mouth, ear and eye irritation, rashes, myalgia, abdominal pains, pulmonary consolidation, visual disturbances, kidney damage, and liver damage (Codd et al., 2005).

Cyanotoxins can be grouped according to the chemical structure as cyclic peptides (microcystin and nodularin), alkaloids (anatoxin-a, anatoxin-a(s), saxitoxin, cylindrospermopsin, aplysiatoxins, lyngbyatoxin-a) and lipopolysaccharides, however are more commonly classified in terms of their pathology: neurotoxins, hepatotoxins, cytotoxins and irritant toxins (Kaebernick and Neilan, 2001) (Fig. 1). Among them, the neurotoxins and hepatotoxins have major implications to public health (Pearson and Neilan, 2008).

Neurotoxins have been responsible for several animal poisonings worldwide. The most common neurotoxin, anatoxin-a (ATX), and its analogue homoanatoxin-a (HTX) are postsynaptic, cholinergic neuromuscular blocking agents, which act by molecular mimicry of the neurotransmitter acetylcholine, while the anatoxin-a(s) inhibits acetylcholinesterase activity. Saxitoxins (STX) block the sodium channels and are part of a greater group of neurotoxins involved in paralytic shellfish poisoning (PSP), called PSPs (Carmichael 1997; Carmichael 2001; Codd et al. 2005). The final effect of all the neurotoxins is the interference with the transmission of signals in neurons or across the neuromuscular junction, leading to muscular paralysis and, at worst, death due to respiratory failure (Hoff et al., 2007).

Hepatotoxins have been most often associated to toxicoses, concerned animals deaths and have been responsible for human illness and death, reported from India, China, Australia and Brazil (Carmichael, 1992). This group include the cyclic heptapeptide microcystins (MCs), of which over 100 structural variants have been described (Puddick et al., 2014). All the chemical forms share the common structure, cyclo (-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-) and are mostly variable in the L-amino acids at positions 2 and 4, with demethylation at positions 3 and/or 7 (Sivonen and Börner, 2008; Metcalf and Codd, 2012). Microcystins and nodularin (NOD), a cyclic pentapeptide, inhibit eukaryotic protein phosphatases causing excessive phosphorylation of cytoskeletal filaments and changes in hepatocyte membrane integrity and conductance, eventually leading to liver failure. Besides, these toxins are tumour promoters (Carmichael, 1992; Falconer, 1998). A third common hepatotoxin, the alkaloid cylindrospermopsin (CYN), suppresses glutathione and protein synthesis. Although the liver results the main organ affected, this toxins has cytotoxic effect and is able to affect a wide variety of organ and tissue. Moreover, is able to cause dermatotoxicity and genotoxicity, including the breakage of double stranded DNA (Runnegar et al. 1995; de la Cruz et al. 2013).

The exact role of the production and excretion of secondary metabolites, such as cyanotoxins, it is not yet understood, however it has been suggested that they may play a role in attracting beneficial bacteria while at the same time repelling antagonistic ones and higher order grazers (Paerl and Millie, 1996).

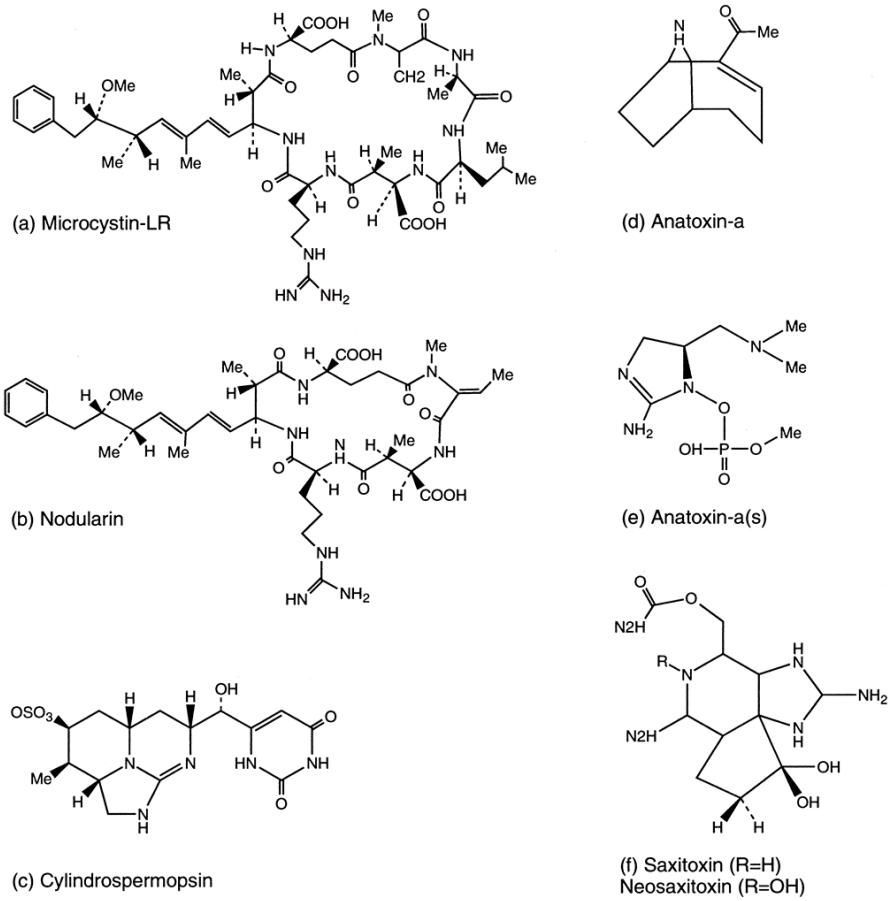

Figure 1. Chemical structures of the most common cyanobacterial hepatotoxins (a,b,c) and neurotoxins (d,e,f) (Kaebernick and Neilan, 2001).

1.3 Cyanotoxin gene clusters

Biosynthetic pathways have been assigned to the majority of known cyanobacterial toxins and revealed unique biochemical features. Cyanotoxins are produced by multifunctional enzyme complexes containing both non-

ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules (Dittmann et al., 2013).

The gene clusters encoding the biosynthetic enzymes, *mcy* (microcystin), have recently been sequenced and partially characterised in several cyanobacterial species, including *Microcystis, Anabaena, Planktothrix* and *Nodularia* (Tillett et al., 2000; Christiansen et al., 2003; Moffitt and Neilan, 2004; Rouhiainen et al., 2004) (Fig. 2). In all genera, MCs biosynthesis follows the same string of reactions, despite some differences in domain specificities, organization of operons and genes, and open reading frames (ORFs) arrangement (Neilan et al., 2013). In the microcystin biosynthetic gene cluster, the *mcyE* is a mixed NRPS-PKS gene (Tillett et al., 2000) encoding the glutamate-activating adenylation domain (ADDA), which is responsible for toxic properties. Despite the presence of the *mcyE* gene it was described even in non-MCs-producing cyanobacteria, this is a very sensitive molecular marker for the determination of potential hepatotoxic cyanobacteria in environmental samples (Rantala et al., 2006; Mankiewicz-Boczek, 2012).

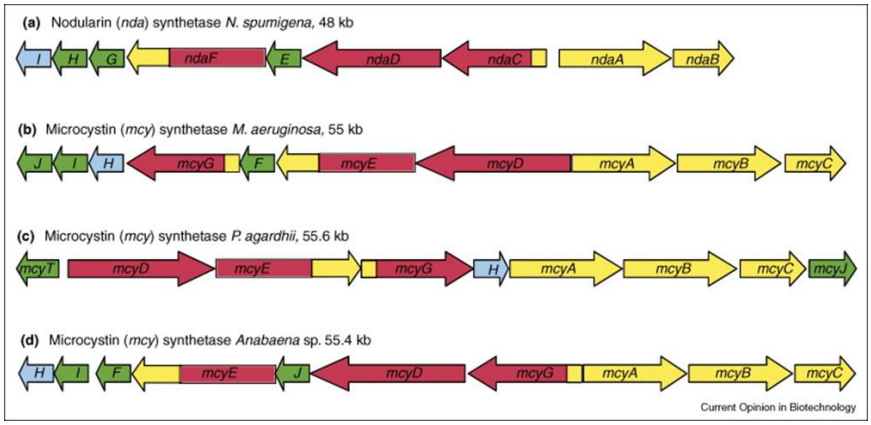

Figure 2. Hepatotoxin gene clusters from various cyanobacteria. Structures of the microcystin and nodularin gene clusters of **(a)** *N. spumigena*, **(b)** *M. aeruginosa*, **(c)** *P. agardhii* and **(d)** *Anabaena* sp. 90, showing genes encoding polyketide synthases (red), non-ribosomal peptide synthetases (yellow), tailoring enzymes (green) and ABC transporters (blue) (Pearson and Neilan, 2008).

The biosynthesis gene cluster for anatoxin-a (*ana*) was described in *Oscillatoria* sp. and in *Anabaena* sp. (Cadel-Six et al., 2009; Méjean et al., 2009; Rantala-Ylinen et al., 2011) (Fig. 3). In the two genera, the *ana* gene cluster encode seven biosynthetic proteins, suggesting a common biosynthetic

pathway. The main differences were determined in the genetic organization of the cluster, which differed for the orientation of some genes (Méjean et al., 2009; Rantala-Ylinen et al., 2011). The *anaC* and *anaF* genes were successfully used for the identification of ATX production in cyanobacteria (Ballot et al., 2010a; Rantala-Ylinen et al., 2011; Shams et al., 2015; Salmaso et al., 2016). The *anaC* gene encodes a NRPS type adenylation domain protein, whereas the *anaF* gene contain a PKS domain (Méjean et al., 2010).

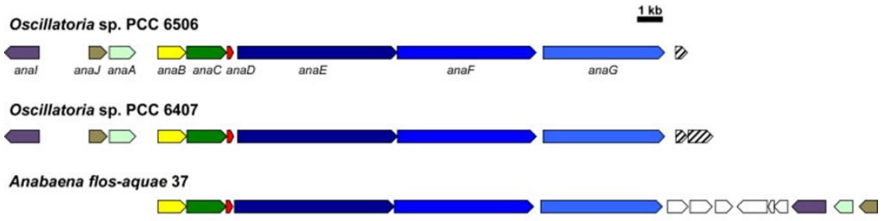

Figure 3. The *ana* clusters so far identified in cyanobacteria. The genes are colour coded for clarity, and the clusters were aligned starting from *anaB* (yellow). Transposase genes are hatched. Genes that have no function in the biosynthesis of anatoxins, in *Anabaena flos-aquae* 37, are white (Méjean et al., 2014).

1.4 The deep lakes south of the Alps

The large and deep lakes south of the Alps (Garda, Iseo, Como, Lugano, and Maggiore) represent the most important lake district in Italy, including over 80% of the surface freshwater resources (Fig. 4). They are narrow and elongated, with a maximum depth comprised between 251 and 410 m (Tab. 1), which brings to a complete mixing only during cold winters (oligomixis). These lakes are located at different altitudes, ranging between the 65 m of Lake Garda and the 271 m of Lake Lugano. The broad hydrographic network of which they belong, that discharges in the River Po, is extensively used in hydropower plants, agriculture, industry, and as drinking water supply. The economic value of this renowned region is farther increased by tourism and the growing numbers of visitors and residents results in a wide use of these lakes for recreational activities (Salmaso and Mosello, 2010).

During the post-war economic growth of the 1960s, the boom of intensive agriculture and tourism industry in the subalpine region led to a significant increase of nutrients (Salmaso & Mosello 2010; Milan et al. 2015). At the end of the 1980s, the eutrophication process had already reached conditions of oligomesotrophy in Lake Garda, meso-eutrophic in Lake

Figure 4. Geographical location of the deep lakes south of the Alps in northern Italy (Salmaso and Mosello, 2010)

Maggiore, eutrophy in lakes Como and Iseo, and eutrophy-hypereutrophy in Lake Lugano. The following restoration measures, like waste water treatment, partially or completely recovered lakes from eutrophication, inducing a general decrease in nutrients. At present, the lakes have stabilised around conditions of oligotrophy (Maggiore), oligo-mesotrophy (Garda, Como), meso-eutrophy (Iseo), and eutrophy (Lugano) (Salmaso and Mosello, 2010; Salmaso et al., 2012). Besides the increase of phosphorus content, in the last 40 years the deep subalpine lakes experienced a water temperature warming. This rising trend (0.1–0.2 °C per decade) is coherent with those observed in other large lakes in the northern side of the Alps, in Europe and in North America (Salmaso et al., 2007). Climate change had a reinforcing effect on anthropogenic pressure in the modification of composition and structure of phytoplankton community (Salmaso et al., 2007; Salmaso and Mosello, 2010).

The deep subalpine lakes sustain a rich aquatic biodiversity (Salmaso and Mosello, 2010) and the development of phytoplankton groups were influenced by concurrent factors, as the large-scale atmospheric patterns over the Atlantic and the Mediterranean regions, the winter air and spring water temperature, the extent of the spring lake overturn, and the extent of nutrient enrichment (Salmaso et al., 2012). Nevertheless, the composition of the phytoplankton community is highly comparable among the lakes, and the main differences are represented by the dominance relationships among the more abundant taxa. In general, Lake Como shows intermediate characteristics between eastern lakes (Garda and Iseo) and the western ones (Lugano and Maggiore). Besides, they share a common seasonal pattern, with the development of large diatoms during spring, a diversified community in summer, and cyanobacteria and Chlorococcales in summer and autumn (Salmaso and Mosello, 2010).

Table 1. Morphometric and hydrological characteristics of the deep lakes south of the Alps (Salmaso and Mosello, 2010).

	Garda	Iseo	Como	Lugano[1]	Maggiore
Altitude (m a.s.l.)	65	186	198	271	193
Area (km^2)	368	62	146	28	213
Maximum depth (m)	350	251	410	288	370
Mean depth (m)	133	123	154	171	178
Volume (km^3)	49.03	7.57	22.5	4.69	37.5
Catchment area (km^2)	2290	1842	4508	297	6599
Mean outflow discharge ($m^3 s^{-1}$)	58.4	58.7	158.0	12.0	291.3
Renewal time (years)	26.6	4.1	4.5	12.4	4.1
Cryptodepression (m)	285	65	212	17	177

[1]Northern basin.

1.5 Cyanobacteria and cyanotoxins in the deep lakes south of the Alps

In the deep subalpine lakes, cyanobacteria represent one of the most dominant phytoplankton groups. The lists of the more abundant cyanobacteria species were highly comparable among these lakes (Salmaso and Mosello, 2010). During the 1970s, *Planktothrix rubescens* appeared in Lake Garda, and are now among the dominant taxa in all the subalpine lakes communities, with the highest biovolumes in lakes Iseo and Como (Salmaso and Mosello, 2010; D'ALELIO et al., 2011; Salmaso et al., 2014). Except for Lake Lugano, a bloom-forming species, *Dolichospermum lemmermannii*, appeared in this lake district from the 1990s and, at the same time, a spreading of *Microcystis aeruginosa* blooms was observed in lakes Como, Iseo and Lugano. Another differences was represented by the presence of *Aphanizomenon flos-aquae* in Lake Maggiore and its strong development in Lake Lugano (Salmaso & Mosello 2010; Salmaso 2000.; Salmaso et al. 2003). The presence of *Tychonema bourrellyi* was documented since the second half of the 2000s only in Lake Maggiore (International Commission for the Protection of Italian-Swiss Waters reports) (Salmaso et al., 2016).

In the deep subalpine lakes, a large number of MCs congeners were widespread and the presence of ATX was reported in all the lakes, except Lugano. Besides, NOD and CYN were not detected. A study undertaken in 2009 in this lake district, showed levels of MCs ranging between 1 ng l^{-1} to about 200 ng l^{-1} and ATX at concentrations ranging from 45 to about 590 ng l^{-1}. However, cyanotoxins found in pelagic and epilimnetic zones did not reach very high concentrations, compared with lakes with higher trophic status, and the majority of MCs variants recorded were less toxic than LR, which WHO uses as a reference (Cerasino and Salmaso, 2012a). In this lake district, *P. rubescens* was considered the main responsible for MCs production, as one of the dominant species, while the ATX producers resulted unknown.

1.5.1 Tychonema bourrellyi

The genus *Tychonema* (Oscillatoriales) include cold-stenotherm filamentous species characterized by keritomized cell content (Anagnostidis and Komárek, 1988; Komárek and Anagnostidis, 2005), able to live in environments ranging from oligotrophic to eutrophic conditions (Skulberg and Skulberg, 1985; Komárek et al., 2003). *Tychonema bourrellyi* is a planktic species first described by Lund (1955) in the Windermere lake, Lake District, in Northern England. It grows as solitary, free-floating trichomes, and develop mass populations usually in metalimnetic layers (Komárek et al., 2003). The presence of large vacuoles irregularly distributed within the cells, instead of gas vesicles, confer to this species a special buoyancy strategy (Komárek and Albertano, 1994) (Fig. 5).

Abundant populations were described in colder lakes of Northern Europe and Canada (Kling and Holmgren, 1972; Skulberg and Skulberg, 1985; Komárek and Albertano, 1994; Komárek et al., 2003). Recently, *T. bourrellyi* was reported in Lake Erhai (1972 m a.s.l.), in China (Wei et al., 2012). Although the authors observed a low cell abundance in the surface, the highest biomass was reached in July and was only slight correlated with water temperature, total phosphorus, total nitrogen, and Chlorophyll-a.

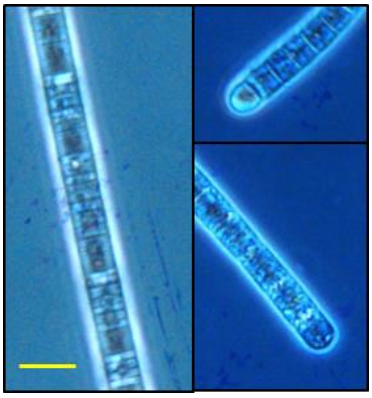

Figure 5. Micrographs of *T. bourrellyi* isolated in Lake Garda in 2014. Observations made at 400X and phase contrast. Scale bars = 10 µm.

1.5.2 Dolichospermum lemmermannii

The genus *Dolichospermum* belongs to nostocalean heterocytous cyanobacteria, the different species of which share the special characters of metameric structure of solitary floating trichomes, with paraheterocytic formation of akinetes and gas vesicles in the vegetative cells (Wacklin et al., 2009). All the Nostocales have the ability to differentiate specialised cells from vegetative cells, adapted for the nitrogen fixation, the heterocytes, and for survival in harsh environments, the akinetes. The latter, can remain quiescent in

bottom sediment and germinate in favourable environmental conditions, therefore can serve as dispersal units for spreading in new environments (Kaplan-Levy & Hadas 2010; Padisák 1998; Stüken et al. 2006). The akinetes shape differs among species and their distribution and position within a filament is used as a taxonomic feature within *Dolichospermum* genus (Kaplan-Levy and Hadas, 2010).

Dolichospermum lemmermannii (Richter) P.Wacklin, L.Hoffmann & J.Komárek, first described in 1903 in Northern Germany (e.g. Grosser Ploner See; Forti, 1907), is reported in temperate and boreal regions (Komárek and Zapomělová, 2007; Lepistö & Holopainen 2008; Skulberg et al. 1994; Willén 2003) in oligo-mesotrophic and deep, stratifying lakes or mesotrophic shallow lakes, with good light conditions (Reynolds et al. 2002; Padisák et al. 2009). Its optimum growth temperature is between 13 to 18° C, however it is higher in specific strains (i.e. between 18°C and 25°C),which underlines the variability to temperature adaptation in this species (Zapomělová et al., 2010). Moreover, the morphological features are quite variable, and the development of the akinetes at both sides of the heterocyte it is the only stable character for the accurate characterization of the species (Komárek & Zapomělová 2007; Zapomělová et al. 2011) (Fig. 6).

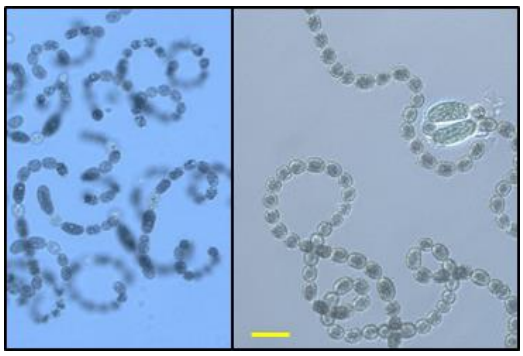

Figure 6. Micrographs of *D. lemmermannii* isolated in Lake Lugano in 2015. Observations made at 400X and phase contrast. Scale bars = 25 µm.

In the deep subalpine lakes, broad surface blooms of *D. lemmermannii* appeared for the first time in Lake Garda at the beginning of the 1990s, between July and September (Salmaso, 2000). Following an altitudinal gradient, likely in relation to the warming of the region (Callieri et al., 2014), blooms were then observed in lakes Iseo (second half of the 1990s), Maggiore (2005), and Como (2006). In this lake district, it generally showed a biovolume increase in epilimnetic layers in summer stratified conditions and during calm weather, when water temperatures were over 16 - 17°C (Salmaso et al., 2012).

2. MATERIALS AND METHODS

2.1 Collection of samples and environmental variables

Field measurements and collection of samples were made at monthly intervals in Lake Garda in the layers 0–2, 9–10 and 19–21 m. The sampling station was located at the deepest point of the lake. Vertical profiles of water temperature were measured with a multi-parameter probe (Idronaut ocean Seven 401 and 316 Plus, and Seabird SBE 19-03). The light attenuation coefficients (Kd) were measured with a submersible irradiance sensor, LiCor 192SA. Concentrations of dissolved inorganic nitrogen (DIN), total and soluble reactive phosphorus, SRP and TP, and pH analyses were carried out using standard methods (Cerasino & Salmaso 2012). Chlorophyll-a, was determined by spectrophotometry. The counting of phytoplankton was undertaken on samples preserved in acetic Lugol's solution following the Utermöhl hl method in sedimenting chambers.

2.2 Isolation of cyanobacteria strains and morphological characterization

Water samples for the isolation of cyanobacteria were collected in different lakes, in the deepest point of the basins, by vertical tows from 30 m to the surface with 25 cm diameter 80 µm mesh plankton nets. Samples were stored at 20°C and processed within 24 h. Single trichomes of the selected cyanobacteria species, were isolated under a macroscope (WILD M420) using a microcapillary. The filaments were washed in Z8 medium (Kotai, 1972) and put in wells on microtiter plates filled with 3 mL Z8 medium. After successful growth, the strains were transferred 2 times and maintained in 300 mL CELLSTAR (Greiner Bio-One GmbH) cell culture flasks containing 150 mL Z8 medium at 20 °C. *T. bourrellyi* strains were growth under continuous light conditions (25 µmol m^{-2} s^{-1}), whereas *D. lemmermannii* strains were growth under 16:8 h light:dark photoperiod (25 µmol m^{-2} s^{-1}). Species were determined on the basis of morphological and morphometrical traits according to Komárek & Albertano (1994), Anagnostidis & Komárek (1988), and Komárek & Zapomělová (2007).

2.3 Transmission electron microscope (TEM)

Cyanobacteria strains were collected together with some of the growth medium and fixed overnight in 1.25 % glutaraldehyde at 4° C in filtered sea water, then post-fixed in 1% OsO_4 in 0.1 M phosphate buffer (pH 6.8) for 1 hr. After dehydration in an ethanol series and a propylene oxide step, the samples were embedded in Spurr's epoxy resin (Spurr, 1969). At each step the cells were sedimented with a 5 minutes centrifuge step at 1500 g and only the sediment was used for the following step. Each solvent was safely substitute with micropipettes without losing cells.

Transverse sections approximately 80 nm thick were cut with a diamond knife and a Reichert-Jung ULTRACUT ultramicrotome. The sections

were stained with uranyl acetate, lead citrate, and then examined with a Philips EM300 TEM at 80 kV.

2.4 Sediment sampling and akinetes analyses

A sediment core was collected with a gravity Kajak corer (UWITEC, Mondsee, Austria) in the deepest point of the basin. The core was vertically extruded and sliced at 0.5 cm intervals (0 to 30 cm) and at 1 cm intervals (31 cm to the bottom). Sediment core dating was parallelised (Thompson et al., 2012) with a core collected previously.

Samples for the akinetes counting were pre-treated with acids, to remove carbonate and silicate crystalline material, and diatom carpets. For each layer, 2 g of sediment were processed subsequently with 10% hydrochloric acid (HCl), 10% KoH, and 10% hydrofluoric acid (modified from Faegri & Iversen, 1989). Samples were fixed in Lugol's solution and akinetes were counted by an inverted microscope at 400×.

The germination of sub-fossil akinetes was obtained inoculating 2-4 g of sediment in 30-60 mL ASM-1 medium(Carmichael & Gorham, 1974), added with cycloheximide (250 mg L–1) (Livingstone & Jaworski, 1980). The cultures were incubated at 20°C under continuous light (85 μmol m^{-2} s^{-1}), for 16-21 days. Cultures were then fixed with Lugol's solution and the germinated cells were counted under an inverted microscope.

Single filaments germinated from akinetes were isolated from the cultures, and transferred in Z8 medium. The strain cultures were grown at 20°C under 16:8 h light:dark photoperiod (25 μmol m^{-2} s^{-1}).

2.5 Cyanotoxins analysis

For the determination of intracellular concentration of cyanotoxins, cyanobacteria cultures (100-250 mL) or environmental samples (2L) were filtered through 1.2 μm GF/C filters (Whatman – GE Healthcare Life Science) and frozen at -20°C. Cyanotoxins were extracted by water:methanol 30:70 v/v – 0.1% formic acid solution and analysed by liquid chromatography-mass spectrometry (LC-MS), using a Waters Acquity UPLC system coupled to a SCIEX 4000 QTRAP mass spectrometer equipped with a turbo ion spray interface. The LC-MS protocols for MC, NOD, CYN are described in details in Cerasino and Salmaso (2012) and Cerasino et al. (2016). The protocol for STX was adapted from Dell'Aversano et al. (2005) and was conducted using an Ascentis Express OH5 (2.7 μm particle size, 50 x 2.1 mm) column (kept at 20 °C). Quantification was performed using calibration curves obtained with commercially analytical standards MC (RR, [D-Asp3]-RR, YR, LR, [D-Asp3]-LR, WR, LA, LY, LW, LF), ATX (Tocris Cookson Ltd), NOD-R, CYN (Vinci Biochem), GTX1/4, C1/2, NeoSTX, GTX5, STX and dcSTX (NRC-CNRC, Canada). The limits of quantitation (LOQ) were between 30 ng L^{-1} and 500 ng L^{-1} (MC congeners), 140 ng L^{-1} (NOD-R), 30 ng L^{-1} (ATX), and 8 ng L^{-1} (CYN).

2.6 Genomic DNA extraction

The cyanobacteria cultures (50-250 mL) were alternatively filtered on 1.2 µm GF/C filters (Whatman – GE Healthcare Life Science) or pelleted by centrifuge, and stored at -20°C. Total genomic DNA was extracted from the filters using the Mo Bio PowerWater DNA Isolation Kit (Mo Bio Laboratories, Inc., CA, USA) or from the pellet using the E.Z.N.A. SP Plant DNA Kit (Omega Bio-Tek Inc., GA, USA), with Glass beads-acid washed (212– 300 mm; 425–600 mm) (Sigma–Aldrich CO., MO, USA). DNA extracted were checked by NanoDrop ND-8000 (Thermo Fisher Scientific Inc., MA, USA).

2.7 PCR amplification and sequencing

Taxonomic identification and phylogenetic analyses of cyanobacteria were undertaken by the amplification of the 16s rRNA gene and housekeeping genes, the *rpoB* gene (β subunit of RNA polymerase) and the *rbcLX* genes (large subunit of ribulose bisphosphate carboxylase/oxygenase). The strains ability to produce MCs and ATX was assessed by the amplification of the *mcyE* gene and *anaC-anaF* genes, respectively.

PCRs were executed on an Eppendorf Mastercycler ep (Eppendorf AG, Germany) using the DyNazyme II DNA Polymerase enzyme (Thermo Scientific). Primers used in PCR reaction are listed in Table 2. The correct amplification of PCR products were checked by 1% agarose gel electrophoresis stained with ethidium bromide, using a commercial DNA ladder (GeneRuler Express, Fermentas). Amplified products were purified by Exonuclease plus Shrimp Alcaline Phosphatase (ExoSAP) and sequenced using the BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Germany), according to the manufacturer's protocols. Primers used for sequencing reaction are listed in Table 2. The reaction products were purified by Agencourt CleanSEQ® Kit (Beckman) and run on an Automated Capillary Electrophoresis Sequencer 3730XL DNA Analyzer (Applied Biosystems, Germany). After trimming of low quality ends by Chromatogram Explorer 3.3.0 (Heracle Biosoft), the sequences were assembled using ClustalW multiple alignment or Contig Assembly Program (CAP) in BioEdit 7.2.5 (Hall, 1999).

2.8 *Tychonema*-specific primers design

A new set of primers specific for *Tychonema* were designed on the region *rbcLX* (Tab. 2), using Primer3Plus software (Untergasser et al., 2007). Potential secondary structures were checked by Oligoanalyzer 3.1 tool (PrimerQuest® program, IDT, Coralville, USA). The primers were tested for specificity, in silico and by PCR against *M. aeruginosa*, *A. flos-aquae*, *P. rubescens*, and *Anabaena* sp. (unpublished).

The primers rbcLX-tyc were developed for PCR reactions, and allowed a rapid identification of *T. bourrellyi* in pelagic samples, amplifying a fragment of ca. 600 bp. The reaction mixture contained DyNazyme II DNA Polymerase (0.5 U), PCR Buffer (10x), deoxynucleoside triphosphate mix (0.25 mM), each

forward and reverse primer (0.5µM), and 2-10 ng genomic DNA. The PCR protocol used is displayed in Table 3. PCR was run on an Eppendorf Mastercycler ep (Eppendorf AG, Germany).

Table 2. Primers used in this study for PCR, sequencing, and real-time qPCR.

Target locus	Primer set	Primer sequence (5'-3')	Product (bp)	Application	References
16s rRNA	CYA359F	GGGGAATYTTCCGCAATGGG	450	PCR/Sequencing	(Nübel et al., 1997)
	CYA781R(a)	GACTACTGGGGTATCTAATCCCATT			
	CYA781R(b)	GACTACAGGGGTATCTAATCCCTTT			
16s rRNA	PA	AGAGTTTGATCCTGGCTCAG	1800	PCR	(Rajaniemi et al., 2005)
	B23S	CTTCGCCTCTGTGTGCCTAGGT			
16s rRNA	16S544R	ATTCCGGATAACGCTTGC		Sequencing	(Rajaniemi-Wacklin et al., 2006)
	16S1092R	GCGCTCGTTGCGGGACTT			
	16S979F	CGATGCAACGCGAAGAAC			
rbcLX	CW	CGTAGCTTCCGGTGGTATCCACGT	800	PCR/Sequencing	(Rudi et al., 1998)
	CX	GGGGCAGGTAAGAAAGGGTTTCGTA			
rpoB	rpoBanaF	AGCMACMGGTGACGTTCC	600	PCR/Sequencing	(Rajaniemi et al., 2005)
	rpoBanaR	CNTCCCARGGCATATAGGC			
anaC	anaC-oscF	CTCTATTCTCACAAGTTTGGTCT	250	PCR/Sequencing	(Rantala-Ylinen et al., 2011)
	anaC-oscR	GTTAGTTCAATATCAAGTGGTGGA			
anaF	atxoaf	TCGGAAGCGCGATCGCAAATCG	300	PCR/Sequencing	(Ballot et al., 2010b)
	atxaR	ACCTCCGACTAAAGCTAGGTCG			
mcyE	mcyE-F2	GAAATTTGTGTAGAAGGTGC	800	PCR/Sequencing	(Rantala et al., 2004)
	mcyE-R4	AATTCTAAAGCCCAAAGACG			
rbcLX	rbcLX-tycF	GTCCAAGCACGTAACGAAGG	600	PCR	This study
	rbcLX-tycR	TTGAATGCCAGTACGAACCA			
rbcX	rbcX-tycQF	ATCCTCCTCTGGCAATCTGG	120	qPCR	This study
	rbcX-tyc_QR	CGCACAGTCATGATCCGAAA			

The primers rbcX-tycQ amplify a region of ca. 120 bp of the *rbcX* gene. These primers are suitable for real-time qPCR application and were developed for the quantification of *T. bourrellyi* in environmental samples. The real-time qPCR protocol was performed for SYBR Green, with KAPA SYBR® FAST qPCR Kit Optimized for LightCycler® 480, following the manufacturer's protocol with an annealing temperature of 59°C. Measurements were carried out on a Lightcycler 480 (Roche, Almere, the Netherlands), with LightCycler software version 1.5 (Roche). Serial dilution of the DNA of *T. bourrellyi* was used as standard sample.

2.9 Phylogeny and statistical analysis

Phylogenetic analyses were realised by R software (R Core Team 2013). Sequences were aligned by MUSCLE (Edgar, 2004), and poorly aligned positions and divergent regions were removed by Aliscore (Misof and Misof, 2009). Phylogenetic trees were computed by Maximum Likelihood (ML), using PhyML 3.1 (Guindon et al., 2010) and approximate likelihood-ratio test based branch support (aLRT, SH-like; Anisimova & Gascuel 2006) or using the package phangorn with 1000 bootstrap (BS) replicates. The best-fitting

evolutionary models, were evaluated by the phymltest function in the R package ape (Guindon et al. 2010; Paradis et al. 2004).

Table 3. PCR protocol for the amplification of the *rbcLX* region in *Tychonema*, using the rbcLX-tyc primers.

Step	T (◦C)	Time	Cycle
Initial denaturation	94	5 min	1
Denaturation	94	30 Sec	30
Annealing	54	40 Sec	
Elongation	72	1 min	
Final elongation	72	7 min	1

The phylogeography of *D. lemmermannii* was analysed applying statistical tests (R software). A matrix of pairwise distances between sequences and a principal coordinate analysis (PCoA) was performed by package ape (Paradis et al. 2004).The dissimilarity matrices of the geographic distances and genetic distances between strains were computed by, respectively, packages gdistance (van Etten, 2015) and ape (Paradis et al. 2004). A Mantel statistic, based both on Pearson's and Spearman's correlations, was used to evaluate the significance of the correlation between the two dissimilarity matrices, using the package vegan (Oksanen et al., 2015).

3. RESULTS

3.1 *Tychonema bourrellyi*

In a study carried out from February to March 2014 (Paper I), *T. bourrellyi* was identified in Lake Garda for the first time. In collected water samples, this species appeared as solitary, free floating and pale red trichomes, with clearly visible large vacuoles, produced by thylacoids. Over 65 cultures were obtained from the isolation of single strain and the morphological determination was supported by phylogenetic analyses on 16S rRNA gene (bp). All the strains isolated in Lake Garda, clustered together with *T. bourrellyi* strains isolated in Norway and maintained by the Norwegian Institute for Water Research Culture Collection of Algae (NIVA-CCA), and with *Tychonema* sequences derived from public repositories. This cluster resulted closely related to *Phormidium autumnale* and *Microcoleus antarcticus* strains. The taxonomic identification was further confirmed by a BLAST homology search on the *rbcLX* gene regions, sequenced from the newly isolated strains. The analysis showed a homology higher than 99% to other *T. bourrellyi* strains from public repositories.

Moreover, 24 strains isolated in Lake Garda were analysed by LC-MS and 11 of them produced ATX (>0.1 mg L^{-1}). On a cell basis, the ATX

concentration was in the range of 0.01-0.35 pg cell^{-1}. To confirm these evidences, in toxic strains the *anaC* gene was amplified, whether it wasn't detected in non-toxic strains. The ATX production was confirmed also in one NIVA-CCA *T. bourrellyi* strain. On the contrary, none of the strains from Lake Garda and NIVA-CCA were positive for MCs, which was also confirmed by the failed amplification of the *mcyE* gene.

A second study aimed at evaluating the development of *T. bourrellyi* in all the deep lakes south of the Alps (Paper II), allowed to identify this species even in lakes Iseo, Como and Maggiore. A wide analysis was undertaken on 49 strains isolated in lakes Garda, Iseo, Como and Maggiore, from May to November 2014, which were taxonomically identified by morphological characterization and phylogenetic analyses on the 16s rRNA gene. These strains formed a compact cluster with other *T. bourrellyi* strains from Norway and Northern Ireland. Further, the BLAST homology search on the *rbcLX* gene of these strains showed >99% similarity to other *T. bourrellyi strains* from public repositories. The discrimination from *P. rubescens*, one of the most common cyanobacteria in the deep subalpine lakes, was fully supported both by morphological analysis under the stereomicroscopes and phylogenetic analysis on the 16S rRNA gene.

Overall, 26 *T. bourrellyi* isolated strains tested positive for the amplification of the *anaC* and *anaF* genes, which was confirmed by the observation of ATX production by LC-MS. Almost all the toxic strains resulted also HTX producers. Usually, HTX was produced with concentrations from 0.2% to 2% compared to those of ATX. On a cell basis, the ATX and HTX concentration varied in the range of 0.03-2.74 pg cell^{-1} and 0.001–0.025 pg cell^{-1}, respectively. The *anaF* gene of toxic strains was sequenced, showing total identity in strains within and among the subalpine lakes and resulted well conserved in Oscillatoriales. A selection of strains were also tested for MCs production giving negative results. On the contrary, *P. rubescens* strains isolated in Lake Garda were confirmed as MCs producers.

To deepen the ecological characteristics of *T. bourrellyi*, in Lake Garda was undertaken a detailed analysis of its environmental distribution between April and November. This species showed a clear distribution, mostly limited to spring and summer months. Its maximum biovolumes were recorded between 10 and 30 m, with peaks in spring. The depth × time development of ATX and HTX followed the same pattern. Whereas, both *Planktothrix* and MCs were strictly localised in the metalimnetic layer in the period between half July and half September. Overall, the mean annual values of MCs in the first 20 m (33 ng L^{-1}) were around 10 times lower than the corresponding ATX averages (308 ng L^{-1}). Moreover, the study of the interannual development (2009-2015) of ATX and MCs in the euphotic layers showed a slight increase of ATX and a significant decrease of MCs. Their maximum concentrations were 2 months out of phase, with maxima around June and August–September, respectively.

The primers specific for *Tychonema* designed in the present study, were able to selective amplify the *rbcLX* genes of *T. bourrellyi* in pelagic environmental samples of Lake Garda, both by PCR and qPCR (data not shown).

3.2 *Dolichospermum lemmermannii*

Considering the wide spread of *D. lemmermannii* in the deep lakes south of the Alps, the first study on this species (Paper III) included a detailed investigations to clarify its taxonomic position, ecology and potential toxicity. In 2013-2014, 19 strains of *D. lemmermannii* were isolated from lakes Garda, Iseo, Como and Maggiore.

The microscopic determination of the isolated strains was supported by phylogenetic analyses based on 16S rRNA and *rpoB* genes. Both sequences underlined slight differences in strain from Lake Maggiore, compared to the other subalpine lakes. However, the Italian strains clustered together with other *D. lemmermannii* strains isolated in Finland. Moreover, none of the strains from the deep subalpine lakes resulted producers of MCs, ATX, HTX, NOD, and CYN.

The long-term and seasonal development of *D. lemmermannii* in the epilimnetic layers (0–20 m) was deeply studied in Lake Garda (1993-2013). Since the beginning of the observations, the abundances of *D. lemmermannii* showed a little tendency to increase until the beginning of 2000s. Its development was characterized by a strong cyclical component, with most of the population developing in the warmest months, generally between June and October. The higher growth of this species was favoured by thermal stability and higher water temperatures, above 15°C. Nevertheless, after 2004, its growth between July and August underwent a strong decrease, with the population mostly developing in early summer and early autumn. Excluding a peak measured in July 2012, its biovolume values were always low (<50 mm3 m^{-3}), usually contributing less than 5% to the total annual cyanobacterial biovolumes.

Despite the appearance of blooms in the deep subalpine lakes from the 1990s, the establishment historical period of *D. lemmermannii* remained unclear and was evaluated by the study of sub-fossil akinetes preserved in deep sediments of Lake Garda (Paper IV). The analysis were undertaken on a sediment core collected in 2014, both by direct counting of sub-fossil akinetes and filaments germinated from viable akinetes.

The direct counting of akinetes was possible till the layer corresponding to 1965. Repeated counts made on deeper layers relative to the previous period (1935-1961) were negative. Since the 1970s akinetes abundance suddenly and rapidly increased until 2007, then began to decrease. Similarly, the viable filaments germinated from akinetes, were observed until the layer corresponding to 1971, and their density steadily increased from 1985 to the end of 2000s. The taxonomic position of *D. lemmermannii* sub-fossil populations was confirmed by phylogenetic analysis of germinated strains corresponding to

the years 1989, 2000, 2005 and 2012. The *rpoB* gene resulted conserved in this time period, until nowadays.

A broad study of the global distribution of *D. lemmermannii* (Paper V) pointed out the differences among populations, deepening the biogeography of this species. As integration to the first study described previously, thirteen more strains, attributed to *D. lemmermannii* by morphological and morphometrical analysis, were isolated from different water bodies in southern and northern side of the Alps (e.g. lakes Maggiore, Lugano, Caldonazzo, Ammersee, and Starnberger See). To increase the studied geographic area, 13 more *D. lemmermannii* strains, isolated in Norway and Northern Germany and maintained by NIVA-CCA, were analysed.

Two representative strains of the deep subalpine lakes populations, isolated in lakes Garda and Como, were analysed by TEM. The ultrastructural analysis showed quite dilated thylakoids distributed in whole vegetative cell, peripheral polyhedral bodies, and gas vesicles widespread or concentrated at the free pole in the apical cell of the filament. A common feature, was a wide, central, electron dense area, containing strongly electron dense granules. Cyanophycin granules increased in number in akinetes, and in the heterocytes no real honeycomb structure was observed.

Among all the strains tested, 5 strains from Norway resulted producers of MCs. Their *mcyE* gene was positive amplified and sequenced. The sequences shared a 100% similarity to each other and a Blast homology search showed 100% similarity to others *D. lemmermannii*, and 99% similarity to *Dolichospermum* and *Anabaena* genera.

Differences among populations were determined by phylogenetic analysis on the 16s rRNA gene, comparing the newly isolated strains with NIVA-CCA strains, and strains obtained from public repositories. Overall, *D. lemmermannii* formed a distinct clade, separated from other *Dolichospermum* spp.. Not all the MCs producing strains clustered together, but a relation with their geographic origin was suggested. While non-MCs producing strains were distributed from northern to southern Europe, MCs-producers were confined in Northern Europe (Norway and Finland). However, information about toxicity was not available for several strains retrieved from public repositories, allowing therefore only a partial description of the toxic potential of the species.

Within the *D. lemmermannii* clade, 4 distinct sub-clusters with evident genetic differences were observed. Each sub-cluster included strains isolated from the subalpine lakes (except Lake Maggiore), New Zealand, United Kingdom, and Northern Norway, respectively. These ones were far from a main group formed by closely related strains isolated in Central and Northern Europe (Germany, Czech Republic, Norway, and Finland). The results were supported by a PCoA based on pairwise genetic distances between strains. Both the analytical approaches, revealed genetic differences between the strains isolated in Lake Maggiore and in the others subalpine lakes, which showed a closer relation to the central-north European group. The geographic isolation

hypothesis and the effect of dispersal barrier in the differentiation of *D. lemmermannii* strains were examined applying a Mantel test, which showed a significant correlation between the geographic and genetic distances.

4. DISCUSSION

4.1 *Tychonema bourrellyi*

This study demonstrated the presence of *T. bourrellyi* in Lake Garda. This species is considered a cold-stenotherm genus, particularly abundant in northern Europe and Canada (Komárek et al., 2003). These features are consistent with the presence of this species in Lake Garda in the colder months. Moreover, the study on *T. bourrellyi* populations in Lake Garda revealed its ability to produce ATX. This was the first evidence of a planktonic genus belonging to the Oscillatoriales able to produce ATX. This feature was further confirmed by the observation of ATX producing strains isolated in Norway, therefore, the ability to produce ATX does not seem to be restricted to specific climatic regions or isolated populations. After the first discovery in Lake Garda, the presence of toxic populations of *T. bourrellyi* was demonstrated also in the other deep subalpine lakes (e.g. Iseo, Como and Maggiore). Besides ATX, the ability of this species to produce the analogue HTX was also observed. Both are potent neurotoxins that represent a potential serious risk for human and animal health (Onodera et al., 1997; Wood et al., 2007).

The predominant Oscillatoriales in the deep lakes south of the Alps was *P. rubescens* (Salmaso et al., 2012). The discrimination between the two species in samples preserved with Lugol's solution is not always easy, therefore the exact contribution of *T. bourrellyi* to the cyanobacterial community before their isolation from natural samples cannot be determined with certainty. In Lake Maggiore rare filaments of *T. bourrellyi* were identified by microscopic observations in the monitoring campaigns since 2006 (C.N.R.-I.S.E. Sede di Verbania 2007), and recently was documented its increase, with biovolumes similar or greater than those of *P. rubescens* (C.N.R.-I.S.E. Sede di Verbania 2015). The analyses undertaken in Lake Garda proved a stronger development of *T. bourrellyi* compared to *P. rubescens*. Both species mostly developed in a layer of 20–30 m around the euphotic depth. After a higher spring growth, *T. bourrellyi* decreased, growing together with *P. rubescens* during the summer months. This behaviour can be interpreted considering that, contrary to *P. rubescens*, *T. bourrellyi* does not possess gas-vesicles, and therefore it is not able to control buoyancy and vertical position, which represents a negative selective characteristic in deep lakes during the stratification months. The conversed long-term trends in the ATX and MCs concentrations suggested a colonisation process of Lake Garda by *T. bourrellyi*, though a conclusive evaluation should require longer time series of cyanotoxins analyses. The proper and fast identification of *T. bourrellyi* and its distribution in environmental samples can be deepened using the specific primers designed in

this study. The preliminary analysis showed their powerful potential use for surveillance and research purposes and the protocols will be developed for further uses.

The causes of ongoing and rapid changes in cyanobacterial populations occurring in the deep subalpine lakes remain to be explored. However, it can be suggested a role for oligotrophication processes documented in this lake district.

4.2 *Dolichospermum lemmermannii*

This study firstly confirmed the taxonomic position of the *Dolichospermum* species appeared as blooms in the deep subalpine lakes from the 1990s, thanks to the association of morphological and phylogenetic approaches. In Lake Garda, the contribution of *D. lemmermannii* to the total biovolumes of cyanobacteria in the trophogenic layers (0–20 m) was always very low. As it was described for this kind of gas-vacuolated species (Reynolds and Walsby, 1975), the blooms are the result of a physical surface accumulation of filaments already present in the trophogenic layers, and not the effects of in situ growth processes. Nevertheless, the seasonal development of this species resulted primarily connected with water temperature.

Most of the records of *D. lemmermannii* were made after the 1990s, between the 40th and 60th parallels. Therefore, its habitat looks circumscribed to cold and temperate environments. Its spread toward south is harder to interpret compared with other warm-adapted Nostocales whose expansion toward north was explained by the recent widening of new suitable habitats due to global warming (Sukenik et al., 2012). To clarify the causes that favoured the introduction and the successive rapid spread of this species in the subalpine lakes, the colonisation pattern of *D. lemmermannii* in Lake Garda was reconstructed through the analyses of the distribution of sub-fossil akinetes preserved in deep sediment. The study of akinetes, allowed to identify the period of establishment of self-sustaining and naturalised populations, after a first introduction of the species, representing a powerful tool for the reconstruction of the long-term temporal development of Nostocales in water bodies. The analyses indicated the beginning of colonisation between the middle of the 1960s and 1970. However, it cannot be excluded the possible presence of a few other akinetes in the older sediment layers. The results strongly suggested that this species increased their importance during the 1980s and 1990s, coinciding with the appearance of the first surface blooms (Salmaso et al., 1994). Along with the increase of water temperatures recorded since the 1960s, the establishment of *D. lemmermannii* appeared favoured by the growth of anthropogenic pressure and changes in trophic status. The genetic structure of strains germinated from sub-fossile akinetes in Lake Garda, compared with modern strains, did not show any mutation or recombination signal, suggesting the feature of clonal population.

The *D. lemmermannii* populations actually present in the deep subalpine lakes shared common morphological characteristics of vegetative and

specialized cells. The analysis of ultrastructure revealed particular characteristics compared as related species, as a large area of medium electron density present in many vegetative cells containing electron dense granules. These features, as well as the thylakoidal pattern within vegetative cells, can be used to further evaluate phylogenetic relations in cyanobacterial classifications.

The wide study aimed at evaluate the biogeography of *D. lemmermannii*, showed the existence of genetic differences and capability to synthesize microcystins among populations from different geographical regions. The strains isolated in lakes south of the Alps, tested negative for the production of a wide range of toxins, as well as strains from Germany. On the contrary, five Norwegian strains isolated between 1981 and 2000 and maintained in long-term culturing at NIVA were MCs producers. Other MCs producing strains were previously identified in Finland (Sivonen et al., 1992), therefore MCs production emerged as an exclusive ability of North European strains, suggesting a geographical segregation. The partial *mcyE* sequences amplified from MC-producing Norwegian strains were identical to each other and resulted well conserved in the genera *Dolichospermum* and *Anabaena*. In Northern Europe, toxic populations coexisted along with non-toxic populations in lakes of the same regions and even in a single lake (Lake Storavatn, Lindås, Hordaland, Norway). The *mcy* gene loss can be speculated in non-toxic strains, however whether the non-toxic strains conserve remnants of *mcy* gene cluster should be verified.

The phylogenetic analysis on the 16s rRNA gene showed a clear separation between *D. lemmermannii* and other *Dolichospermum* species. Furthermore, it confirmed the genetic stability of the populations settled in the deep subalpine lakes, except Lake Maggiore, which showed slight genetic differences from this group and a closer relation with Norwegian strains. In summer, the deep subalpine lakes showed strong differences in the mean epilimnetic temperatures. In lakes Maggiore and Lugano temperatures were 2 to 3 °C lower than other lakes (Salmaso et al., 2012). Hence, this genetic differences can be the result of the environmental selection of ecotypes with different temperature optima. Further, the inability to achieve high biomass growth rate in Lake Lugano can be explained by water temperature lower than the growth optima of this ecotypes and higher mixing in the epilimnion (Salmaso et al., 2012).

The genetic variation highlighted within *D. lemmermannii* species, was in part explained by geographic distance. Similarly to the Italian group, a distinct clustering was observed for strains from New Zealand, United Kingdom and Northern Norway, whereas strains from central and northern Europe were more closely related. This spatial pattern can be explained by founder effects, likely stabilised over the years by geographic isolation reinforced by physical barriers (e.g. Alps, Sea, ice cover). In Central Europe (Germany, Czech Republic) and in the southern part of Norway and Finland, these effects may have been mitigated by multiple colonisation events. Overall, in this process,

environmental selective factors were influential in the successful establishment of genotypes adapted to particular geographical niches. Nevertheless, whether the current distribution pattern of *D. lemmermannii* has ancient or recent origin still needs to be disclosed, albeit the low degree of genetic diversity among strains suggests a quite recent spread. The role of human activities and water birds which followed migratory flyways across Europe, as dispersal mechanism driving the distribution of *D. lemmermannii*, and akinetes, as dispersal units, can be speculated.

5. CONCLUSIONS

This study highlighted the rapid changes in cyanobacterial populations occurring in the deep subalpine lakes. Though the causes remain to be explored, the appearance and development of two nuisance cyanobacteria species, *T. bourrellyi* and *D. lemmermannii*, raised serious concerns about the safety of these water resources.

In the deep subalpine lakes, the populations of *T. bourrellyi* demonstrated their ability to produce ATX and HTX. Whereas, *D. lemmermannii* resulted unable to produce a wide range of toxins. Considering the growing development of *T. bourrellyi*, these evidences underlined the eventuality of a synergic toxic effects caused by the presence of MCs, produced by *P. rubescens*, and ATX. On the other side, the excessive development of *D. lemmermannii* blooms in summer and the consequent occurrence of tastes and smells, can undermine the attractiveness of these lakes, considerably influencing their value. Moreover, *D. lemmermannii* showed its spreading potential over dispersal barriers, thanks to adaptability to temperature variation. This feature, associated with the ability of North European populations to produce MCs, raises an issue on the appearance of harmful algal blooms in other temperate areas, in southern Europe. This hypothesis is further strengthened by the low degree of genetic diversity among strains from different geographical regions, which suggests a quite recent spread of this species.

Considering the reinforcing effects of current climate change on eutrophication and cyanobacterial development, and the consequent implications for water management, this study provided useful information for reducing the impact of invasive cyanobacteria on the subalpine lakes ecosystem.

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LIST OF PAPERS

This doctoral thesis is a summary of the following papers:

I. Shams, S., Capelli, C., Cerasino, L., Ballot, A., Dietrich, D.R., Sivonen, K., and Salmaso, N. 2015. Anatoxin-a producing *Tychonema* (cyanobacteria) in European waterbodies. *Water Research*, **69**: 68-79 DOI:10.1016/j.watres.2014.11.006.

II. Salmaso, N., Cerasino, L., Boscaini, A., and Capelli, C. 2016. Planktic *Tychonema* (Cyanobacteria) in the large lakes south of the Alps: phylogenetic assessment and toxigenic potential. *FEMS Microbiology Ecology*, **92**(10).

III. Salmaso, N., Capelli, C., Shams, S., Cerasino, L. 2015. Expansion of bloom-forming *Dolichospermum lemmermannii* (Nostocales, Cyanobacteria) to the deep lakes south of the Alps: colonisation patterns, driving forces and implications for water use. *Harmful Algae*, **50**: 76-87.

IV. Salmaso, N., Boscaini, A., Capelli, C., Cerasino, L, Milan, M., Putelli, S., and Tolotti, M. 2015. Historical colonization patterns of *Dolichospermum lemmermannii* (Cyanobacteria) in a deep lake south of the Alps. *Advances in Oceanography and Limnology*, **6**, 1/2.

V. Capelli, C., Ballot, A., Cerasino, L, Papini, A., and Salmaso, N. 2016. Biogeography of bloom-forming toxic and non-toxic populations of *Dolichospermum lemmermannii* (Cyanobacteria). *Harmful Algae*, submitted.

I.

WATER RESEARCH 69 (2015) 68–79

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/watres

Anatoxin-a producing *Tychonema* (cyanobacteria) in European waterbodies

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ARTICLE INFO

Article history:
Received 22 August 2014
Received in revised form
14 October 2014
Accepted 7 November 2014
Available online 18 November 2014

Keywords:
Anatoxin-a
Tychonema
Cell quota
Polyphasic approach
Phylogenetic analysis
European waterbodies

ABSTRACT

In order to identify the cyanobacterial species responsible of anatoxin-a (ATX) production in Lake Garda (Northern Italy), an intensive isolation and culturing of filamentous cyanobacteria were established since 2014 from environmental samples. In this work, we report a detailed account of the strategy adopted, which led to the discovery of a new unexpected producer of ATX, *Tychonema bourrellyi*. So far, this species is the first documented example of cultured Oscillatoriales able to produce ATX isolated from pelagic freshwater ecosystems. The isolated filaments were identified adopting a polyphasic approach, which included microscopic species identification, genetic characterisation and phylogenetic analyses based on 16S rRNA genes. The taxonomic identification was further confirmed by the high (>99%) *rbcLX* sequence similarities of the *T. bourrellyi* strains of Lake Garda with those deposited in DNA sequence databases. More than half of the isolates were shown to produce a significant amount of ATX, with cell quota ranging between 0.1 and 2.6 μg mm^{-3}, and 0.01 and 0.35 pg $cell^{-1}$. The toxic isolates were tested positive for *anaC* of the anatoxin-a synthetase (*ana*) gene cluster. These findings were confirmed with the discovery of one ATX producing *T. bourrellyi* strain isolated in Norway. This strain and a further non-ATX producing Norwegian *Tychonema bornetii* strain tested positive for the presence of the *anaF* gene of the *ana* gene cluster. Conversely, none of the Italian and Norwegian *Tychonema* strains were positive for microcystins (MCs), which was also confirmed by the absence of *mcyE* PCR products in all the samples analysed. This work suggests that the only reliable strategy to identify cyanotoxins producers should be based on the isolation of strains and their identification with a polyphasic approach associated to a concurrent metabolomic profiling.

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http://dx.doi.org/10.1016/j.watres.2014.11.006
0043-1354/

1. Introduction

The long evolutionary history is the basis of the high competitive ability that characterizes cyanobacteria. They are distributed in most aquatic and terrestrial habitats, including extreme environments (Paerl et al., 2003; Boyer and Zimba, 2007; Kleinteich et al., 2012). In waterbodies characterized by high concentrations of nutrients, limited water exchange and high temperatures and thermal stability, cyanobacteria can develop with high biomasses, giving rise to the formation of blooms at the surface, euphotic zone or in the metalimnic layers, largely depending on the respective species (Paerl and Paul, 2012). Cyanobacteria represent one of the major causes of ecosystem degradation and impairment of the economical value of freshwater resources. Specific strains produce a wide range of powerful toxins, with important implications for health risks associated with the human exploitation of recreational and drinking waters (Meriluoto and Codd, 2005; Mankiewicz-Boczek et al., 2011; Zamyadi et al., 2012). The principal classes of cyanotoxins are microcystins, nodularins, anatoxin-a and homoanatoxin-a, anatoxin-a(S), saxitoxins and cylindrospermopsins (Metcalf and Codd, 2012; Méjean et al., 2014).

Compared with microcystin (MC) producers, only a few anatoxin-a (ATX) producing taxa have been distinctly isolated and characterized (Table 1). Other reports, based on analyses carried out on bulk environmental samples, suggest the existence of a wide spectrum of potential cyanobacterial taxa able to produce ATX (see, among the others, Carrasco et al., 2007; Van Apeldoorn et al., 2007; Aráoz et al., 2010; Metcalf and Codd, 2012; Quiblier et al., 2013). Many reports, however, were not confirmed by analyses made on isolated strains. Toxic species can be detected using direct analytical chemical approaches (Meriluoto and Codd, 2005; Humpage et al., 2012; Metcalf et al., 2012) as well as molecular methods able to detect the presence of toxin biosynthetic genes (Pearson and Neilan, 2008; Sivonen, 2008; Rantala-Ylinen et al., 2011a). Nevertheless, until a few years ago, a genetic molecular approach to identify ATX encoding genes was not feasible because of the unknown biosynthetic pathway leading to the production of anatoxin. Biosynthetic genes coding for ATX have been characterized only recently in a benthic *Oscillatoria* PCC 6506 (Méjean et al., 2009, 2010) and planktonic *Anabaena* sp. strain 37 (Rantala-Ylinen et al., 2011b), opening the way to the design and use of primers for the detection of genes coding ATX in *Oscillatoria*, *Phormidium*, *Aphanizomenon* and *Anabaena* strains (Cadel-Six et al., 2009; Ballot et al., 2010; Wood et al., 2010; Rantala-Ylinen et al., 2011b).

In a recent work, Cerasino and Salmaso (2012) documented a widespread presence of ATX in the lake district south of the Alps. Based on analyses carried out on environmental samples collected during the warmer months, detectable concentrations of ATX ranging between 0.1 and 0.6 μg L^{-1} were found in the lakes Garda, Iseo, Como and Maggiore, i.e. the largest lakes that experienced a recent colonization and summer surface blooms of *Dolichospermum lemmermannii* (Salmaso et al., 2012). However, a clear identification of producers in the different seasons was not possible because biological analyses on isolated strains were not available.

Based on the hypothesis that filamentous cyanobacteria could possibly be amongst the ATX producers, cultures of Oscillatoriales were established from environmental samples collected since 2014 in Lake Garda with the aim to isolate potential new producers. Owing to the very low abundance of cyanobacteria usually recorded in the winter months (Salmaso, 2011), samples were collected using plankton nets and initial cultures established. The isolated cyanobacteria were then examined and identified following a polyphasic approach (Vandamme et al., 1996; Lee et al., 2014), which included microscopic species identification, genetic and phylogenetic analyses. Culture strains were further screened for cyanotoxins, particularly ATX and MCs, and tested for the presence of ATX and MCs biosynthesis encoding genes. Above approach led to the discovery and characterization of a new unexpected filamentous cyanobacterial producer of ATX.

2. Methods

2.1. Study site

Lake Garda is located at the southern border of the north eastern Italian Alps, at 65 m a.s.l. With a volume of more than 49 × 10^9 m^3, a maximum depth of 350 m and a surface of

Table 1 – Cyanobacterial anatoxin-a producers. The list, at the genus level, includes only the results obtained from analyses carried out on isolated strains in culture conditions.

	Genus	Selected references
Heterocystous genera	*Dolichospermum/Anabaena*	Sivonen et al. (1989), Lakshmana Rao et al. (2002) and Rantala-Ylinen et al. (2011b)
	Aphanizomenon	Sivonen et al. (1989) and Osswald et al. (2009)
	Cuspidothrix (*Aphanizomenon*)	Wood et al. (2007a), Ballot et al. (2010) and Hodoki et al. (2013)
	Cylindrospermum	Sivonen et al. (1989)
Oscillatoriales	*Oscillatoria*[a]	Sivonen et al. (1989), Edwards et al. (1992), Aráoz et al. (2005) and Rantala-Ylinen et al. (2011b)
	Phormidium[b]	Wood et al. (2012) and Harland et al. (2013, 2014)
	Tychonema	This work

[a] Including *O. limnetica* (*Pseudanabaena limnetica*).
[b] Populations of *Phormidium* producing ATX were observed for the first time in benthic river mats (Wood et al., 2007b).

368 km^2, Lake Garda is one of the largest freshwater bodies in Europe. From the 1970s to the 1990s the average concentrations of total phosphorus in the whole water column doubled, from 10 to 20 μg L^{-1} and beyond. Present concentrations are decreasing and stabilising around 18 μg L^{-1}. Information on the lake and previous investigations were reported in Salmaso and Mosello (2010).

2.2. Collection of samples and environmental variables

The sampling station was located at the deepest point of the lake (350 m), between the villages of Brenzone and Gargnano (45.69 N, 10.72 E). Field measurements and collection of samples were made between February and April 2014 (Table 2). Owing to the very low abundance, in February and March 2014 phytoplankton was collected by single vertical tows from 10 to 15 m to the surface with a 25 cm diameter 80 μm mesh plankton net, which resulted in 0.5–0.7 m^3 of filtered water. In April 2014, filamentous cyanobacteria were collected with plankton nets and Niskin bottles. Vertical profiles of water temperature were carried out with a multi-parameter probe (Idronaut Ocean Seven 316). The light attenuation coefficients (K_d) were measured with a submersible irradiance sensor, LiCor 192SA. The euphotic depth was computed as $Z_{eu} = \ln(100) \times K_d^{-1}$ (Kirk, 1994). Concentrations of dissolved inorganic nitrogen (DIN), soluble reactive phosphorus (SRP) and total phosphorus (TP) were carried out using standard methods (APHA et al., 2000; Cerasino and Salmaso, 2012).

2.3. Isolation of strains, culture conditions and morphological characterization

Single filaments of cyanobacteria were isolated from diluted net-phytoplankton samples under a stereomicroscope (Leica M125) and a macroscope (WILD M420) using a micropipette. The single filaments were washed 3 times and placed in microwell plates containing 3 mL Z8 medium (Kotai, 1972). After initial growth, as assessed by visual inspection and the macroscope, single strains were first transferred to 30 mL Z8 medium and, upon successful growth, to 150 mL medium Z8 CELLSTAR (Greiner Bio-One GmbH) cell culture flasks. The flasks were maintained at 20 °C under continuous light conditions (25 μmol m^{-2} s^{-1}). From each single culture, after careful homogenization, 15 mL were preserved with Lugol's solution for biovolume determinations, whereas 250 mL were filtered with a 0.45 μm GF/C filter (Whatman – GE Healthcare Life Sciences) for subsequent cyanotoxin and genetic analyses.

Depending on abundances, cell densities and biovolumes were estimated by measuring cell sizes and length of filaments from 1 to 3 transects at 200 magnification in 10 mL sedimentation chambers of 25 mm diameter. Morphological features were analysed using an inverted microscope (Zeiss Axiovert 135). Single specimens were identified following morphometric and morphological criteria described in Komárek and Albertano (1994) and Komárek and Anagnostidis (2007).

Over 65 single filament cultures were analysed microscopically, while 24 randomly selected cultures were analysed for the presence of cyanotoxins and for preliminary sequencing of 16S rRNA genes with reverse primer. Complete analyses of toxins, cyanotoxins encoding genes, and sequencing of 16S rRNA and *rbcLX* genes (with forward and reverse primers) were carried out in 4 single filament cultures selected randomly from each of the 3 sampling time points (12 isolates).

Table 2 – Chemical and physical characteristics at the surface and around the upper boundary of the euphotic layer (Z_{eu}, 20 m) in the three sampling dates. DIN, dissolved inorganic nitrogen; SRP, soluble reactive phosphorus; TP, total phosphorus; K_d, vertical light attenuation coefficient.

Variables		11 Feb		11 March		8 April	
Depth	m	0	20	0	20	0	20
Temperature	°C	9.1	9.1	10.0	9.2	12.4	9.9
pH		7.6	7.9	7.7	8	8.4	8.2
Conductivity	μS cm^{-1} at 20 °C	211	214	212	213	211	214
NO_3–N	μg N L^{-1}	273	256	337	341	227	268
DIN	μg N L^{-1}	280	261	342	346	232	275
SRP	μg P L^{-1}	9	8	3	4	2	<2
TP	μg P L^{-1}	14	11	10	16	15	12
K_d	m^{-1}	0.17		0.21		0.19	
Z_{eu}	m	27		22		24	

2.4. Cyanotoxins analyses

2.4.1. Toxin extraction

To measure intracellular concentrations of toxins, 250 mL of cyanobacterial cultures were filtered with a 0.45 μm GF/C filter. The filter was frozen and stored at −20 °C until further processing (within one week of filtration). For toxin extraction, the filter was homogenized for 5 min after addition of 7 mL of extraction solvent (70% methanol containing 0.1% formic acid) in a homogenization tube. After centrifugation (9850 G), the supernatant was transferred and the pellet was re-extracted again with an additional 7 mL of extraction solvent. The evolving supernatants were combined. A 2 mL aliquot of the filter-extract was filtered on Phenex-RC syringe filter (0.2 μm pore size, Phenomenex) and used for the determination of MCs and nodularin (NOD-R) content via liquid chromatography–mass spectrometry (LC–MS). The remaining filter-extract was reduced under vacuum and filtered with a 0.2 μm pore size RC syringe filter and the filtrate subjected to alkaloid (ATX, and cylindrospermopsin, CYN) LC–MS analytics.

2.4.2. LC–MS analysis

LC–MS analyses were carried out on a Waters Acquity UPLC system directly coupled to an AB SCIEX 4000 QTRAP mass spectrometer equipped with a turbo ion spray interface. Standard injection volume was 2 μL. The analysis of MC and nodularin-R was carried out using a Phenomenex Kinetex XB-C18 column (1.7 μm particle size, 2.1 × 50 mm) at 40 °C. The mobile phase consisted of water (A) and acetonitrile (B), both containing 0.1% formic acid. A linear gradient scheme was employed: the starting eluent was 80% A, decreased to 30% A at 4.5 min, and finally restored at 80% A at 6.5 min (hold

0.5 min). The total run time was 7 min with a flow rate of 0.25 mL min^{-1}. The analysis of CYN and ATX was carried out using a Phenomenex Kinetex HILIC column (1.7 μm particle size, 2.1 × 50 mm) at 30 °C. The mobile phase consisted of water with 1% acetonitrile (A), containing ammonium acetate (10 mM) and acetic acid (10 mM), and acetonitrile (B). A linear gradient scheme was employed: the starting eluent was 10% A (hold 0.5 min), raised to 25% A at 1 min (hold 1 min), raised to 60% A at 3.5 min (hold 2 min), and finally returned to 10% A at 8 min (hold 2 min). The total run time was 10 min with a flow rate of 0.25 mL min^{-1}.

The mass detector was operated in positive Electro Spray mode (ESI+) using the Multiple Reaction Monitoring (MRM) scanning mode. General settings were as follows: ion spray voltage 5000 V, entrance potential 10 V, cell exit potential 10 V, interface heater temperature 300 °C. For each target compound, two transitions were monitored. Toxin identification was achieved by comparing the chromatographic retention time and relative intensity of the two transitions with those of the commercial standards. Toxin quantification was performed with the external standard procedure, in which, for each compound, a calibration curve was obtained using the most intense transitions. Compound-specific settings of the mass detector and performance data of the methods are listed in Table 3.

Homoanatoxin-a and other degradation products e.g. dihydro- and epoxy-homoanatoxin-a were not quantified because analytical protocols were still under evaluation.

Microcystins (RR, [D-Asp^3]-RR, YR, LR, WR, LA, LY, LW, LF), NOD-R and CYN analytical standards were purchased from Vinci Biochem, ATX from Tocris Cookson Ltd. All solvents and reagents used in this procedure were LC–MS grade. The limits of quantitation (LOQ) were between 30 and 500 ng L^{-1} (different MCs congeners), 140 ng L^{-1} (NOD-R), 30 ng L^{-1} (ATX), and 8 ng L^{-1} (CYN).

Table 3 – Compound-specific parameters in LC–MS analysis of toxins. All toxins are reported in the same table for clarity, but MC/NOD and CYN/ATX are analysed in different chromatographic conditions. RT = chromatographic retention time; DP = declustering potential; CE = collision energy.

Toxin variant	RT (min)	MS acquisition parameters			
		MRM transitions [a] (m/z)	Ratio [b]	DP (V)	CE (V)
[D-Asp3]RR	1.20	512.8/135	17	85	44
		512.8/213		85	50
RR	1.43	520.1/135	15	85	44
		520.1/213		85	50
NOD-R	2.20	825.6/135	1	140	83
		825.6/70		90	100
YR	2.63	523.6/135	6	45	20
		523.6/911		45	20
LR	2.78	498.6/135	17	40	19
		498.6/213		40	43
WR	3.06	535.0/135	18	40	18
		535.0/213		40	42
LA	4.30	911.6/135	2	85	90
		911.6/213		85	65
LY	4.40	1002.6/135	2	106	96
		1002.6/213		106	75
LW	4.80	1025.6/135	2	111	100
		1025.6/213		111	80
LF	4.93	986.6/135	2.5	96	95
		986.6/213		96	73
CYN	1.90 (HILIC)	416.3/194	1.5	80	53
		416.3/336		80	53
ATX	4.10 (HILIC)	166.1/149	1.6	60	21
		166.1/131		60	24

[a] For all compounds the most intense transition is reported in the first line, the less intense in the second line.
[b] The relative ratios between the intensities of the two MRM transitions are reported in this column.

2.5. *DNA extraction, PCR amplification and sequencing*

Total genomic DNA was extracted from the filters using the Mo Bio PowerWater® DNA Isolation Kit (Mo Bio Laboratories, Inc., CA, USA) following manufacturer's instructions. The quantity and quality of DNA was measured by spectrophotometry with a NanoDrop ND-8000 (Thermo Fisher Scientific Inc., MA, USA).

Taxonomic identification and phylogenetic analyses of filamentous cyanobacteria were carried out by the amplification of a short fragment (ca. 420 bp) of the 16S rRNA gene using the forward primer CYA359F and an equimolar mixture of the reverse primers CYA781Ra and CYA781Rb (Nübel et al., 1997) synthesized commercially (Sigma–Aldrich Co. LLC). The PCRs were carried out on an Eppendorf Mastercycler ep (Eppendorf AG, Hamburg, Germany). The reaction mix, with a final volume of 25 μl, contained 1× Optimized DyNAzyme PCR Buffer (Thermo Scientific), 0.2 mM dNTPs mix (Thermo Scientific), 0.1 μM forward primer, 0.05 μM each of the two reverse primers, 1U of DyNAzyme II DNA Polymerase (Thermo Scientific), and 1 μl of DNA templates. Genomic DNA concentrations were in the range 4.9–20.3 ng μL^{-1}. The cycling protocol consisted of a first denaturation step at 94 °C for 3 min followed by 35 cycles of DNA denaturation at 94 °C for 30 s, primer annealing at 53 °C for 30 s, strand elongation at 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. PCR products were checked and separated by 1.5% agarose gel electrophoresis stained with ethidium bromide. Sizing of DNA fragments were first evaluated with a commercial DNA ladder (GeneRuler Express, Fermentas).

Besides 16S rRNA phylogenetic analyses, taxonomic identification of isolates presented in Table 4 was further checked using the *rbcLX* gene region. *rbcLX* products (878 bp) were amplified using the primers CW and CX, following the protocols in Rudi et al. (1998), with the exception of the number of cycles in the second cycle step (35 instead of 38).

PCR products (16S rRNA and *rbcLX* genes) were cleaned with Exonuclease plus Shrimp Alcaline Phosphatase (ExoSAP). The same primers as in the PCR (CYA359F and CYA781Ra for 16S rRNA) were used with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems), according to the manufacturers' protocols. After purification in automation using the Agencourt CleanSEQ® Kit (Beckman), products were run on an Automated Capillary Electrophoresis Sequencer 3730XL DNA Analyzer (Applied Biosystems). In order to trim the low quality ends, sequences were checked with Chromatogram Explorer 3.3.0 (Heracle Biosoft). Forward and reverse chromatograms were further evaluated and

Table 4 – (a) Codes of *Tychonema bourrellyi* strains isolated in Lake Garda (Northern Italy) between February and April 2014, and corresponding ENA accession numbers of 16S rRNA and *rbcLX* genes. (b) Polymerase chain reaction amplification of ATX and MCs biosynthesis encoding genes (*anaC* and *mcyE*), and concentrations of anatoxin-a (ATX) and total microcystins (MCs); "+" and "–" indicate the presence and absence of expected amplicons using agarose gel electrophoresis. "*nd*", not detectable.

(a)					(b)			
Date	Isolate number	Isolate code	Accession number		*anaC*	ATX	*mcyE*	MCs
			16S rRNA	*rbcLX*		$\mu g\ L^{-1}$		$\mu g\ L^{-1}$
11 Feb	1	FEM_GaT0214-3	LM997416	LM997428	+	6.44	–	*nd*
	2	FEM_GaT0214-12	LM997417	LM997429	+	10.46	–	*nd*
	3	FEM_GaT0214-16	LM997418	LM997430	+	11.32	–	*nd*
	4	FEM_GaT0214-21	LM997419	LM997431	+	5.76	–	*nd*
11 Mar	5	FEM_GaT0314-2	LM997420	LM997432	+	2.92	–	*nd*
	6	FEM_GaT0314-4	LM997421	LM997433	–	*nd*	–	*nd*
	7	FEM_GaT0314-13	LM997422	LM997434	–	*nd*	–	*nd*
	8	FEM_GaT0314-17	LM997423	LM997435	+	2.91	–	*nd*
08 Apr	9	FEM_GaT0414-14	LM997424	LM997436	+	1.48	–	*nd*
	10	FEM_GaT0414-16	LM997425	LM997437	+	2.02	–	*nd*
	11	FEM_GaT0414-26	LM997426	LM997438	+	1.55	–	*nd*
	12	FEM_GaT0414-27	LM997427	LM997439	+	0.48	–	*nd*

assembled using the BioEdit 7.2.5 sequence alignment editor (Hall, 1999). Sequences were deposited to the European Nucleotide Archive (ENA) and analysed with Megablast (NCBI) against 16S rRNA and *rbcLX* gene sequences.

In addition to the toxins analyses (Section 2.4), the isolated strains were analysed for the presence of MCs and ATX encoding genes. The presence of *mcyE* genes was evaluated according to the PCR protocols of Rantala et al. (2006) using general primers (mcyE-F2/R4) and *Anabaena* 90 as a positive control (Ana-90; UHCC). The presence of anatoxin-a synthetase gene (*anaC*) was determined using the primer pairs anaC-osc, Osc-193 (UHCC) as positive control, and the methods described in Rantala-Ylinen et al. (2011b).

2.6. Comparison with Norwegian Tychonema strains

Eight *Tychonema bornetii* and *Tychonema bourrellyi* strains isolated in Norway between 1976 and 1982 from phytoplankton samples collected in the River Glåma and Lake Mjøsa were included in the study. The eight Norwegian *Tychonema* strains are cultivated in the culture collection of algae of the Norwegian Institute for Water Research (NIVA-cca). DNA extraction was conducted according to Ballot et al. (2014), PCR and sequencing of the 16S rRNA gene was conducted using the methods described in Ballot et al. (2008). The Norwegian *Tychonema* sp. strains were investigated for the production of MC and ATX using the Abraxis Microcystins/Nodularins (ADDA), ELISA Kit and the Abraxis Anatoxin-a Receptor-Binding Assay (Biosense, Bergen, Norway) respectively. The same strains were tested for the *anaF* encoding gene using the primers atxoaf and atxoar and the protocol according to Ballot et al. (2010, 2014). The presence of *mcyE* genes was evaluated according to the PCR protocols of Rantala et al. (2006) using general primers (*mcyE*-F2/R4). Sequences were submitted to ENA.

2.7. Phylogenetic analysis

The 16S rRNA genes of the 20 *Tychonema* strains listed in Tables 4 and 5 were analysed using molecular sequence assembly software Seqassem version 04/2008 (SequentiX – Digital DNA processing, Klein Raden, Germany). The Align

Table 5 – (a) Codes of *Tychonema bourrellyi* strains isolated in Lake Mjøsa and River Glåma (Norway), and corresponding ENA accession numbers of 16S rRNA genes. (b) Polymerase chain reaction amplification of *anaF* and *mcyE* genes, and positive or negative detection of anatoxin-a (ATX-a) and total microcystins (MCs) measured using ELISA Kit and Anatoxin-a Receptor-Binding Assay. "+" and "–" indicate the presence and absence of expected amplicons using agarose gel electrophoresis (*anaF* and *mcyE*), and the presence and absence of corresponding toxins (ATX, MCs), respectively.

(a)					(b)			
Code	Year of isolation	Species	Origin	Accession number 16S rRNA	*anaF*	ATX	*mcyE*	MCs
NIVA-CYA 33/1	1976	*T. bourrellyi*	Lake Mjøsa	LM651410	–	–	–	–
NIVA-CYA 33/3	1976	*T. bourrellyi*	Lake Mjøsa	LM651411	–	–	–	–
NIVA-CYA 33/4	1976	*T. bourrellyi*	Lake Mjøsa	LM651412	–	–	–	–
NIVA-CYA 33/5	1976	*T. bourrellyi*	Lake Mjøsa	LM651413	–	–	–	–
NIVA-CYA 60	1978	*T. bornetii*	Lake Mjøsa	LM651414	+	–	–	–
NIVA-CYA 95	1982	*T. bornetii*	River Glåma	LM651415	–	–	–	–
NIVA-CYA 96/1	1982	*T. bourrellyi*	Lake Mjøsa	LM651416	–	–	–	–
NIVA-CYA 96/3	1982	*T. bourrellyi*	Lake Mjøsa	LM651417	+	+	–	–

(version 03/2007) MS Windows-based manual sequence alignment editor (SequentiX – Digital DNA processing, Klein Raden, Germany) was used to obtain DNA sequence alignments, which were then corrected manually. Segments with highly variable and ambiguous regions and gaps making proper alignment impossible were excluded from the analyses. A 16S rRNA gene set containing 405 bp was used. *Gloeobacter violaceus* (AF132790) was employed as outgroup in the 16S rRNA tree. Thirty-three additional Oscillatoriales from GenBank were included in the 16S rRNA gene sequence analysis.

A phylogenetic tree for 16S rRNA gene sequences was constructed using the maximum likelihood (ML) algorithm with 1000 bootstrap replicates. In the ML analysis, evolutionary substitution model was evaluated in MEGA version 6 (Tamura et al., 2013) and K2 + G + I was found to be the best-fitting evolutionary model for the 16S rRNA gene.

3. Results

3.1. Environmental samples

In the first 20 m, water temperatures in the three sampling dates ranged between 9.1 and 12.4 °C (Table 2). An incipient stratification was apparent beginning in April. DIN and TP concentrations were in the range 232–342 μg N L^{-1} and 10–16 μg P L^{-1}. The euphotic depth was between 22 and 27 m.

3.2. Microscopic examinations

Over 65 cultures were obtained from the isolation of single filaments collected in February, March and April. In the original samples, the filaments were solitary and free floating, pale red, sometimes longer than 2 mm. All the specimens looked quite different from the filaments of *Planktothrix rubescens*, the predominant cyanobacterium in Lake Garda, and more similar to other Phormidioideae, namely *Tychonema* spp. (Komárek and Albertano, 1994; Komárek and Anagnostidis, 2007). A first preliminary round of PCR and sequencing with the reverse primer CYA781Ra on 24 cultures matched well to various *Tychonema* species (similarity between 99% and 100%), as well as to sheathed Oscillatoriales (*Phormidium*, *Microcoleus*) (see Section 3.3).

In the algal cultures, filaments were colourless or pale purplish/brown, rarely green, unbranched and without apparent firm sheaths. Cells usually were more or less isodiametric, with widths between 4.5 and 7 μm (Fig. 1). In some cultures, cells looked shorter (<5 μm) than wide (e.g. Fig. 1d, e). Filaments looked immotile or slightly trembling, with rounded apical cells, without calyptra and with no or very slight attenuation at the ends. In some specimens, the width changed slightly along the filaments (e.g., Fig. 1a, c, d). Sometimes, the cells had clearly visible large holes, similar to "vacuoles" (quite apparent in Fig. 1a, c), which, actually, are widened thylakoids (Komárek and Albertano, 1994). The centripetal formation of the cross walls was often easily detectable (e.g., arrows in Fig. 1b). These characteristics were consistent with the diacritical features described for *T. bourrellyi*. However, taking into account that planktonic populations of *Tychonema tenue* could possibly be identical to *T. bourrellyi* (Komárek and Anagnostidis, 2007), the microscopic discrimination of these two species in pelagic environments is not straightforward.

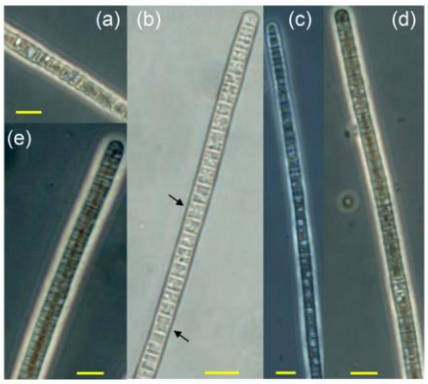

Fig. 1 – Micrographs of *Tychonema* isolated from samples collected in Lake Garda in (a) February, (b, c) March and (d, e) April. Scale bars = 10 μm. From (a) to (e), filaments are 5.7, 5.8, 5.2, 5.3 and 6.8 μm wide, respectively. The arrows in (b) indicate the centripetal formation of the cross wall. Observations made at 400× and phase contrast.

3.3. Sequences and phylogenetic analyses

The morphological determination of the isolated strains was supported by phylogenetic analyses based on 16S rRNA performed on the 12 selected strains in Table 4. Phylogenetic relationships of the investigated strains are presented in the ML tree of the 16S rRNA region of Oscillatoriales strains (Fig. 2). All 16S rRNA sequences from the Italian and Norwegian *Tychonema* strains (Tables 4 and 5) were grouped in a distinct cluster together with *Tychonema* sequences derived from GenBank. The *Tychonema* cluster was very closely related to a *Phormidium autumnale* and a *Microcoleus antarcticus* strain (Fig. 2). The whole cluster was supported by a bootstrap value of 96%.

These findings were further confirmed by a BLAST (NCBI) homology search. Results showed that the *rbcLX* gene regions sequenced in the 12 selected strains (Table 4) were >99% similar to *T. bourrellyi* (7 strains) and *T. bornetii* (1 strain).

3.4. Toxicity of the single strains

Of the 24 strains of *Tychonema* isolated from Lake Garda and submitted to LC–MS analyses, 14 produced ATX, although in some cases, at very low concentrations (3 isolates with ATX < 0.1 μg L^{-1}). A typical LC–MS chromatogram, showing the analyses of standards (ATX and CYN) and of a representative sample is reported in Fig. 3. The molecular analyses

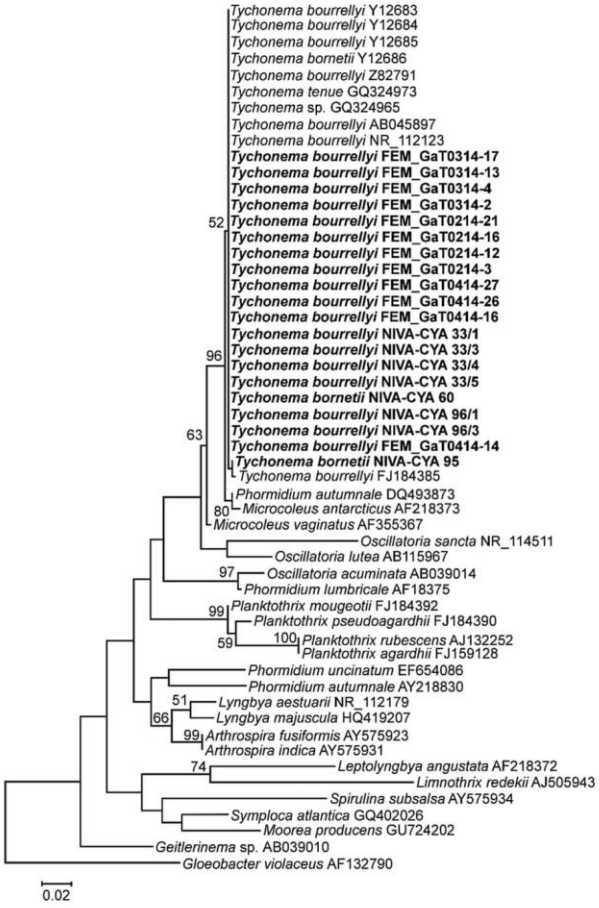

Fig. 2 – Maximum likelihood tree determined on the basis of partial 16S rRNA gene sequences of 53 Oscillatoriales strains. Outgroup = *Gloeobacter violaceus* (AF132790). Strains from this study are marked in bold. Bootstrap values above 50 are included. The scale bar indicates 2% sequence divergence.

allowed amplifying the *ana*C encoding gene fragment of the anatoxin-a synthetase (*ana*) gene cluster. In the same group of 24 isolates, PCR products were identified in 11 strains. No *ana*C PCR products were identified in all the non-ATX producing strains, and in the 3 culture samples with very low concentrations of ATX (<0.1 μg L^{-1}).

A representative picture of the *ana*C products amplified with anaC-osc primers in the 12 selected strains subjected to phylogenetic analysis and included in Table 4 is reported in Fig. 4. In these 12 selected strains, the cell quota of ATX on a biovolume basis were between 1.3 and 2.6 μg mm^{-3} in February, 0.1 and 0.3 μg mm^{-3} in March, and 0.2 and 1.8 μg mm^{-3} April. On a cell basis, corresponding values were in the range 0.18–0.35 pg $cell^{-1}$ (February), 0.01–0.04 pg $cell^{-1}$ (March) and 0.02–0.20 pg $cell^{-1}$ (April).

As for the strains isolated in the Norwegian freshwaters (Table 5), *T. bourrellyi* strain NIVA-CYA 96/3 was confirmed as ATX producer using the Abraxis Anatoxin-a Receptor-Binding Assay, while the other seven strains from NIVAcca tested negative for ATX. *T. bourrellyi* strain NIVA-CYA 96/3 and *T.*

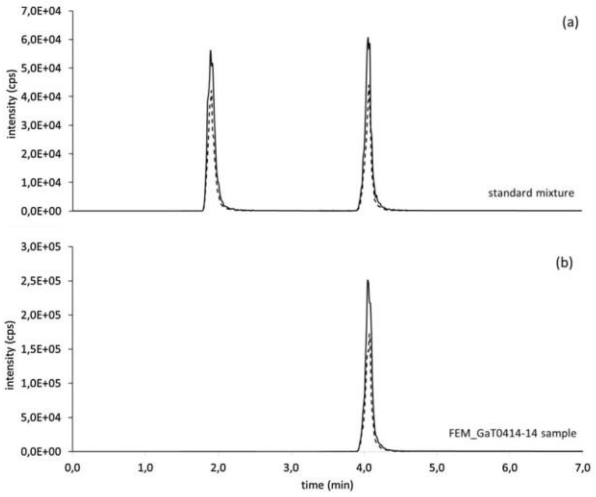

Fig. 3 – Liquid chromatography–mass spectrometry chromatograms resulting from the injections of a mixture of pure standards of cylindrospermopsin (50 ng/mL) and anatoxin-a (165 ng mL^{-1}) (panel a) and of an extract of a *Tychonema* culture (panel b). For each toxin, the traces of the two monitored MRM transitions are shown (solid and dashed lines).

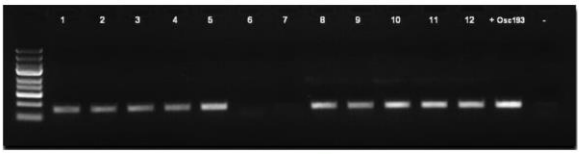

Fig. 4 – PCR products amplified with anac-osc primers (Rantala-Ylinen et al., 2011b). Samples from 1 to 12 are coded as in Table 4. "+" positive (Osc-193 UHCC strain) and "–" negative controls. Size of the ladder (in base pairs): 100, 300, 500, 750, 1000, 1500, 2000, 3000, 5000. The amplicon products are located between the bands 100–300 bp.

bornetii strain NIVA-CYA 60 tested positive for *anaF* of the ATX synthetase (*ana*) gene cluster (accession numbers LM651418 and LN555581, respectively).

None of the Italian and Norwegian *Tychonema* strains were positive for MC, which was also confirmed by the absence of *mcyE* PCR products in all the samples analysed (Tables 4 and 5).

4. Discussion

After establishing several cultures of potentially toxic filamentous Cyanobacteria collected in Lake Garda, we discovered that *Tychonema* is able to produce ATX. This is the first discovery of a planktonic genus belonging to the Oscillatoriales able to produce ATX. These findings were confirmed with the discovery of ATX producing *Tychonema* strains isolated in Norway.

The discovery of *Tychonema* in Lake Garda was quite unexpected. The predominant Oscillatoriales in this lake and in the other large lakes south of the Alps (Iseo, Como, Lugano and Maggiore) is *P. rubescens* (Salmaso et al., 2012). Nevertheless, the high number of isolates of *P. rubescens* analysed so far by molecular methods (16S rRNA, *rpoC1*, *rbcLX* genes) in these lakes referred to samples mostly collected during the late spring and summer months, i.e. when the biomass development of this species was at its seasonal maximum (*P. rubescens* accession numbers are reported in D'Alelio et al., 2012, 2013). During winter, the abundances of *P. rubescens* and of the other Oscillatoriales (*Limnothrix* sp., *Planktolyngbya limnetica*) in Lake Garda are very low (Salmaso, 2011). In the work presented here, the isolation of filaments of *Tychonema* was possible on

samples collected by means of plankton nets, and filtering 0.5–1 m^3 of lake water. Filaments in the samples collected with the Niskin bottles were rare and difficult to isolate. The presence of *Tychonema* appeared almost exclusive, because only a few filaments of *P. rubescens* were isolated from the net samples. On the other hand, the concurrent presence of *P. rubescens* was confirmed by molecular analysis carried out by checking the presence of *rbcLX* PCR products (see D'Alelio et al., 2013) on the environmental samples collected from February and April between the surface and 60 m (data not shown).

Tychonema is considered a cold-stenotherm genus of northern temperate regions (Komárek et al., 2003). Abundant populations of *T. bourrellyi* were documented particularly in northern Europe and Canada (Lund, 1955; Skulberg and Skulberg, 1985; Rudi et al., 1998; Komárek et al., 2003). In Lake Garda, the presence of this species in the colder months is consistent with these features. On the other side, the absence of gas vesicles, and therefore the inability to control buoyancy and vertical position, can represent a negative selective characteristic in deep and large lakes during the stratification months. The ecological role of the large "vacuoles", which are particularly apparent in the older cells, is still unclear. The large intracellular "holes" are due to the widened thylacoid membranes enveloping the enlarged intrathylacoidal spaces. Electron microscopy showed that these spaces were filled with electron dense fibrillar structures or globules similar to polyphosphate bodies (Komárek and Albertano, 1994). It is interesting to observe that another species – *Tychonema sequanum* – was identified by microscopic methods in Lake Maggiore (Kamenir and Morabito, 2009). This taxon, however, has smaller dimensions (2.5–5 μm width) compared to *T. bourrellyi*. Recently, *T. bourrellyi* was recorded also in Lake Erhai, in China. Comparative analyses of the 16S rDNA gene sequences determined from filaments isolated in this lake confirmed that the Chinese strains were grouped with *T. bourrellyi*/*T. tenue* (Wei et al., 2012).

The number of genera known to produce ATX is quite limited, especially when the list of producers is restricted to the only cases determined on species isolated and analysed in culture (Table 1). In Italy, reports of ATX are quite rare, referring exclusively to environmental samples (e.g., Cerasino and Salmaso, 2012). The production of ATX was associated with blooms or higher development of *Anabaena planctonica* (*Dolichospermum planctonicum*) (Bruno et al., 1994), *Anabaena crassa* (*D. crassum*) (Messineo et al., 2009) and "a peculiar *P. rubescens* population" (Viaggiu et al., 2004; Messineo et al., 2009). However, the ability of isolated populations of these species to produce ATX in culture conditions was not confirmed.

So far, the only Oscillatoriales proven to produce ATX in isolated populations belonged to the genera *Oscillatoria* and *Phormidium* (Table 1). These taxa are mostly detected in benthic or periphytic substrates. In contrast, *T. bourrellyi* is known to develop pelagic populations (as in our study), opening new perspectives about the ability of pelagic Oscillatoriales to produce ATX. Taking into consideration the positive amplification of *anaF* genes in the strains of *T. bourrellyi* and *T. bornetii* isolated in Norway, the ability to produce ATX does not seem to be restricted to specific climatic regions or isolated populations. On the other hand, the high variability of the ATX quota in the isolates of *T. bourrellyi* grown under standard conditions suggests the existence of differences in the ability to produce ATX in strains isolated in different months. Overall, differences in the cell quota in the isolates of Lake Garda were between 0.01 and 0.35 pg $cell^{-1}$, i.e. within more than 1 order of magnitude, but well within the range estimated in cultures of *P. autumnale* grown under different iron and copper stress conditions (between ca. 0 and 1.2 pg $cell^{-1}$; Harland et al., 2013), and within the variations observed in natural benthic *Phormidium* mats (100 fold differences in ATX quota; Wood et al., 2012).

The primers anaC-osc were specifically designed to amplify the *anaC* gene in the *Oscillatoria* genus (Rantala-Ylinen et al., 2011b). These primers proved to be useful also in the amplification of the *anaC* genes in *Tychonema*, suggesting the potential for a wider application of these specific protocols also to other Oscillatoriales. Similar considerations apply to the atxoaf-r primers, which were originally designed to detect *Aphanizomenon* species and other cyanobacteria (Ballot et al., 2010, 2014). In perspective, the comparison of the *ana* gene cluster encoding ATX in *Tychonema* and in the other cyanobacteria will provide further insight for the design of protocols tailored for the detection of pelagic ATX producers (cf. Méjean et al., 2014).

Overall, our findings open new perspectives in the study of the ecology of phytoplankton and cyanotoxins producers in Lake Garda and in the deep alpine and subalpine lakes. The new discovery of *Tychonema* in Lake Garda will require to be studied in detail by evaluating the seasonal and spatial dynamics, distribution and diversity, as well as toxic potential assessed both in environmental and isolated strains. An open question that needs to be dealt with is the significance of this appearance and its potential evolution particularly in relation with the very recent oligotrophication of the lake and the decrease of *P. rubescens* populations (Salmaso and Cerasino, 2012).

5. Conclusions

In the work presented here, we identified a new pelagic cyanobacterium belonging to the Oscillatoriales able to synthesize ATX. This species – *T. bourrellyi* – was isolated for the first time in the largest Italian lake (Lake Garda) during the winter and spring months.

- The identification of the species was carried out using a polyphasic approach, based on the microscopic identification of diacritical characters, molecular methods (16S rRNA and *rbcLX* genes) and phylogenetic analyses.
- Isolates of *Tychonema* were able to produce consistent amounts of ATX. The identification of this new ATX producer was also verified by the amplification of the *anaC* genes involved in the biosynthesis of ATX. These new findings were confirmed by the concurrent analyses of *Tychonema* strains isolated in Norway.
- The significance and impact of *Tychonema* producing ATX will require additional evaluation by studying the seasonal dynamics and toxic potential of populations in relation to

the development of other toxic cyanobacteria as well as in a wider geographical context.

- This study further highlights how the number of cyanotoxins producers in the freshwater environments is possibly still underestimated. On the other hand, the way in which these results originated, suggests that the only reliable strategy to identify cyanotoxins producers should be based on the isolation of strains and their identification with a polyphasic approach associated to a concurrent metabolomic profiling performed with advanced analytical techniques.

Acknowledgements

Investigations in Lake Garda were made in the framework of the LTER (Long Term Ecological Research) Italian network, site "Southern Alpine lakes", IT08-000-A (http://www.lteritalia.it/). The activity was supported by PhD fellowships to S.S. and C.C. from the E. Mach Foundation – Istituto Agrario di S. Michele all'Adige (FIRST FEM International Research School). We thank the European Cooperation in Science and Technology COST Action ES1105 CYANOCOST for networking and knowledge-transfer support as well as the Marie Curie International Research Staff Exchange Fellowship within the 7th European Community Framework Program (PIRSES-GA-2011-295223) for supporting researcher exchanges.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.11.006.

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FEMS Microbiology Ecology, 92, 2016, fiw155

doi: 10.1093/femsec/fiw155
Advance Access Publication Date: 10 July 2016
Research Article

RESEARCH ARTICLE

Planktic *Tychonema* (Cyanobacteria) in the large lakes south of the Alps: phylogenetic assessment and toxigenic potential

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One sentence summary: This work highlights and discusses the implications of the ongoing replacement of *Planktothrix* and microcystins (hepatotoxins) by *Tychonema* and anatoxin-a (and homoanatoxin-a) (neurotoxins) in the large lakes south of the Alps.
Editor: Riks Laanbroek

ABSTRACT

This work allowed assessing a widespread occurrence of *Tychonema bourrellyi* in the largest lakes south of the Alps (Garda, Iseo, Como and Maggiore). The taxonomy of the species was confirmed adopting a polyphasic approach, which included microscopic examinations, molecular (16S rRNA and *rbcLX* sequences) and (Lake Garda) ecological characterisations. Over 70% of the 36 isolates of *Tychonema* sampled from the four lakes tested positive for the presence of genes implicated in the biosynthesis of anatoxins (*anaF* and/or *anaC*) and for the production of anatoxin-a (ATX) and homoanatoxin-a (HTX). A detailed analysis carried out in Lake Garda showed strong ongoing changes in the cyanobacterial community, with populations of *Tychonema* developing with higher biovolumes compared to the microcystins (MCs) producer *Planktothrix rubescens*. Moreover, the time × depth distribution of *Tychonema* was paralleled by a comparable distribution of ATX and HTX. The increasing importance of *Tychonema* in Lake Garda was also suggested by the opposite trends of ATX and MCs observed since 2009. These results suggest that radical changes are occurring in the largest lakes south of the Alps. Their verification and implications will require to be assessed by extending a complete experimental work to the other large perialpine lakes.

Keywords: *Tychonema*; cyanobacteria; anatoxins; phylogenetic analysis; toxic algae; perialpine lakes

INTRODUCTION

The high ecological plasticity and adaptability account for the wide distribution of cyanobacteria in almost every aquatic and terrestrial habitat (Sompong *et al.* 2005; Seckbach 2007; Whitton 2012). On the other hand, it has been recognised that not all the single species of cyanobacteria can occur everywhere and that different genotypes can be distinctly restricted to specific habitat types (Reynolds 2006; D'Alelio *et al.* 2011; Komarek 2011). The recent documented episodes of introduction and invasion of heterocytous cyanobacteria from the tropics to the northern subtropical and temperate regions has been interpreted as the consequence of environmental changes induced by global warming (Sukenik *et al.* 2012). Nevertheless, along with different explanatory hypotheses about the interpretation of the invasion routes and patterns of migration (Gugger *et al.* 2005), the establishment of new populations of cyanobacteria is universally recognised as the consequence of environmental changes regarding, in particular, climate and hydrological alterations, trophic status changes, or both (O'Neil *et al.* 2012; Paerl and Otten 2013; Salmaso *et al.* 2015a).

Received: 25 February 2016; **Accepted:** 8 July 2016

The production of several secondary toxic metabolites (cyanotoxins) is a distinctive feature of most cyanobacterial species (Metcalf and Codd 2012). The use of freshwater resources for drinking and recreational purposes, irrigation and fishing is seriously impaired when waters are contaminated by the presence of toxic populations (Mohamed and Al Shehri 2009; Ibelings *et al.* 2014). The colouring of surface waters, the increase of turbidity and the development of surface blooms of cyanobacteria further contribute to undermine the economic value of water resources (Hamilton *et al.* 2014).

The most studied cyanotoxins are the microcystins (MCs). With over 100 congeners (Puddick *et al.* 2014), MCs are produced by several cyanobacteria (Metcalf and Codd 2012). Their effects have been studied on a large number of living organisms, including humans (Svircev *et al.* 2009; Sukenik, Quesada and Salmaso 2015). The discovery of the biosynthetic route and gene clusters encoding MCs permitted to design primers to be used for the detection of toxic strains in environmental or unispecific samples (e.g. Vaitomaa *et al.* 2003). Conversely, the biosynthetic routes of many other cyanotoxins were elucidated only in more recent years (Dittmann, Fewer and Neilan 2013; Méjean and Ploux 2013), as in the case of the group of neurotoxins anatoxin-a (ATX) and homoanatoxin-a (HTX). The gene clusters (*ana*) coding for anatoxins have been described for a few Oscillatoriales and Nostocales, including *Oscillatoria* spp., *Anabaena* sp. and *Cylindrospermum stagnale* (Méjean *et al.* 2009, 2014; Rantala-Ylinen *et al.* 2011). These discoveries allowed designing specific primers for the detection of *anaC* and *anaF* genes, which code for an adenylation protein and a polyketide synthase, PKS, respectively. These primers were successfully used to test the presence of potentially neurotoxic cyanobacteria strains in unispecific cultures and environmental samples by using PCR (e.g. Ballot *et al.* 2010, 2014; Wood *et al.* 2010; Rantala-Ylinen *et al.* 2011) and quantitative real-time PCR (Wang *et al.* 2015).

The extensive surveys conducted since 2009 in Lake Garda and in the other large lakes south of the Alps (Maggiore, Como, Iseo and Lugano) demonstrated, besides a widespread presence of MCs, the occurrence of measurable concentrations of ATX (Cerasino and Salmaso 2012). Nevertheless, while the MCs were clearly produced by *Planktothrix rubescens*, i.e. the dominant cyanobacterium typical of these lakes (Salmaso *et al.* 2014; Shams *et al.* 2014), the producers of ATX remained unknown. In 2014, Shams *et al.* (2015) isolated, for the first time, several strains of *Tychonema bourrellyi* (Oscillatoriales) from the environmental samples collected in Lake Garda during the spring months. Until then, and based on microscopic observations, the presence of *T. bourrellyi* in the southern subalpine lakes was documented since the second half of the 2000s only in the reports describing the monitoring activities of International Commission for the Protection of Italian-Swiss Waters in Lake Maggiore (C.N.R.-I.S.E. Sede di Verbania 2015; Morabito, pers. comm.). In Lake Garda, most of the strains isolated in 2014 tested positive for the presence of the *anaC* gene of the *ana* gene cluster and for the production of ATX. The discovery raised a number of important questions regarding the spread of this species, the changes in the cyanobacterial structure and the production of cyanotoxins in the Lake District south of the Alps. Following the first discovery of ATX-producing strains of *Tychonema* in Lake Garda, the objectives of this work include (i) the assessment of the presence of *Tychonema*, and the taxonomic characterisation and comparison of the populations of *Tychonema* and *Planktothrix* in the largest lakes south of the Alps (Garda, Iseo, Como and Maggiore); (ii) the toxicity assessment and the phylogenetic analyses of *Tychonema* compared with other cyanobacteria bearing the *ana* cluster genes, based on the comparison of *anaF* sequences; and (iii) the potential impact of the presence of *Tychonema* on the cyanobacterial structure and toxicological fingerprint of Lake Garda.

MATERIALS AND METHODS

Study sites

The largest water bodies in the Lake District south of the Alps are the lakes Garda, Iseo, Como and Maggiore. Lake surface area and water volumes range between 62 and 368 km^2, and 7.6 and 49 billion m^3, respectively (Table 1). Owing to their large depth (z_{max} between 251 and 410 m), these lakes are characterised by long periods of incomplete winter mixing, which are interrupted by full vertical water circulation only after the occurrence of harsh winters (Mosello *et al.* 2010). Originally ultraoligotrophic (Milan *et al.* 2015), during the 1960s and 1970s, total phosphorus (TP) in these four lakes underwent a strong increase (Mosello, Calderoni and de Bernardi 1997; Salmaso *et al.* 2007). During and after the 1990s, recovery interventions have led to a strong reduction (Maggiore, Como) or stabilisation (Garda, Iseo) of nutrients. At present, concentrations of TP in the epilimnetic layers and in the whole water column suggest conditions of oligotrophy (Maggiore), oligo-mesotrophy (Garda, Como) and mesoeutrophy (Iseo) (Table 1).

Table 1. Main morphometric, hydrological, physical and chemical characteristics of the largest lakes south of the Alps. [a]Salmaso *et al.* (2012); [b]Mosello *et al.* (2010); [c]Salmaso *et al.* (2007).

		Garda	Iseo	Como	Maggiore
Lake altitude	m	65	186	198	193
Surface (S)	km^2	368	62	146	213
Maximum depth (z_{max})	m	350	251	410	370
Volume (V)	km^3	49.0	7.6	22.5	37.5
Renewal time (τ_w)	years	26.6	4.1	4.5	4.0
[a]Temperature 0–20 m, annual range	°C	7.9–20.1	6.1–20.3	6.5–20.1	6.5–17.9
[a]TP 0–20 m, annual averages	$\mu g\ P\ L^{-1}$	13	24	16	8
[b]TP at spring overturn, 0 m-z_{max}	$\mu g\ P\ L^{-1}$	18	50–60	25	10
[c]NO_3-N at spring overturn, 0 m-z_{max}	$\mu g\ N\ L^{-1}$	370	640	850	850
[c]Alkalinity at spring overturn, 0 m-z_{max}	$meq\ L^{-1}$	2.1	1.9	1.2	0.8
[c]Conductivity at spring overturn, 0 m-z_{max}	$\mu S\ cm^{-1}\ 20°C$	220	273	167	146

Isolation of strains, culturing methods and density estimation

Samplings for the isolation of cyanobacterial strains were carried out in 2014 in the deepest zones of the four lakes, as described in Salmaso *et al.* (2015b). Samples were collected by vertical tows from 20 to 30 m to the surface with plankton nets (mesh size 80 μm). In Lake Garda, strains were isolated in samples collected from late spring to summer. In lakes Iseo, Como and Maggiore, the isolation of strains was made on samples collected in June, October and November, respectively. The isolates were identified by analysing morphological characteristics following Komárek and Anagnostidis (2005). Among the Oscillatoriales, in the four lakes the microscopic examination allowed to identify filaments belonging to both *Tychonema bourrellyi* and *Planktothrix rubescens*.

Single filaments of Oscillatoriales were isolated using micropipettes under stereomicroscopes within 2 days after the sampling of the fresh material. The inocula were made after washing repeatedly with Z8 medium (Kotai 1972) the filaments. After initial growth in plates containing 3 mL Z8 medium, single filaments were transferred to 30 mL flasks and, finally, 150 mL medium Z8 CELLSTAR (Greiner Bio-One GmbH, Germany) culture flasks. The flasks were incubated at 20°C under continuous light illumination (25 μmol m^{-2} s^{-1}) in climatic simulation chambers (ProClimatic, Italy) (Shams *et al.* 2015).

After homogenisation, 12–14 mL of the final cultures of *Tychonema* were preserved with Lugol's solution for density and biovolume determinations (Rott, Salmaso and Hoehn 2007). The microscope counting was made on 2–5 transects at 200× magnification in 10 mL Utermöhl sedimentation chambers. The number of cells was computed by dividing the total length of filaments with the average length of the cells (around 5 μm; cf. Shams *et al.* 2015). Biovolumes were calculated from the total length and average width (5.8 μm) of filaments, assuming cylindrical shapes (cf. Komárek and Albertano 1994). In conjunction with cyanotoxins analyses, densities and biovolumes were used to estimate the cell quota (Q) of ATX of the cultured *Tychonema* strains.

The material for genetic analysis of *Tychonema* and *Planktothrix* was obtained by collecting 50 mL of each culture in Falcon tubes, successively stored at −20°C. After 2 cycles of freezing and thawing at 65°C, samples were centrifuged at 4000 *g* for 15 min. After the supernatant was discarded, the pellets were centrifuged (Eppendorf 5415D; Eppendorf AG, Hamburg, Germany) in Eppendorf tubes for 5 min at 16 000 *g* and stored at −20°C.

Lake Garda: sampling, and field and laboratory analysis

In this work, detailed analyses in Lake Garda were carried out monthly from April to November 2015 in the Long Term Ecological Research station of Lake Garda, between the villages of Brenzone and Gargnano (350 m depth; 45.69 N, 10.72 E). Samples for chemical, cyanobacterial and cyanotoxins analyses were collected in the layers between 0 and 2 m (1 m), 9 and 10 m (10 m), 19 and 21 m (20 m) and at 60 m. In the field, water temperatures were measured with a multiparameter probe (Idronaut Ocean Seven 316 Plus). The euphotic depth (z_{eu}) was estimated using the formula $z_{eu} = \ln(100) \times K_d^{-1}$ (Kirk 2011); the light attenuation coefficients, K_d, were measured with a submersible irradiance sensor, LiCor 192SA (Lincoln, USA).

In laboratory, determinations of nitrate nitrogen (NO_3-N), total and soluble reactive phosphorus, pH and conductivity were carried out using standard methods (APHA-AWWA-WPCF 1998).

Owing to the difficulty to discriminate the filaments of *Tychonema* and *Planktothrix* in samples fixed with Lugol's solution, the day after the sampling the quantitative estimation of these two taxa was made on fresh, natural samples. Aliquots of 10 mL were vacuum filtered onto 5 μm pore size, 25 mm diameter Whatman Cyclopore transparent polycarbonate membrane filters (Graham 1999), using a Whatman 1.2 μm pore size glass microfibre GF/C as support filter (Whatman—GE Healthcare Life Sciences, UK). Filtration was carefully made at low pressure to avoid the damage of filaments. The filters were then transferred to 75 × 25 mm glass slides for the discrimination and quantification of *Tychonema* and *Planktothrix* under an upright light microscope (Olympus BX51) using 200× magnifications. The filaments were measured on the whole area of the filter. Densities and biovolumes were computed as described above.

Besides samplings in 2015, the long-term development of ATX and MCs in the epilimnetic layers (0–20 m) was evaluated using the results obtained since 2009 from samplings carried out every 4 weeks or monthly (from 10 to 13 samplings per year). The sampling strategy and analytical methods throughout the whole sampling period were the same as those described in 2015 (see also next section). Further details have been reported in Cerasino *et al.* (2016).

Cyanotoxins analyses

To measure intracellular concentration of toxins in cyanobacteria, 2 L of environmental water samples and 250 mL of cyanobacterial cultures were filtered on 1.2 μm pore size GF/C filters, which were stored at −20°C. Toxin extraction followed the methods described in detail by Shams *et al.* (2015), which allowed to obtain concentrated solutions for LC-MS analysis (achieved concentration factors were around 300 and 30, for environmental samples and cultures, respectively).

The quantification of toxins extracted from the environmental samples and cultures was performed by LC-MS analysis, using a Waters Acquity UPLC system coupled to a SCIEX 4000 QTRAP mass spectrometer equipped with a turbo ion spray interface. The toxins analysed include MCs, ATX and HTX, nodularin (NOD), and cylindrospermopsin (CYN). The analysis was performed using calibration curves obtained with commercially analytical standards MC (RR, [D-Asp3]-RR, YR, LR, [D-Asp3]-LR, WR, LA, LY, LW, LF), ATX (Tocris Cookson Ltd, Avenmouth, UK), HTX (Novakits, Nantes, France), NOD-R, CYN (Vinci Biochem, Vinci, Italy). Semi-quantitative determination of other toxin variants (demethylated MC, deoxyCYN) was carried out assuming analytical response factors similar to the reference compounds. The limits of quantitation (LOQ) were between 30 and 500 ng L^{-1} (MC congeners), 140 ng L^{-1} (NOD-R), 30 ng L^{-1} (ATX), 200 ng L^{-1} (HTX), and 8 ng L^{-1} (CYN). A detailed description of the LC-MS protocols was reported in Cerasino and Salmaso (2012) and Cerasino *et al.* (2016).

DNA extraction, PCR amplification and sequencing

Total genomic DNA of the single strains of *Tychonema* and *Planktothrix* was extracted from the pellets using Glass beads-acid washed (212–300 μm; 425–600 μm) (Sigma-Aldrich CO., MO, USA) and the E.Z.N.A. SP Plant DNA Kit (Omega Bio-Tek Inc., GA, USA). DNA concentrations, in the range 10–190 ng μL^{-1}, were measured with a NanoDrop ND-8000 (Thermo Fisher Scientific Inc., MA, USA).

The 16S rRNA fragments of *Tychonema* and *Planktothrix* were amplified with primers pA (5′-AG

AGTTTGATCCTGGCTCAG-3′) (Edwards *et al.* 1989) and B23S (5′-CTTCGCCTCTGTGTGCCTAGGT-3′ (Lepère, Wilmotte and Meyer 2000; Taton *et al.* 2003) following the PCR protocol in Gkelis *et al.* (2005). The 16S rRNA was sequenced with the four internal primers 16S544R, 16S1092R and 16S979F (Rajaniemi-Wacklin *et al.* 2005), and CYA781Ra (Nübel, Garcia-Pichel and Muyzer 1997). The taxonomic position of the *Tychonema* strains isolated in the four lakes was further assessed by analysing the *rbcLX* genes (RuBisCO large subunit and chaperone). *rbcLX* were amplified and sequenced using the primers CW and CX, following the protocols in Rudi, Skulberg and Jakobsen (1998), as modified in Shams *et al.* (2015). The presence of the ATX synthetase gene cluster was assessed by analysing two genes encoding an adenylation protein (*anaC*) and a PKS (*anaF*), and using toxic strains of *Tychonema* previously checked both by PCR/sequencing and LC-MS as positive control (e.g. Shams *et al.* 2015). The *anaC* gene was qualitatively determined by PCR using the primer pairs anaC-gen and the methods described in Rantala-Ylinen *et al.* (2011). The *anaF* gene was analysed by PCR and sequenced using the primer pairs atxoaf and atxoar, and protocols described in Ballot *et al.* (2010, 2014). The isolated strains were analysed by PCR also for the presence of MCs-encoding genes (*mcyE*) using general primers (mcyE-F2/R4) according to the PCR protocols of Rantala *et al.* (2006).

All the PCRs were carried out on an Eppendorf Mastercycler ep (Eppendorf AG, Hamburg, Germany). The PCR products were checked and separated by 1% agarose gel electrophoresis stained with ethidium bromide. The size of the DNA amplicons was evaluated with a commercial DNA ladder (GeneRuler Express, Thermo Fisher Scientific, MA, USA). Before DNA sequencing of the 16S rRNA, *rbcLX* and *anaF* genes, PCR products were cleaned with Exonuclease plus Shrimp Alkaline Phosphatase. The sequencing methods were described in Shams *et al.* 2015. DNA chromatograms were screened with Chromatogram Explorer 3.3.0 (Heracle Biosoft), and further checked manually, whereas forward and reverse sequences were assembled using the Contig Assembly Program in BioEdit 7.2.5 (Hall 1999). The sequences were deposited to the European Nucleotide Archive with accession numbers LT546458–LT546478 (16S rRNA, *Tychonema*, 1408–1478 bp), LT546519–LT546532 (16S rRNA, *Planktothrix*, 1397–1478 bp), LT546479–LT546498 (*rbcLX*, *Tychonema*, 823–876 bp) and LT546499–LT546518 (*anaF*, *Tychonema*, 444 bp) (Table S1, Supporting Information).

Phylogenetic analyses

Excluding a few isolated single-nucleotide polymorphisms in the 16S rRNA of *Tychonema* (four sites) and *Planktothrix* (two sites), the sequences of the strains isolated from the four lakes looked identical. Similarly, the *rbcLX* and *anaF* showed identical sequences in the four lakes. Therefore, one sequence per lake was included in the phylogenetic analyses computed using the 16S rRNA and *anaF* sequences. The analyses based on the 16S rRNA were made adding other 47 sequences of Oscillatoriales from The International Nucleotide Sequence Database Collaboration, and using *Gloeobacter violaceus* PCC 7421 as outgroup. Reference sequences of *Tychonema* and *Planktothrix* were also checked in the SILVA ribosomal RNA gene database (Quast *et al.* 2013). The trees built using *anaF* sequences, which included representatives of all the species for which the gene was sequenced, were unrooted. Species were renamed following the most updated literature (Komárek and Anagnostidis 2005; Strunecký, Elster and Komárek 2011; Komarek *et al.* 2013; Strunecký *et al.* 2013; Guiry and Guiry 2016). Molecular sequences were aligned with MUSCLE called from the ape package in R 3.2.3 (Paradis 2012; R Core Team 2015). After trimming both ends, the DNA sequence alignments, poorly aligned positions and divergent regions were checked and eliminated using Aliscore (Misof and Misof 2009; R Core Team 2015). Phylogenetic trees were computed by maximum likelihood (ML) using specific scripts and calling PhyML 3.1 (Guindon *et al.* 2010) from R 3.2.3. Branch support was estimated by the approximate likelihood-ratio test (aLRT, SH-like option; Anisimova and Gascuel 2006); the selection threshold for SH-like supports should be in the 0.8–0.9 range (Guindon *et al.* 2010). DNA substitution models were analysed, calling PhyML 3.1, with the phymltest function in the R package ape; GTR + I + G and K80 + I were found to be the best-fitting evolutionary models for the 16S rRNA and *anaF* genes, respectively. The Newick rooted and unrooted trees obtained with the ML analyses were imported and plotted with the R package ape.

Statistical analyses

The relationships between the concentrations of ATX and the abundances (biovolumes and densities) of *Tychonema* were evaluated using quantile regressions using the package quantreg in R 3.2.3 (Koenker 2006; R Core Team 2015). Quantile regressions allow estimation of parameters when the assumption of normality in the residuals might not be satisfied (Koenker and Bassett 1978). The 50% quantile regression ($\tau = 0.50$, the median value) provides an estimate in which half of the observations are expected to fall below and above the regression line. Similarly, the 25% and 75% quantile regressions ($\tau = 0.25$ and $\tau = 0.75$) allow estimating the values of the response variable below and above which the 25% and 75%, and 75% and 25% of cases are expected to fall, respectively. The significance of relationships was tested using the bootstrap option (xy-pair method) in the package quantreg. The regression slope with $\tau = 0.50$ is an estimate of the quantity of toxins on a per unit biovolume or cell (cell quota, Q) of the populations in the field (Salmaso *et al.* 2014).

The long-term trends of ATX and MCs based on the data recorded between 2009 and 2015 in the 0–20 m layer of Lake Garda were evaluated using generalized additive mixed models (GAMM). In this work, GAMM were based on smoothing splines, using the package gamm in R (R Core Team 2015). The models allowed testing the estimated annual cycle and the estimated long-term trend of ATX and MCs. Autocorrelation in the residuals was checked with the inclusion of an autoregressive process of order 1 (AR1) within year (Wood 2006). The respective models were described by the effective degrees of freedom (edf); the higher was the edf, the more non-linear was the smoothing spline (Zuur *et al.* 2009).

RESULTS

Microscopic examination and phylogenetic analysis of strains

The isolation of the single filaments of Oscillatoriales in lakes Garda, Iseo, Como and Maggiore allowed to obtain 49 strains with the same microscopic and diacritical characteristics of *Tychonema* spp. described in Shams *et al.* (2015). Of these, the taxonomic position of 23 strains was also evaluated by the analysis and sequencing of the 16S rRNA and/or *rbcLX* genes (Table 2; Table S1A, Supporting Information). Similarly, a total of 21 other filaments were isolated and identified microscopically as *Planktothrix rubescens*. The taxonomic position of

Table 2. Polymerase chain reaction amplification of *anaF*, *anaC* and *mcyE* genes, and concentrations of anatoxin-a (ATX), homoanatoxin-a (HTX) and total microcystins (MCs) analysed in the strains of *Tychonema*. '+' and '−' indicate the presence and absence of expected PCR amplicons using agarose gel electrophoresis. Q, cell quota of ATX, computed either on a biovolume or cell basis. 'nd', not detectable. 'a' indicate the strains included in the phylogenetic analyses.

Lake	Date	Strain	*anaF* +/−	*anaC* +/−	*mcyE* +/−	ATX $\mu g\ L^{-1}$	HTX $\mu g\ L^{-1}$	MCs $\mu g\ L^{-1}$	Q ATX $\mu g\ mm^{-3}$	Q ATX pg $cell^{-1}$
Garda	06/05/2014	FEM_GT511	+	+						
Garda	06/05/2014	FEM_GT529[a]	+	+		60.90	0.09	nd	4.16	0.55
Garda	03/06/2014	FEM_GT604	−	−						
Garda	03/06/2014	FEM_GT606	+	+		4.41	nd	nd	2.20	0.29
Garda	03/06/2014	FEM_GT609	+	+		67.68	0.44	nd	2.18	0.29
Garda	03/06/2014	FEM_GT612	−	−						
Garda	09/07/2014	FEM_GT703	+	+	−	1.42	nd	nd	0.25	0.03
Garda	05/08/2014	FEM_GT806	+	+	−	94.60	0.23	nd	5.02	0.66
Iseo	24/06/2014	FEM_IT0608	+	+	−	94.45	0.49	nd	16.59	2.19
Iseo	24/06/2014	FEM_IT0609[a]	+	+	−	137.41	0.99	nd	11.37	1.50
Iseo	24/06/2014	FEM_IT0614	+	+	−	101.99	0.79	nd	14.55	1.92
Iseo	24/06/2014	FEM_IT0615	+	+	−	154.23	0.90	nd	20.77	2.74
Iseo	24/06/2014	FEM_IT0621	+	+	−	43.87	0.58	nd	2.87	0.38
Como	20/10/2014	FEM_CT1001[a]	+	+	−	6.32	0.11	nd	0.43	0.06
Como	20/10/2014	FEM_CT1003	+	+	−	28.67	0.49	nd	3.66	0.48
Como	20/10/2014	FEM_CT1005	+	+	−	41.85	0.15	nd	2.59	0.34
Como	20/10/2014	FEM_CT1007	+	+	−	16.88	0.08	nd	1.75	0.23
Como	20/10/2014	FEM_CT1009	+	+	−	50.17	0.22	nd		
Maggiore	11/11/2014	FEM_MT1115	+	+		92.56	0.20	nd	3.55	0.47
Maggiore	11/11/2014	FEM_MT1122[a]	+	+		55.44	0.19	nd	2.72	0.36
Maggiore	11/11/2014	FEM_MT1127	+	+		50.69	0.19	nd	3.96	0.52
Maggiore	11/11/2014	FEM_MT1128	+	+		104.49	1.53	nd	13.15	1.74
Maggiore	11/11/2014	FEM_MT1130	+	+		39.73	0.22	nd	1.13	0.15

Planktothrix was further evaluated by the analysis of the 16S rRNA in 14 strains (Table S1B, Supporting Information). In both *Tychonema* and *Planktothrix*, many isolates collected in a same sample probably belonged to a same population. Nevertheless, it is worth highlighting the coexistence of potentially toxic and non-toxic strains in the sample collected in Lake Garda in June 2014 (Table 2). The discrimination of the two species of Oscillatoriales was possible from the first step of the isolation of filaments from the environmental samples under the stereomicroscopes. The filaments of *Planktothrix* appeared brown and, in general, with a more intense colour compared to *Tychonema* (Fig. 1). These characteristics were less apparent in samples fixed with Lugol's solution.

The taxonomic classification of the isolated filaments of *Tychonema* and *Planktothrix* was fully supported by phylogenetic analyses based on partial 16S rRNA gene sequences (1361 bp; Fig. 2). The selected 16S rRNA sequences of lakes Garda, Iseo, Como and Maggiore (Table 2; Table S1, Supporting Information) formed a compact cluster with other *Tychonema bourrellyi* strains isolated in Norway and Northern Ireland and, in a slight different cluster, Lake Erhai, China (strain HAB663). This common cluster, however, included also representatives of *T. bornetii* and *T. tenue* isolated in Norway (Lake Mjøsa and River Glåma) (Fig. 2). The identity of the *Tychonema* isolated in the Italian lakes was confirmed by a BLAST homology search, which showed that the *rbcLX* sequences were >99% similar to at least seven other different strains of *T. bourrellyi* (query cover 87%–100%).

Similarly, the *P. rubescens* strains isolated in the four large Italian lakes were included in a compact clade with other *P. rubescens* strains isolated in Norway, Germany and Switzerland. This clade was separated from a very near cluster including *P. agardhii* and *P. suspensa* (Fig. 2).

Toxicity of the isolated strains

A selection of 36 strains of *Tychonema* were analysed for the presence of genes encoding ATX. Of these, 26 strains (>70%) tested positive for either the *anaC* or the *anaF* of the ATX synthetase (*ana*) gene cluster and for the presence of ATX and—generally—HTX. Only two additional strains, characterised by small concentrations of ATX (0.1–0.4 $\mu g\ L^{-1}$), tested negative for the presence of *anaC* and/or *anaF* amplicons. A selection of toxic and non-toxic strains is listed in Table 2, which includes only the taxa classified also by means of genetic and phylogenetic analyses (previous section and Table S1, Supporting Information). The selection of the isolates is biased towards the toxic strains to allow a significant number of ATX and HTX determinations, and the computation of the cell quota. In Table 2, though dependent from the abundance of filaments in the analysed cultures, the concentrations of ATX and HTX provide a clear indication on the relative importance of the two toxins in the populations of *Tychonema* in the four lakes. Usually, HTX was produced with concentrations from 0.2% to 2% compared to those of ATX. The corresponding cell quota, computed from the concentrations of ATX and the densities and biomasses estimated on the same cyanobacterial cultures, varied in the range 0.3–20.8 $\mu g\ mm^{-3}$ and 0.03–2.74 pg $cell^{-1}$ (Table 2; Table 3A and B). In the four lakes, the lower and upper quartiles, which provide an indication of data distribution after disregarding extreme observations, were 2.18–11.37 $\mu g\ mm^{-3}$ and 0.29–1.50 pg $cell^{-1}$ (Table 3B). The cell quota of HTX was in the range 0.01–0.19 $\mu g\ mm^{-3}$ and 0.001–0.025 pg $cell^{-1}$. Of all the 49 available strains of *Tychonema* isolated from the four large lakes, 25 and 22 were also tested for the production of MCs by LC-MS and the presence of *mcyE* genes, respectively. All the analyses provided negative results (see also Table 2 for a selection of analysed strains).

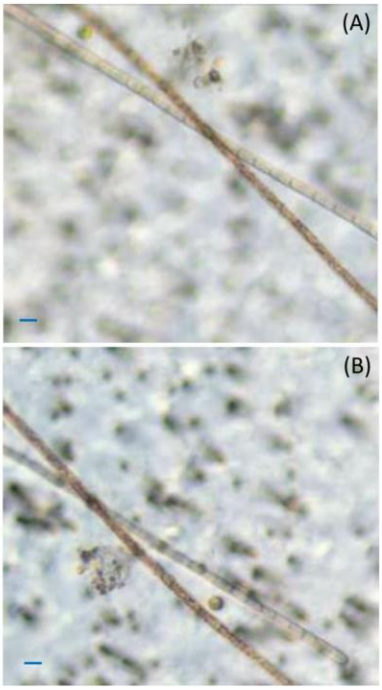

Figure 1. Micrographs of *T. bourrellyi* and *P. rubescens* (with reddish filaments) collected in Lake Garda and concentrated on 5 μm Whatman Cyclopore transparent polycarbonate membrane filters. (**A**) and (**B**), scale bars 10 μm.

The potential toxicity of *P. rubescens* was tested in 11 strains isolated from samples collected in Lake Garda. All the strains tested negative for the presence of the *anaC* gene and, with the exclusion of one isolate, positive for the presence of the *mcyE* gene of the *ana* and *mcy* gene clusters, respectively. Moreover, the available analyses confirmed the production of MCs (2–10 μg L^{-1}, mostly RRdm) and the absence of ATX in four analysed strains.

Phylogenesis of the *anaF* gene

The *anaF* gene amplicon sequencing of the strains of *Tychonema* isolated in lakes Garda, Iseo, Como and Maggiore (Table 2; Table S1A, Supporting Information) provided identical sequences of 444 bp. The *anaF* sequences of the Italian lakes formed a unique cluster with a strain of *T. bourrellyi* (NIVACYA_96/3) isolated from Lake Mjøsa (Fig. 3A). This group of planktic *T. bourrellyi* sequences was included in a larger cluster including other Oscillatoriales (i.e. *T. bornetii*, *Phormidium* cf. *uncinatum* and *Microcoleus autumnalis*). *Oscillatoria* sp. and the strains belonging to the Nostocales were included in other groups. The tree topology in Fig. 3A, which was based on a multialignment of 157 bp, was confirmed also considering a phylogenetic analysis based on a multialignment of 404 bp of a smaller group of species (Fig. 3B).

Environmental variables and seasonal dynamics of *Tychonema* and ATX/HTX in Lake Garda

From April to November, temperature values in the first 60 m of the water column of Lake Garda were between 9°C and 25°C (Fig. 4A). The lake showed a developing stratification in April and May, whereas a strong stratification, with a well-extended metalimnion, was apparent from June to September. The euphotic depth was between 18 and 33 m (Fig. 4A). In the whole period, TP and DIN concentrations in the first 20 m and at 60 m were in the range <5–14 μg P L^{-1} and 90–380 μg N L^{-1}, and <5–14 μg P L^{-1} and 340–495 μg N L^{-1}, respectively. Between 0 and 60 m, pH and conductivity were in the range 7.9–8.8 and 204–229 μS cm^{-1} at 20°C, respectively.

Tychonema showed a clear and localised distribution, which was mostly limited to the spring months and, partly, summer. Maximum biovolumes of this species were localised between 10 and 30 m (Fig. 4B). The depth × time development of ATX and HTX followed the same pattern of *Tychonema* abundances (Fig. 4C and D). These two toxins were significantly linked by the relationship HTX = −0.37 + 0.0047 × ATX (n = 32, r^2 = 0.95). The slopes of the quantile regressions between ATX and the biovolumes of *Tychonema*, estimated from the analyses of the environmental samples collected in Lake Garda, (Fig. 5; Table 4) were very close to the corresponding cell quota computed from the analyses of the single strains isolated from the same lake (Table 3A). The use of the ordinary least square regression provided an almost coincident result with that obtained with the quantile regression (Fig. 5; Table 4). The good relationship between ATX and the biovolume of *Tychonema* over the whole range of data was further confirmed after logarithmic transformation of the data (Fig. S1, Supporting Information). The two variables showed a good agreement also in the lower range of values.

Both *Planktothrix* and MCs were strictly localised in the metalimnetic layer in the period between half July and half September (Fig. 4E and F). Overall, the mean annual values of MCs in the first 20 m (33 ng L^{-1}) were around 10 times lower than the corresponding ATX averages (308 ng L^{-1}). The cell quota of MCs in *P. rubescens* estimated from the environmental samples was 2.2 μg mm^{-3} (τ = 0.50).

Interannual development of ATX and MCs

In the euphotic layers, peaks of ATX >500–1000 ng L^{-1} were regularly observed since 2011 (Fig. 6A). Excluding an isolated large value in 2015, MCs showed a tendency to develop with lower peaks (Fig. 6B). Though not significant (approximate P-value > 0.05), the GAMM analyses suggested a slight increase of ATX (Fig. 6C) and a significant decrease of MCs ($P < 0.05$) (Fig. 6D). The estimated annual cycles ($P < 0.01$) clearly showed that the maximum concentrations of ATX and MCs were out of phase by 2 months, with maxima around June and August–September, respectively (Fig. 6E and F). The significance of trends ($P > 0.05$ and $P < 0.05$ for ATX and MCs, respectively) and annual cycles ($P < 0.01$) was confirmed also including the AR model for the residuals. The analyses of the data collected in the 0–60 m layer provided practically the same results (GAMM trend and annual cycles; figures not shown). The only difference consisted in the lower average annual peaks of ATX (500–2200 ng L^{-1}) and MCs (25–260 ng L^{-1}) detected in the 0–60 m layers compared to the euphotic layers.

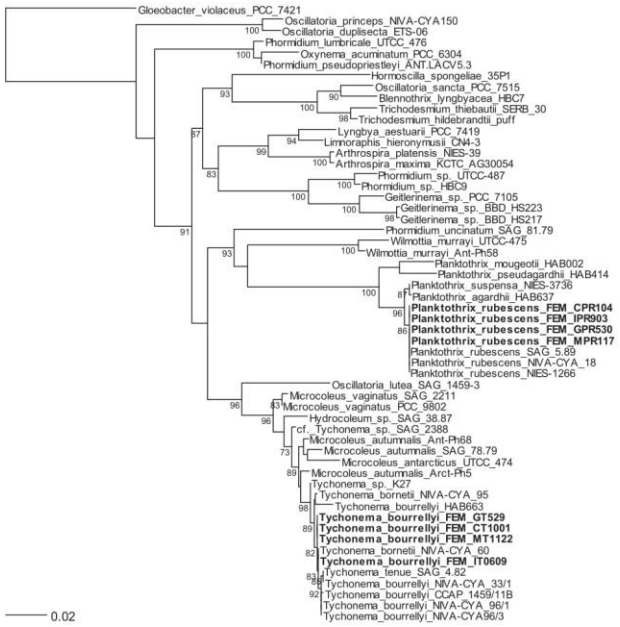

Figure 2. ML rooted topology of different species of Oscillatoriales based on partial 16S rRNA gene sequences. The strains of *Tychonema* and *Planktothrix* isolated in lakes Garda, Iseo, Como and Maggiore are highlighted in bold. Branch support aLRT-SH-like option values (×100) <70 are not shown. The complete list of strains and accession numbers is reported in Table S1A–C, Supporting Information.

Table 3. Means, standard deviations (SD) and medians of Q, the cell quota of ATX (biovolume or density based) estimated from the analysis of the strains of *T. bourrellyi* isolated from the samples collected in (A) Lake Garda ($n = 5$) and (B) in the whole group of lakes ($n = 19$) (see Table 2).

(A)	Strains (Garda)		Biovolume based $\mu g\ mm^{-3}$	Density based $pg\ cell^{-1}$
	Average	Q	2.76	0.36
	SD	Q	1.87	0.25
(B)	Strains (Garda, Iseo, Como, Maggiore)		Biovolume based $\mu g\ mm^{-3}$	Density based $pg\ cell^{-1}$
	Average	Q	5.94	0.78
	SD	Q	6.10	0.81
	Median	Q	3.55	0.47
	Lower quartile	Q	2.18	0.29
	Upper quartile	Q	11.37	1.50

DISCUSSION

After the first discovery in Lake Garda, this research demonstrated that toxic populations of *Tychonema bourrellyi* were present also in the other largest lakes south of the Alps, namely the lakes Iseo, Como and Maggiore. Besides ATX, the LC-MS analyses for the first time proved that populations of this species were also able to produce HTX. This discovery contributed to interpret, in a wider perspective, the ongoing and rapid changes occurring in the largest lakes on the foothills of the Alps.

The distinction between the three established and accepted *Tychonema* species in the manual by Komárek and Anagnostidis (2005) (i.e. *T. bourrellyi*, *T. bornetii* and *T. tenue*) is based on differences in the width of filaments, the habitat (periphytic/mats or pelagic) and pigments. *Tychonema bourrellyi* is characterised by possessing thin filaments (4–6 μm wide) with phycoerythrin, and by exclusively colonising pelagic habitats. This species is also known to form metalimnetic maxima (Komárek, Komárková and Kling 2003). The other two species develop in periphyton, also forming benthic mats. However, contrary to *T. bornetii* (8/12–16 μm wide), the dimensions of *T. tenue* (5.5–7 μm wide) overlap with those of *T. bourrellyi*; moreover, *T. tenue* can be also found with tychoplanktic filaments in the plankton of small lakes, swamps and ponds. Actually, planktic populations of this species could be possibly identical with *T. bourrellyi* (Komárek and Anagnostidis 2005). Therefore, a clear morphological distinction among the three species could be possible only between *T. bourrellyi*/*T. tenue* and *T. bornetii*. The presence in the plankton communities cannot be considered an exclusive ecological feature to be used for the specific attribution of filaments to *T. bourrellyi*. For example, in shallower lakes, the

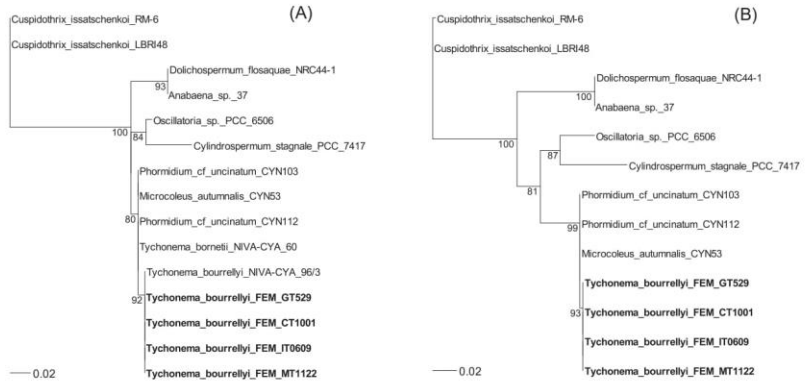

Figure 3. ML unrooted topology of different cyanobacteria species based on *anaF* gene sequences of (**A**) 157 bp and (**B**) 404 bp. The strains of *Tychonema* isolated in lakes Garda, Iseo, Como and Maggiore are highlighted in bold. Branch support aLRT-SH-like option values (×100) <70 are not shown. The complete list of strains and accession numbers is reported in Table S1D, Supporting Information.

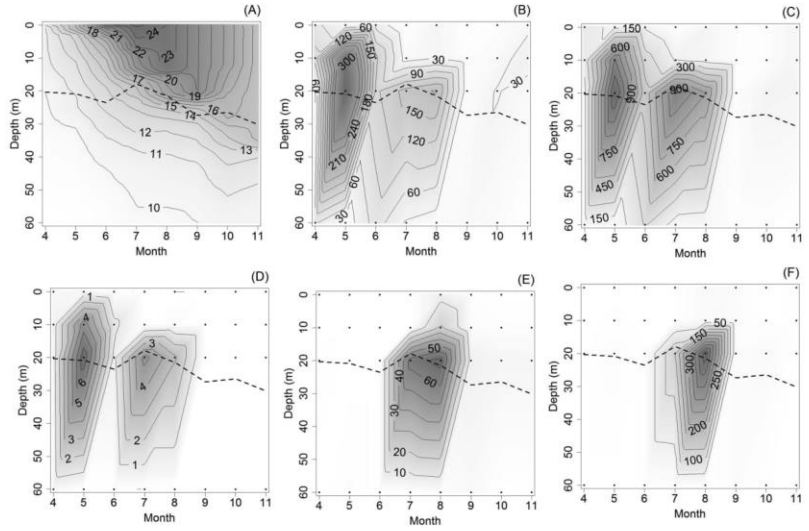

Figure 4. Depth × time distribution of (**A**) water temperature (°C), (**B**) biovolume of *T. bourrellyi* ($mm^3\ m^{-3}$), (**C**) ATX ($ng\ L^{-1}$), (**D**) HTX ($ng\ L^{-1}$), (**E**) biovolume of *P. rubescens* ($mm^3\ m^{-3}$) and (**F**) total MCs ($ng\ L^{-1}$). The dashed line (A–F) and the black dots (B–F) indicate the euphotic depth and the sampling depths, respectively. Data refer to Lake Garda, from April to November 2015.

larger (≥10 μm) filaments of *T. bornetii* could be present also with occasional tychoplanktic habit.

Historically, *T. bourrellyi* (and its closer species, *T. tenue*) was recorded in northern regions, mostly Northern Europe and Canada (Kling and Holmgren 1972; Berglind, Holtan and Skulberg 1983; Skulberg and Skulberg 1985, 1991; Komárek and Albertano 1994; Butterwick, Heaney and Talling 2005). In most cases, taxonomic determinations were confirmed also by genetic analyses (Ostensvik *et al.* 1998; Rudi, Skulberg and Jakobsen 1998; Suda *et al.* 2002; Shams *et al.* 2015). More recently, 16S rRNA sequences of several clones of uncultured cyanobacteria collected in a benthic mat community on King George Island

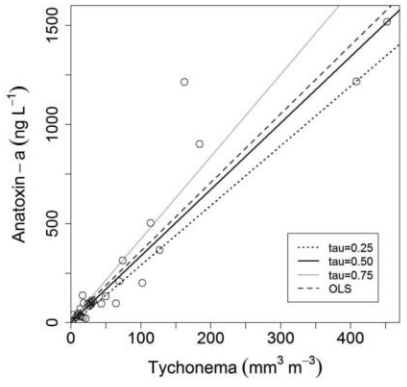

Figure 5. Relationship between anatoxin-a and the biovolume of *T. bourrellyi* in Lake Garda. The lines are ordinary least square regressions (OLS) and quantile regressions computed for $\tau = 0.5$ (median), $\tau = 0.25$ and $\tau = 0.75$.

Table 4. Quantile ($\tau = 0.5$) and ordinary least square (OLS) regressions between anatoxin-a (ATX; ng L^{-1}) and the biovolume (mm^3 m^{-3}; Fig. 5) and density (cells mL^{-1}) of *T. bourrellyi* estimated from the samples collected in Lake Garda ($n = 32$); the slopes give an estimate of the increase of ATX per unit increase of biovolume (μg mm^{-3} = fg μm^{-3}) and density (pg cell^{-1}).

		ATX versus biovolume	ATX versus density
Quantile regression	Intercept	2.15	2.15
	Slope	3.36	0.44
	P	<0.001	<0.001
OLS regression	Intercept	14.9	14.9
	Slope	3.46	0.46
	P	<0.001	<0.001

(Antarctica) were found to share high similarity with the corresponding 16S rDNA gene sequences of organisms classified as members of the genus *Tychonema* (Callejas *et al.* 2011). Morphotypes of *Tychonema* were also reported in environmental samples from the High Arctic regions (de-los-Rios *et al.* 2015). Overall, the historical and new records induce to consider *Tychonema* as a cold-stenotherm genus (Komárek, Komárková and Kling 2003).

The identification of *T. bourrellyi* in the group of the largest lakes south of the Alps was unforeseen. The discrimination between filaments of *Planktothrix rubescens* and *T. bourrellyi* in samples preserved with red Lugol's solution is not always easy, so the exact contribution of *Tychonema* to the cyanobacterial community before their isolation from natural samples cannot be determined with certainty. Nevertheless, the wide collection of environmental samples from several lakes in the Lake District south of the Alps carried out from late spring to early autumn in 2009 and 2010 allowed to isolate only filaments of *P. rubescens* (D'Alelio *et al.* 2011; D'Alelio, Salmaso and Gandolfi 2013), with the only exception of Lake Idro, where an uncommon species of *Planktothrix* was identified (D'Alelio and Salmaso 2011). A few rare and undetermined filaments of Oscillatoriales were however occasionally reported in the analyses of fixed samples of Lake Garda. In Lake Maggiore, the presence of undetermined '*Oscillatoria* sp.' or *T. sequanum* was documented by Ruggiu *et al.* (1998) and Kamenir and Morabito (2009). In this lake, rare filaments of *T. bourrellyi* were moreover identified by microscopic observations in the monitoring campaigns since 2006 (C.N.R.-I.S.E. Sede di Verbania 2007). The more recent reports appeared to document an increase of *T. bourrellyi*, which, in 2014, developed with biovolumes similar or greater than those of *P. rubescens* (C.N.R.-I.S.E. Sede di Verbania 2015: fig. 4.2).

The analyses carried out on the fresh samples collected in 2015 in the largest of the four lakes examined in this work (Lake Garda) allowed giving evidence of a stronger development of *Tychonema* compared to *Planktothrix*. Both species mostly developed, in different periods, in a layer of 20–30 m around the euphotic depth. After an initial and higher spring growth, *Tychonema* decreased, growing together with *Planktothrix* during the summer months. This behaviour can be interpreted considering that, contrary to *P. rubescens*, *T. bourrellyi* does not possess gas-vesicles, and therefore it is not able to control its vertical positioning in stable, stratified conditions. The development of *Planktothrix* in 2015 was much more limited compared to *Tychonema*. This observation widely contrasts with the present paradigm stating that the dominant Oscillatoriales in the deep lakes south of the Alps are represented by *Planktothrix rubescens* (Salmaso *et al.* 2012). The above considerations did not contrast with the hypothesis of a process of colonisation of Lake Garda by *T. bourrellyi*. This could be also confirmed by the opposite long-term trends in the ATX and MCs concentrations, though a conclusive evaluation should require longer time series of cyanotoxins analyses. The extent and the causes of this expansion in the whole group of large lakes south of the Alps remain to be explored. However, the apparent increasing spread of *T. bourrellyi* in Northern Italy does not contrast with the optimum of temperature for growth measured for some specific strains. Butterwick, Heaney and Talling (2005) showed that a strain of *T. bourrellyi* isolated from the Cumbrian lakes grew poorly or not at 5°C and 30°C, whereas the optimum temperature for growth was between 11°C –17°C and 25°C. Moreover, we cannot exclude a link between changes in the cyanobacterial populations and the oligotrophication processes documented in many lakes south of the Alps. In particular, the strong decrease in the concentrations of TP documented since the 1980s in Lake Maggiore and since the second half of 2000s in Lake Garda were both followed by a significant decrease of cyanobacteria and *P. rubescens* (Fastner *et al.* 2015; Salmaso *et al.* 2015a). Nevertheless, the potential significance of these changes on the development of *Tychonema* remains to be explored.

Many strains of *Tychonema* isolated from the four lakes tested positive for the presence of the *anaC* and *anaF* genes, and for the production of ATX and HTX. Though HTX is usually synthesised together with ATX (Aráoz 2005; Aráoz, Molgó and Tandeau de Marsac 2010), its detection and quantitative evaluation in the environmental samples begun only recently (Wood *et al.* 2010; Mann *et al.* 2011). The reasons were probably due to the generally lower concentrations of HTX compared to ATX and the lack of commercial standards in some laboratories (e.g. Shams *et al.* 2015). The *ana* biosynthetic gene clusters so far identified in *Oscillatoria*, *Anabaena* and *Cylindrospermum* showed a common pattern and a few differences, suggesting a common evolutionary origin (Méjean *et al.* 2014). In this work, the partial *anaF* amplicons of *T. bourrellyi* isolated in the large Italian lakes were identical to those sequenced in strains of the same species in

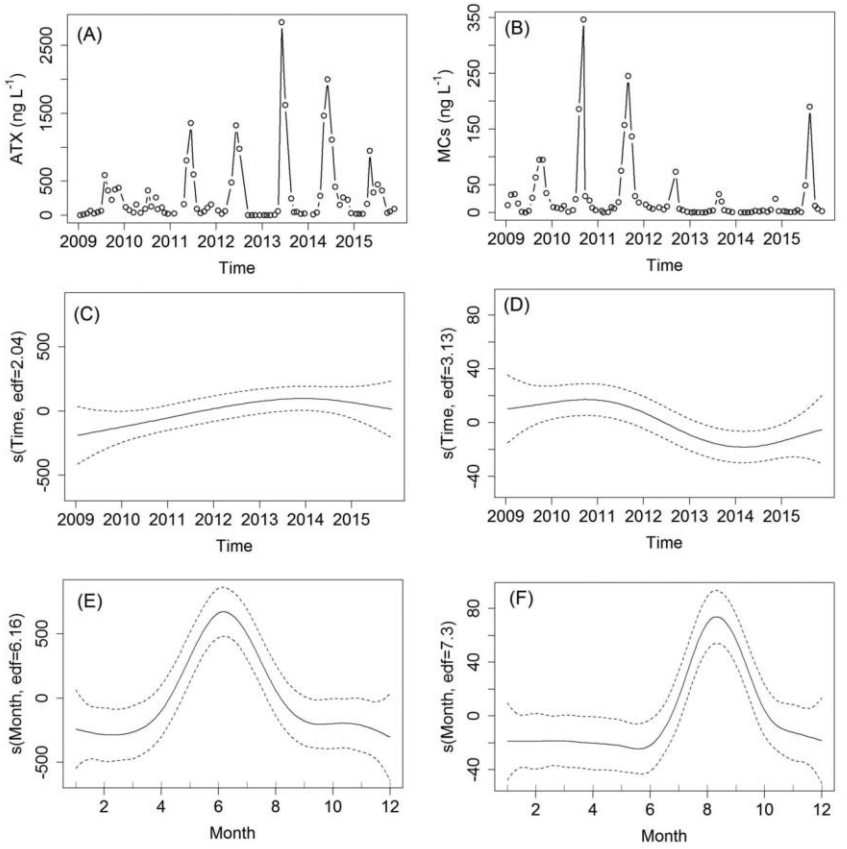

Figure 6. Temporal development of (**A**) ATX and (**B**) total MCs in the 0–20 m layer of Lake Garda from January 2009 to November 2015. Long-term trends of (**C**) ATX and (**D**) MCs, and annual cycles of (**E**) ATX and (**F**) MCs estimated by GAMM (Wood 2006).

Sweden by Shams *et al.* (2015) and, except for a single nucleotide, to the *anaF* amplicons in *Phormidium* and *Microcoleus*. Conversely, it is worth to observe that the *anaF* of *T. bornetii* was identical to that of *Phormidium* and *Microcoleus*, i.e. two species with typical periphytic habitat. Excluding *Cylindrospermum*, the remaining Nostocales formed a separate cluster, in analogy with the phylogenetic tree and taxonomy obtained from a wider analysis of 31 conserved proteins (Komárek *et al.* 2014). The phylogenetic relationships based on these relatively short sequences will require to be confirmed considering larger portions of the *ana* synthetase gene cluster.

Excluding the higher cell quota values found in many strains of Lake Iseo and (one strain) Lake Maggiore (up to 2.7 pg cell^{-1}), the cell quota of ATX on a cell basis in the four lakes was comparable with those previously found in Lake Garda (Shams *et al.* 2015) and within the 100-fold variation in ATX content estimated in *Phormidium* spp. populations (Wood *et al.* 2012; Harland *et al.* 2013). Though part of the variations in the data collected in this work could have been also influenced by uncertainties especially in the measurement of lower biovolume and ATX values, these observations confirm the wide range of cell quota values and toxicity that can characterise different cyanobacterial strains. The presence of two strains of *Tychonema* characterised by minor concentrations of ATX but negative to the amplification of *anaF* and/or *anaC* genes could be due to the failure of PCR in samples with low concentrations of ATX, or to the presence of sequences requiring different PCR optimisation. This aspect, however, would require to be analysed in more detail, especially

after considering the factors that control the degree of toxicity in the different strains.

ATX and HTX are potent neurotoxins (Lilleheil *et al.* 1997) that represent a potential serious risk for human and animal health (Onodera *et al.* 1997; Wood *et al.* 2007). However, present observations indicated that the concentrations of HTX in Lake Garda and in the isolated strains of all the analysed lakes were a very minor component compared with ATX (generally <1%–2%). At present, indications about the concentrations of ATX that can be tolerated in drinking waters were provided in New Zealand and Quebec (Canada). Since no WHO guidelines are available, a Provisional Maximum Acceptable Concentration (PMAC) of 3.7 μg L^{-1} (Quebec) and a Provisional Maximum Acceptable Value (PMAV) of 6 μg L^{-1} (New Zealand) were indicated (Chorus 2012; Ibelings *et al.* 2014). Regarding HTX, in New Zealand a PMAV of 2 μg L^{-1} was suggested. PMAVs are informed by current scientific understanding, even if incomplete (Ibelings *et al.* 2014). In the absence of WHO guidelines, in recreational waters the proposed limits for ATX are higher and quite different, ranging e.g. in the USA between <10 and 300 μg L^{-1}, and with values generally higher than other cyanotoxins (MCs, CYNs and saxitoxins) (see www.epa.gov, guidelines and recommendations). In this and previous studies (e.g. Cerasino and Salmaso 2012; Manganelli *et al.* 2014), the concentrations of ATX in the large lakes south of the Alps were usually below 4 μg L^{-1}, i.e. around the PMAC limits, and well below the thresholds generally suggested to avoid swimming and wading in many US states. Nevertheless, special care should be done to this class of neurotoxins, which should be added to the cyanotoxins to be evaluated in the monitoring plans by environmental agencies. This is urgent also considering that the largest Italian lakes are also used as sources of drinking water (e.g. Sorlini, Gialdini and Collivignarelli 2013), and that in the smaller lakes of the southern subalpine region ATX was measured with concentrations up to 290 μg L^{-1} (Manganelli *et al.* 2014). In the absence of accepted guidance levels, the potential impact of ATX and HTX on animal and human health, alone and in combination with other toxins, would require to be further evaluated.

The evidences collected in this work indicate that *T. bourrellyi* was responsible for the production of ATX and HTX in Lake Garda. Taking into account the results obtained from the analysis of the isolated strains, this same species could be responsible for the production of anatoxins also in the other large lakes south of the Alps. This deduction, however, should be confirmed by an appropriate experimental work carried out at least in the lakes where strains of this species were isolated. A further indication that emerged from this work is that ATX and HTX were not produced by *P. rubescens*. This species, which is widespread in many deep (not shallow) European lakes, is sometimes reported as an ATX producer. Nevertheless, at present, this indication is supported by a unique observation carried out in a small (0.01 km^2) and shallow (z_{max} = 8 m) lake, with microscopic and cyanotoxins analyses made on environmental samples (Viaggiu *et al.* 2004). Actually, the ultimate inclusion of *P. rubescens* among the ATX producers (Shams *et al.* 2015) will be possible only after the isolation of strains and their analyses by molecular methods.

CONCLUSIONS

This work allowed assessing a widespread occurrence of *Tychonema bourrellyi* in the largest lakes south of the Alps. The taxonomic identity of this species in lakes Garda, Iseo, Como and Maggiore was assessed using a polyphasic approach, which included microscopic, molecular and (Lake Garda) ecological characterisations. Most of the strains of *Tychonema* isolated in the large Italian lakes tested positive for the presence of the genes implicated in the biosynthesis of ATX and for the production of ATX. Compared to *Planktothrix rubescens*, which is able to synthetise MCs, the quantitative determination of the Oscillatoriales carried out on fresh samples collected in Lake Garda allowed to prove the greater importance of *Tychonema*. Since 2009, the increasing role of this species in Lake Garda was confirmed by an increase in ATX peaks and a decreasing trend of MCs. These findings induce to change an important paradigm in the phytoplankton ecology of the southern perialpine lakes. In fact, until now, *Planktothrix* was considered the dominant cyanobacterium, and the only producer of MCs. The confirmation of the presence of *Tychonema* also in the other northern large Italian lakes could solve the paradox of the presence of measurable concentrations of ATX not supported—at least until now—by the identification of potential cyanobacterial producers. However, the final confirmation of this hypothesis will require an appropriate experimental work (which is partially still in progress). Besides ATX, many strains of *T. bourrellyi* isolated from the large Italian lakes tested positive, for the first time, for the production of HTX. This discovery raises concerns about the potential synergic toxic effects caused by the presence of MCs, ATX and—though present in minor proportions—HTX.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

ACKNOWLEDGEMENTS

Investigations were carried out in the framework of the LTER (Long Term Ecological Research) Italian network, site 'Southern Alpine lakes', IT08-000-A (http://www.lteritalia.it/), with the support of G. Morabito (CNR-ISE), F. Buzzi (ARPA Lombardia) and B. Leoni (University of Milan). Special thanks are due to S. Shams (former PhD student at FEM) and K. Sivonen (University of Helsinki) for their great support during previous investigations on toxic *Tychonema* strains. We are grateful to our colleagues in FEM, in particular L. Ress, M. Tarter and A. Zampedri, for their support in the field and/or laboratory activities, and to the ARPA Veneto (G. Franzini), for logistic support in the field. We thank the European Cooperation in Science and Technology COST Action ES1105 CYANOCOST for networking and knowledge-transfer support. We are indebted to three anonymous reviewers for valuable comments and suggestions on an earlier version of the manuscript.

FUNDING

FIRS>T PhD fellowship was awarded to CC from the E. Mach Foundation—Istituto Agrario di S. Michele all'Adige.

Conflict of interest. None declared.

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Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Expansion of bloom-forming *Dolichospermum lemmermannii* (Nostocales, Cyanobacteria) to the deep lakes south of the Alps: Colonization patterns, driving forces and implications for water use

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ARTICLE INFO

Article history:
Received 14 July 2015
Received in revised form 17 September 2015
Accepted 18 September 2015
Available online 10 November 2015

Keywords:
Cyanobacteria
Dolichospermum
Surface blooms
Phylogenetic analysis
Cyanotoxins
Nuisance algae

ABSTRACT

Since the beginning of the 1990s, the largest lakes south of the Alps (Garda, Iseo, Como and Maggiore) showed a progressive colonization of *Dolichospermum lemmermannii* (Cyanobacteria). The appearance of surface blooms of this species raised serious concerns because of the impacts on the tourist economy and the potential toxigenic effects. Nevertheless, no detailed investigations were done to clarify the taxonomic position, ecology and toxicity of this species. In this work, phylogenetic analyses based on the 16S rRNA and *rpoB* genes demonstrated how the strains isolated from the Italian lakes were clustered together with other *D. lemmermannii* strains isolated in Northern Europe. The expansion of this species in the southern subalpine lakes contrasted with the prevailing south to north dispersion paths typical of other Nostocales (e.g. *Cylindrospermopsis* and *Aphanizomenon*). Conversely, the spread was consistent with the geographical areal of this cyanobacterium, which appears circumscribed between the 40th parallel and the Arctic Circle. This aspect highlights the ecological heterogeneity that characterizes the order Nostocales. In Lake Garda, *D. lemmermannii* always developed in the warmest months (>15 °C) with low abundances (generally <200 cell mL^{-1}). Nevertheless, owing to its ability to form surface water blooms, this species is considered as one of the most nuisance algae in the subalpine lake district. From the other side, different strains isolated from these large lakes tested negative for the biosynthesis of microcystins, anatoxin-a, nodularins and cylindrospermopsins, and for the presence of *mcyE* and *anaC* genes of the microcystins and anatoxin-a gene clusters.

1. Introduction

With a few exceptions, surface water blooms in freshwater habitats are associated with the development of cyanobacteria. Their excessive development has important negative ecological, health and economic impacts, which include the occurrence of tastes and odors, production of toxins, alteration of biogeochemical cycles and trophic webs, loss of water clarity and esthetic values of water bodies (Metcalf and Codd, 2012; Hamilton et al., 2014; Sukenik et al., 2015). Nevertheless, these effects and the associated risks are strongly dependent from the autecology of the dominant cyanobacterial species, their mode of bloom formation, the presence of toxic strains, the type and quantity of toxins produced, and the intended use of aquatic resources (Dietrich and Hoeger, 2005; Manganelli et al., 2010). Surface blooms require particular attention when waters are used for recreational activities, whereas species developing in deeper layers (e.g. metalimnetic blooms of *Planktothrix rubescens*) cause distinctive problems to the supply of drinking water abstracted from pipelines located at discrete depths (Akçaalan Albay et al., 2014).

Eutrophication and global warming are considered the two main factors promoting the development of cyanobacteria (O'Neil et al., 2012; Paerl and Paul, 2012). Since the 1960s, phosphorus (P) has been identified as one of the principal causes of water quality deterioration and one of the critical factors controlling cyanobacterial development. Though nitrogen (N) has been demonstrated as a factor as important as P in the occurrence of toxic, non-diazotrophic cyanobacterial blooms (Lewis and Wurtsbaugh, 2008; Conley et al., 2009; O'Neil et al., 2012) its role is still considered more controversial (Schindler, 2012).

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http://dx.doi.org/10.1016/j.hal.2015.09.008
1568-9883/

More recent studies demonstrated that the development of cyanobacteria can be favored by global warming (Wagner and Adrian, 2009; Carey et al., 2012; Winder and Sommer, 2012). The tendency of lakes to increase water temperatures has been demonstrated in several investigations (Dokulil et al., 2006; Sharma et al., 2015). In warmer lakes, cyanobacteria have been considered to gain competitive advantage due to higher replication rates at higher temperatures compared with many other algal eukaryotes (Jöhnk et al., 2008; Paerl and Huisman, 2009). Nevertheless, the more important advantages seem rather linked to the indirect effects of rising water temperatures, which include the increase in the physical stability of the water column and the widening of the stratification period (Sommer et al., 2012). These factors reinforce and extend the period of cyanobacterial optimal growth, favoring the massive development of species able to regulate their buoyancy and access nutrients in the hypolimnion (Wagner and Adrian, 2009; O'Neil et al., 2012). This view has been strengthened by the experimental work by Lürling et al. (2013) on several species of cyanobacteria and chloropytes. While these two algal groups showed a substantial coincidence in the optimum temperatures for growth, many chlorophytes were characterized by higher growth rates than cyanobacteria at higher temperatures.

Global warming has been considered as a main driving factor controlling the south to north invasion of two Nostocales genera, *Cylindrospermopsis* and *Aphanizomenon*, to lakes and reservoirs in subtropical and temperate zones (Sukenik et al., 2012). Studying the temperature- and light-dependent growth of several Nostocales species, Mehnert et al. (2010) suggested that any further temperature increase would promote the growth and spread of this group in general, and that of the invasive species in particular. In this context, the spread of another Nostocales, *Dolichospermum lemmermannii*, in the large lakes south of the Alps in the 1990s and 2000s (Salmaso et al., 2012) seems to be in contrast with the prevalent south to north dispersion routes. In the northern hemisphere, the numerous works reporting the presence of *D. lemmermannii* allowed to establish the geographic range of this species between the 40th parallel and the Arctic Circle (Fig. S1A). In particular, *D. lemmermannii* has been widely described in Central and Northern Europe, including the boreal regions (Padisák et al., 2003; Lepistö et al., 2005). The presence of this species is of particular concern, because of the identification of several toxigenic populations and of its association with cattle and animal poisonings (Sivonen et al., 1990; Onodera et al., 1997).

The appearance of extended surface blooms of *Dolichospermum lemmermannii* in the large and deep lakes south of the Alps has caused serious concerns, especially for the deterioration of the value of these tourist destinations, which are visited by millions of persons per year (Salmaso et al., 2012; Comunità del Garda, 2013). Nevertheless, a comprehensive taxonomical, ecological, and toxicological account concerning the appearance and spread of this species was never attempted. The specific objectives of this work include: (i) the phylogenetic characterization of *D. lemmermannii* colonizing the largest lakes in the southern subalpine district; (ii) the ecological factors promoting its seasonal development; and (iii) the potential toxicity of populations of *D. lemmermannii* in Lake Garda, the lake where the blooms were identified for the first time during the 1990s. The results will be used to define (iv) hypotheses about the causes of the expansion of *D. lemmermannii* in the southern subalpine lake district.

2. Materials and methods

2.1. Study sites

In this study we will consider the spread of *Dolichospermum lemmermannii* in the largest lakes south of the Alps (deep southern subalpine lakes, DSL), namely the lakes Garda, Maggiore, Como and Iseo. The lakes have maximum depths and volumes ranging between 251 and 410 m, and 7.6 and 49 billions of m^3, respectively (Fig. S1B and C). Considering the climatic location, these lakes should be classified as warm monomictic, with complete winter circulation at or above 4 °C, and stable stratification from spring to early autumn (Wetzel, 2001). Nevertheless, owing to their large depth, these lakes experience long periods of incomplete winter mixing, interrupted by irregular complete overturns following harsh winters (Ambrosetti and Barbanti, 1992). The DSL were originally oligotrophic or ultra-oligotrophic. Since the 1960s and 1970s, total phosphorus (TP) concentrations showed a consistent increase in the four lakes (Mosello et al., 1997). After the 1990s, following recovery interventions, the lakes showed a stabilization (Garda, Iseo) or strong reduction (Maggiore, Como) of nutrients. Present concentrations of TP in the whole water column are representative of oligotrophic (Maggiore, 10 $\mu g\ P\ L^{-1}$), oligo-mesotrophic (Garda, 18 $\mu g\ P\ L^{-1}$; Como, 25 $\mu g\ P\ L^{-1}$) and meso-eutrophic (Iseo, 50–60 $\mu g\ P\ L^{-1}$) conditions (Salmaso et al., 2012).

2.2. Long-term sampling and environmental variables in Lake Garda

In Lake Garda, the sampling station was located at the deepest point of the lake (45.69 N, 10.72 E). Field measurements and collection of samples in the layers 0–2, 9–10 and 19–21 m were made at monthly intervals since 1991. From each layer, constant volumes of water (20 L) were collected for successive subsampling. Detailed data of phytoplankton abundance and nutrients in the epilimnetic layer were available since 1993 and 1995, respectively. Vertical profiles of water temperature were carried out with multi-parameter probes (Idronaut Ocean Seven 401 and 316Plus, and Seabird SBE 19-03; Sharma et al., 2015). Water stability was estimated by the relative thermal resistance (RTR020), which was computed by the density difference in the strata 0–20 m compared to the density difference between 4 °C and 5 °C (Kallf, 2002); water densities were estimated from temperature values. Mixing depths at maximum overturn were estimated following Salmaso (2012). Light attenuation coefficients (K_d) were measured with a submersible irradiance sensor, LiCor 192SA. The euphotic depth was computed as $Z_{eu} = \ln(100) \times K_d^{-1}$ (Kirk, 1994). Nutrients (nitrate nitrogen, NO_3-N, and total and soluble reactive phosphorus, SRP and TP) and pH analyses were carried out using standard methods (Cerasino and Salmaso, 2012). Chlorophyll-*a*, corrected by pheopigments, was determined by spectrophotometry. Phytoplankton counting was carried out on subsamples preserved in acetic Lugol's solution following the Utermöhl method with a constant use of 10 mL sedimenting chambers. For every single taxon, biovolumes were calculated from recorded abundances and specific biovolumes approximated to simple geometrical solids. Species identification followed the more recent monographs of the series Süβwasserflora von Mitteleuropa, established by A. Pascher (Gustav Fisher Verlag, and Elsevier, Spectrum Akademischer Verlag). Further detailed information on the sampling and laboratory methods are reported in Rott et al. (2007), Salmaso and Cerasino (2012), and Salmaso (2012). In this work, relationships between *Dolichospermum* and the environmental variables between 0 and 20 m will be based on counts. Biovolumes will be however considered when evaluating the importance of *Dolichospermum* in the phytoplankton community.

Climatic variables were measured by the Istituto Agrario di S. Michele all'Adige at the meteorological station of Arco (91 m a.s.l.), which is approximately 5 km away from the northern border of the lake. Daily recorded variables (based on hourly frequency) included mean, minimum and maximum air temperatures (°C), atmospheric precipitations (mm) and solar radiation ($MJ\ m^{-2}\ d^{-1}$).

Teleconnection indices (North Atlantic Oscillation, NAO, and East Atlantic pattern, EA) were computed following Salmaso (2012).

2.3. Isolation of strains and culture conditions

Samples for the isolation of *Dolichospermum* strains in the DSL were collected by vertical tows from 20 to 30 m to the surface with 25 cm diameter 80 μm mesh plankton net. The sampling stations were located at the deepest point of the basins, as described in Section 2.2 and Salmaso et al. (2012). The isolates were identified by morphological criteria according to Komárek (2013). In Lake Garda, the isolation was made on samples collected in late autumn 2013 (November and December, 5 strains) and late spring 2014 (May and June, 2 strains), whereas in Lake Iseo samplings were carried out in summer 2014 (June and September, 7 strains). Strain isolation in lakes Como and Maggiore was made on samples collected in October (4 strains) and November 2014 (1 strain), respectively. Within 2 days, single filaments were isolated under a stereomicroscope (Leica M125) and a macroscope (WILD M420) using micropipettes. After washing 3–4 times, the single filaments were placed in microwell plates containing 3 mL Z8 medium (Kotai, 1972). After initial growth, the strains were transferred to 30 mL Z8 medium and, finally, to 150 mL medium Z8 CELLSTAR (Greiner Bio-One GmbH) cell culture flasks. The flasks were maintained at 20 °C under 16:8 h light:dark photoperiod (25 μmol m^{-2} s^{-1}). From each single culture, 250 mL were filtered with 0.45 μm GF/C filters (Whatman – GE Healthcare Life Sciences) for cyanotoxin and genetic analyses. The filters were frozen and stored at −20 °C until further processing. For genetic analysis, in addition to filtration, 50 mL from each culture were collected in Falcon tubes and frozen at −20 °C. After 2 cycles of freezing/thawing at 65 °C, samples were centrifuged at 4000 × *g* for 15 min. After discarding the supernatant, the pellet were centrifuged again for 5 min and finally stored at −20 °C for further analyses.

2.4. Cyanotoxins analyses

The quantification of toxins (MC, microcystins; ATX, anatoxin-a; NOD, nodularin; CYN, cylindrospermopsin) in cultures was performed by LC–MS analysis, using a Waters Acquity UPLC system coupled to a SCIEX 4000 QTRAP mass spectrometer equipped with a turbo ion spray interface. Quantitative analysis of 13 toxins was performed by means of calibration curves built using commercially available analytical standards MC (RR, [D-Asp3]-RR, YR, LR, [D-Asp3]-LR, WR, LA, LY, LW, LF), NOD-R, CYN, (Vinci Biochem), ATX (Tocris Cookson Ltd.). Semi-quantitative determination of other toxin variants (demethylated MC, deoxyCYN, HomoATX), was also conducted, assuming for these compounds analytical response factors similar to the reference compounds. The limits of quantitation (LOQ) were between 30 ng L^{-1} and 500 ng L^{-1} (MC congeners), 140 ng L^{-1} (NOD-R), 30 ng L^{-1} (ATX), and 8 ng L^{-1} (CYN). Detailed descriptions for toxins extraction and LC–MS protocols were reported in Cerasino and Salmaso (2012) and Shams et al. (2015).

2.5. DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from the filters using the Mo Bio PowerWater® DNA Isolation Kit (Mo Bio Laboratories, Inc., CA, USA) or from the pellet using Glass beads-acid washed (212–300 μm; 425–600 μm) (Sigma–Aldrich CO., MO, USA) and the E.Z.N.A. SP Plant DNA Kit (Omega Bio-Tek Inc., GA, USA). DNA concentrations, measured with a NanoDrop ND-8000 (Thermo Fisher Scientific Inc., MA, USA), were in the range 2–60 ng μL^{-1}. Phylogenetic analyses of *Dolichospermum* were carried out by the amplification of a 16S rRNA gene fragment (ca. 1700 bp) and *rpoB* gene (ca. 650 bp) encoding the β subunit of RNA polymerase. The PCRs were carried out on an Eppendorf Mastercycler ep (Eppendorf AG, Hamburg, Germany).

The 16S rRNA fragment was amplified with primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) (Edwards et al., 1989) and B23S (5′-CTTCGCCTCTGTGTGCCTAGGT-3′) (Lepère et al., 2000) following the protocol in Gkelis et al. (2005). *rpoB* was amplified with the primers rpoBanaF and rpoBanaR, following a protocol modified from Rajaniemi et al. (2005). The reaction mix (20 μl), contained 1X Optimized DyNAzyme PCR Buffer (Thermo Scientific), 0.25 mM dNTPs mix (Thermo Scientific), 0.5 μM of the two primers, 0.4 U of DyNAzyme II DNA Polymerase, and 2–20 ng of DNA templates. The cycling protocol included a denaturation step at 94 °C for 5 min; 30 cycles of DNA denaturation at 94 °C for 1 min, primer annealing at 59 °C for 1.5 min, and strand elongation at 68 °C for 2 min; final elongation step at 68 °C for 7 min. PCR products were checked and separated by 1% agarose gel electrophoresis stained with ethidium bromide. DNA sizes were preliminarily evaluated with a commercial DNA ladder (GeneRuler Express, Fermentas).

For DNA sequencing, PCR products were cleaned with Exonuclease plus Shrimp Alcaline Phosphatase (ExoSAP). The 16S rRNA gene was sequenced with the internal primers 16S544R, 16S1092R and 16S979F (Rajaniemi-Wacklin et al., 2006). The *rpoB* gene was sequenced with the same primers used in the PCR. The sequencing methods were described in Shams et al. (2015). DNA chromatograms were checked with Chromatogram Explorer 3.3.0 (Heracle Biosoft). Forward and reverse sequences were assembled using BioEdit 7.2.5 (Hall, 1999). Sequences were deposited to the European Nucleotide Archive (ENA) and analyzed with Megablast (NCBI) against 16S rRNA and *rpoB* gene sequences. Accession numbers are LN871456–LN871469 (16S rRNA) and LN871470–LN871488 (*rpoB*) (Table S1A).

The isolated strains were analyzed by PCR also for the presence of MCs (*mcyE*) and ATX encoding genes following the methods described in Shams et al. (2015). The presence of anatoxin-a synthetase gene (*anaC*) was determined using the primer pairs anaC-gen, and *Anabaena* 37 (UHCC) as positive control (Rantala-Ylinen et al., 2011).

2.6. Phylogenetic analyses

16S rRNA and *rpoB* sequences obtained from *Dolichospermum* strains isolated in the DSL were firstly checked with BioEdit 7.2.5. Excluding isolated single-nucleotide polymorphisms (SNP) in the 16S rRNA in Lake Maggiore and in the *rpoB* genes in lakes Iseo and Maggiore, the sequences looked identical, and 2 of them for each gene and lake were included in the phylogenetic analysis (1 for Lake Maggiore) (Table S1A). The successive analyses were done adding other 37 sequences of Nostocales from GenBank (Table S1B). Taxa were chosen in such a way as to have both 16S rRNA and *rpoB* sequences available in each taxon. We included both planktic and non-planktic species to evaluate on a wider genetic diversity spectrum the phylogenetic position of the new strains isolated from the DSL and to have a more robust basis to compare the two phylogenetic analyses based on the 16SrRNA and *rpoB*. Species were renamed following the most updated literature (Komárek, 2013; Guiry and Guiry, 2015). Most of the sequences were previously analyzed by Lyra et al. (2001) and Rajaniemi et al. (2005). *Microcystis aeruginosa* (KF286992 and EU151907) was included as outgroup. Sequences were aligned with MUSCLE (Edgar, 2004; R Core Team, 2015) and poorly aligned positions and divergent regions of the alignments were checked and eliminated using Aliscore (Misof and Misof, 2009; R Core Team, 2015). Phylogenetic trees were computed by Maximum Likelihood (ML) using PhyML 3.1 (Guindon et al., 2010); branch support was estimated by the approximate likelihood-ratio test (aLRT;

Anisimova and Gascuel, 2006). DNA substitution models were analyzed, calling PhyML 3.1, with the phymltest in the R package ape (Paradis, 2012); GTR+G+I and K80+G+I were found to be the best-fitting evolutionary models for the 16S rRNA and *rpoB* genes, respectively. The Newick rooted trees obtained with PhyML 3.1 were analyzed and plotted with the R package ape.

2.7. Statistical analyses

The long-term trend of *Dolichospermum* abundances was estimated by a seasonal decomposition of the time series by loess (function stl in R; R Core Team, 2015).

To highlight the periods most favorable for the development of *Dolichospermum lemmermannii*, a Principal Components Analysis (PCA) was calculated from the correlation matrix obtained from the monthly epilimnetic averages of selected environmental and biotic variables. PCA was computed including only the period for which full environmental and biological data were available (1995–2013).

The monthly abundances of *Dolichospermum lemmermannii* were related to the monthly values of the environmental variables listed in Section 2.2. As for the daily climatic variables (air temperatures, rain and solar radiation), we considered the mean values recorded during the 3 and 7 days before the sampling dates. Considering that the counts of *D. lemmermannii* were characterized by a high number of zeros (63%), the relationships were analyzed using zero-inflated negative binomial (ZINB) models (Zuur et al., 2009) with the package pscl in R (Zeileis et al., 2008). The ZINB models were compared, and also tested against ZIP (zero-inflated Poisson) models, based on AIC and computing a likelihood ratio test (Zeileis and Torsten, 2002; Zeileis et al., 2008).

Besides monthly frequency, and to evaluate the existence of possible long-term trend effects, the relationships between the same variables described above were also analyzed based on the annual means and on the seasonal means computed for the period of maximum *Dolichospermum* development (June–October). Analyses were based on Pearson and Spearman correlation coefficients; p-values in multiple comparisons were adjusted by the BH (Benjamini and Hochberg, 1995) method. Statistical analyses were made with R (R Core Team, 2015).

3. Results

3.1. First records of Dolichospermum in the DSL

In the deep lakes south of the Alps, *Dolichospermum lemmermannii* was identified for the first time in Lake Garda at the beginning of the 1990s in the form of extended pelagic blooms between July and September (Salmaso, 2000). Following an altitudinal gradient, similar summer blooms of this species made their appearance also in lakes Iseo (second half of the 1990s), Maggiore (2005), and Como (2006). Whereas the first blooms of *D. lemmermannii* in Lake Maggiore were identified at the end of its long-term oligotrophication process (oligotrophy; Callieri et al., 2014), in the other lakes the first appearance of this species was detected when the trophic status was between oligo-mesotrophy and meso-eutrophy (Mosello et al., 2010). The surface blooms were recorded exclusively during the warmest months, between June and early autumn, in the pelagic zones or in sheltered areas (Fig. S2). The formation of the blooms was quite rapid, with irregular daily dynamics and patches that sometimes, during sunny days with lighter winds, were concentrated along the shores. Blooms developed in the first centimeters of the water column, reaching values well over 100000 cells mL^{-1} (e.g. Salmaso, 2000; Callieri et al., 2014). After the recording of these first episodes, measurable quantities of *Dolichospermum* were also detected in the epilimnetic samples collected for phytoplankton analyses.

3.2. Phylogenetic analyses of strains isolated in the DSL

The morphometrical and morphological characteristics of the *Dolichospermum* specimens recorded in the DSL were consistent with the diacritical features of *Dolichospermum lemmermannii*, as resumed in Komárek (2013) (Fig. S3). An important feature, though not always identifiable in every single filament, was the formation of akinetes at both sides of heterocytes (Salmaso et al., 1994). In Lake Garda, where several strains and natural samples were analyzed, the average length of vegetative cells, akinetes and heterocytes was (mean ± SD) 8.6 ± 0.9 μm (n = 40), 18.4 ± 2.7 μm (n = 40), and 8.1 ± 0.7 μm (n = 20), respectively.

The microscopic determination of the isolated filaments was supported by phylogenetic analyses based on 16S rRNA and *rpoB* genes (Fig. 1). The 16S rRNA sequences from the DSL formed a compact clade with other 2 strains of *Dolichospermum lemmermannii* isolated in Finland (Fig. 1A). This group was closely linked with a cluster containing *Dolichospermum flos-aquae*. In the ML tree based on *rpoB* sequences, the distinction between these two species was less apparent, whereas, though clearly included in the cluster, the strain of *D. lemmermannii* isolated in Lake Maggiore was separated from the other DSL strains (Fig. 1B). Based on the *rpoB* sequences, the clade containing *D. lemmermannii* included also *D. flos-aquae* and *Dolichospermum smithii*.

3.3. Factors promoting the seasonal development of Dolichospermum in Lake Garda

The main physical, chemical and biological features of Lake Garda in the layer 0–20 m are reported in Table 1. Water temperatures ranged between 7.7 °C and 23.3 °C. The largest stability (RTR020) of the water column was between May and September. The euphotic depth was always high and typical of deep and clear lakes (13–46 m). TP and SRP were always below 25 μg P L^{-1} and 19 μg P L^{-1}, respectively. Both compounds reached low concentrations during the warmest months, with values around the detection limits in the case of SRP (Fig. 2A). Similarly, in late summer, nitrates reached values below 100–150 μg N L^{-1} (Fig. 2B). NO_3-N was the most abundant nitrogen compound, whereas ammonium rarely reached maximum peaks around 30 μg N L^{-1} (Salmaso, 2000). Since the beginning of the observations, in the epilimnetic layers (0–20 m) the abundances of *Dolichospermum lemmermannii* in Lake Garda showed a little tendency to increase until the beginning of 2000s (Fig. 2C). The temporal development was characterized by a strong cyclical component, with most of the population developing in the warmest months, generally between June and October (Fig. 3). Nevertheless, after 2004, the growth of *Dolichospermum* between July and August underwent a strong decrease, with the population mostly developing in early summer and early autumn (Fig. 3). Excluding a peak measured in July 2012, the biovolume values of *Dolichospermum* were always low (<50 mm^3 m^{-3}) contributing for only a very tiny fraction to the total annual phytoplankton and cyanobacterial biovolumes (usually less than 1% and 5%, respectively).

The PCA results summarize clearly the relationships among the environmental and biotic variables (Fig. 4). The graphs show the first 2 axes, which explain 91% of the total variance (72% and 19%, respectively). The first axis had the highest positive loadings for water temperature, thermal stability, pH, as well as chlorophyll-a and total phytoplankton biovolume, and negative loadings for euphotic depth, SRP and nitrogen. The second axis had positive and negative loadings partially contributed by water temperature and

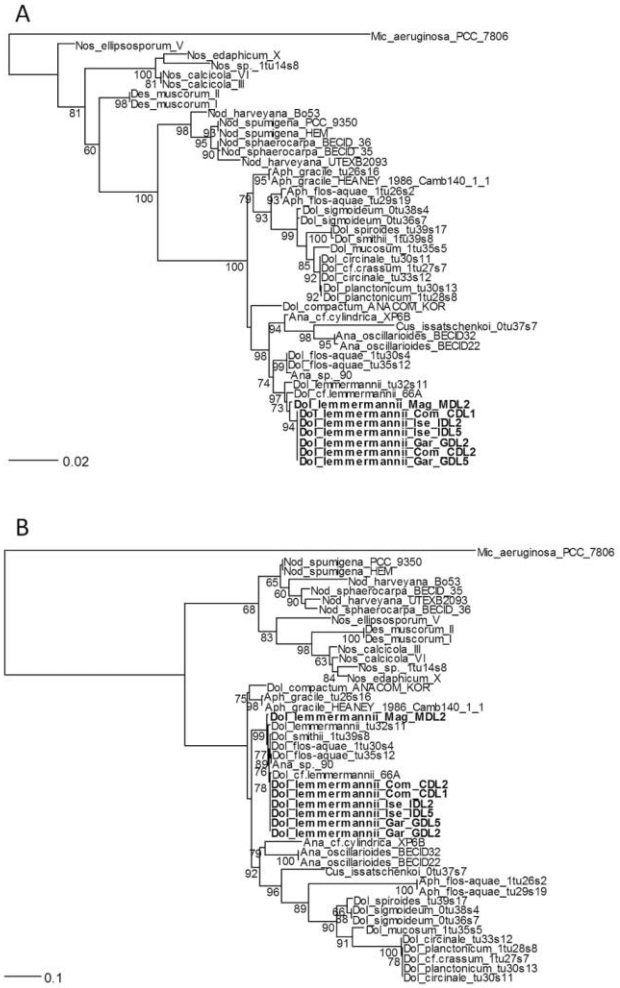

Fig. 1. Maximum likelihood (ML) rooted topology of different species of Nostocales based on alignment of (A) 16S rRNA and (B) *rpoB*. The strains isolated in the lakes Garda, Maggiore and Iseo are highlighted in bold. The list of accession numbers is reported in Table S1. aLRT branch support values <60 were not shown.

phytoplankton abundances, respectively. The single months followed a clear cyclic seasonal pattern (Fig. 4A). The superimposition of the mean monthly abundances to the PCA ordination confirmed the strong temporal delimitation of the development of *Dolichospermum lemmermannii* (Fig. 4B). The higher growth of this species was favored by higher water temperatures and thermal stability. Conversely, *D. lemmermannii* showed high tolerance toward lower availability of light, nutrients, and CO_2, and higher development of other phytoplankton groups.

Both the seasonal development (Fig. 2C) and the results of PCA (Fig. 4) indicate the essential role played by water temperature and thermal structure in the development of *Dolichospermum lemmermannii*. The higher development of this species occurred when water temperatures were above around 15 °C (Fig. 5). After

Table 1
Main physical, chemical and biological features of Lake Garda in the layer 0–20 m. (A) Averages and ranges (minimum and maximum values) over 12 months (1993–2013). (B) Averages and ranges computed for the period of maximum *D. lemmermannii* growth (June–October).

	A		B	
	Mean	Range	Mean	Range
Water temperature (°C)	13.8	7.7–23.3	18.7	13.3–23.3
RTR020	51	−11–282	108	−1–282
SRP (μg P L^{-1})	4	<1–19	2	<1–6
TP (μg P L^{-1})	11	4–24	9	4–16
NO_3-N (μg N L^{-1})	226	57–396	152	57–277
pH	8.36	7.95–8.83	8.50	8.10–8.83
Secchi disk depth (m)	10.2	3.0–25.0	6.9	3.0–12.5
Z_{eu} (m)	23.9	12.7–46.2	19.4	12.7–26.4
Chlorophyll-*a* (μg L^{-1})	3.0	0.6–11.3	3.4	0.8–11.3
Total biovolume (mm^3 m^{-3})	943	178–5314	1210	178–5315
Cyanobacteria (mm^3 m^{-3})	130	1–996	169	1–936
D. lemmermannii (mm^3 m^{-3})	4	0–202	8	0–202

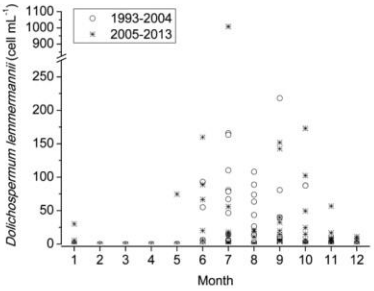

Fig. 3. Monthly distribution of the density of *D. lemmermannii* in Lake Garda.

2005, water temperatures from May to July begun to decrease (Fig. 5A), and this was followed by a decrease of *Dolichospermum* (Fig. 5B), especially from July to August (see also Fig. 3). The direct comparison between water temperature and the development of *D. lemmermannii* showed a strong increase of abundances and a wider spread of values above 15 °C. The application of a Zero Inflated Model confirmed the strong effect of water temperature, which was statistically significant in both the count and inflation portions of the model (Fig. 6; Table 2A). The AIC values and the likelihood ratio test suggested that the ZINB model was a significant improvement over a Zero inflated Poisson model. The remaining single variables (nutrients, Z_{eu}, pH, air temperatures, atmospheric precipitations and solar radiation) did not show any relationship with *Dolichospermum* abundances. A further significant relationship was identified between the *Dolichospermum* abundances, and water temperatures and solar radiation (Table 2B).

As for the mean annual and seasonal (June–October) values, and without the BH adjustments, the only potential relationships were found between the square root transformed abundances of *Dolichospermum lemmermannii* and the atmospheric precipitations ($p < 0.05$), and between the *Dolichospermum* abundances and the water temperatures in the mixolimnetic layer (0–50 m) ($p < 0.05$). Nevertheless, after the BH adjustments, correlations based on the annual and seasonal means did not provide any significant results, so the two above relationships should be interpreted very cautiously.

Contrary to what was observed for the dominant cyanobacterium developing in Lake Garda (*Planktothrix rubescens*; Salmaso and Cerasino, 2012), the abundance of *Dolichospermum lemmermannii*

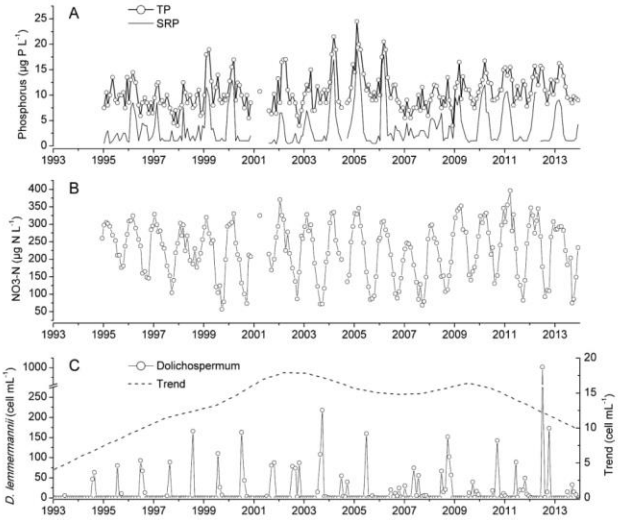

Fig. 2. Long-term trends (1993–2013) in the epilimnetic layer of Lake Garda (0–20 m). (A) Total phosphorus (TP) and soluble reactive phosphorus (SRP). (B) Nitrate nitrogen (NO_3-N). (C) *Dolichospermum lemmermannii* abundances; the long-term trend (dashed line) was estimated excluding the outlier recorded in July 2012.

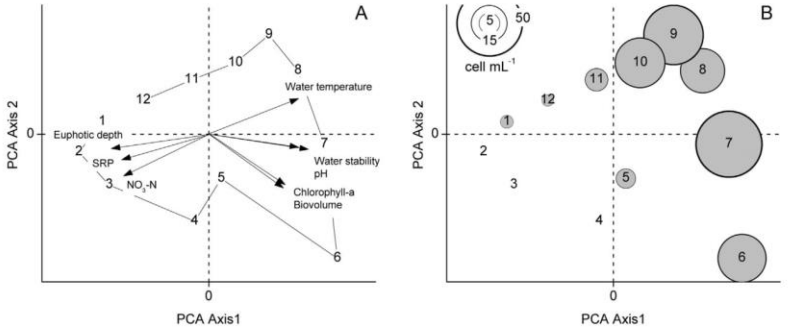

Fig. 4. (A) PCA biplot summarizing the relationships among the monthly average values of the environmental and biotic variables in Lake Garda. (B) Monthly distribution of *Dolichospermum* abundances in the PCA plane; the radius of the disks were transformed by square root. The numbers 1–12 indicate the months.

in the period of its maximum growth (June–October) did not show any dependence from the spring replenishment of nutrients and the related winter climatic and environmental variables (winter East Atlantic pattern, air and water temperatures, and mixing depth).

3.4. Toxicity

None of the 19 *Dolichospermum lemmermannii* strains isolated from the large lakes south of the Alps were positive for the presence of MC, ATX, HomoATX, NOD, CYN and deoxyCYN evaluated by LC–MS. These results were also confirmed by the absence of *mcyE* and *anaC* PCR amplicons in all the cultures analyzed.

4. Discussion

The results obtained in this work have allowed for the first time to verify and confirm the taxonomic position of the *Dolichospermum* species which invaded the large lakes south of the Alps between the 1990s and 2000s. As demonstrated in previous investigations, the taxonomy of coiled *Dolichospermum* species based on morphological characteristics cannot provide clear-cut boundaries to identify species. More specifically, the variability of morphological features of various natural populations of *Dolichospermum lemmermannii* was demonstrated to be quite wide (Komárková, 1988; Komárek and Zapomělová, 2007; Zapomělová et al., 2007). The phylogenetic analyses clearly grouped together the *D. lemmermannii* isolated in the DSL with those isolated in

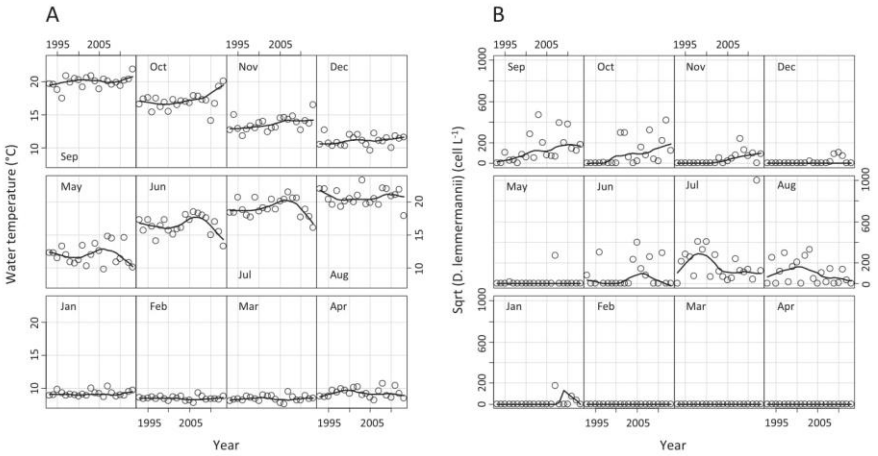

Fig. 5. Coplots showing the temporal development of (A) water temperatures and (B) densities of *D. lemmermannii* (square rooted) in the 0–20 m layer of Lake Garda. The coplot panels are ordered from lower-left (January) to upper right (December).

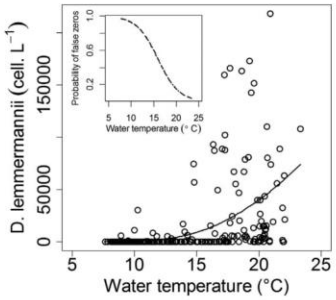

Fig. 6. Zero inflated negative binomial model (ZINB). The main graph reports the relationships between the abundances of *D. lemmermannii* and the water temperatures in the layer 0–20 m in Lake Garda. The fitted curve for the count model is reported with a continuous line. The inset reports the fitted curve for the logistic regression model.

Northern Europe. However, though clearly included in the same clades, a few peculiarities emerged, due to a few differences (<20 bp) in the 16S rRNA sequences of northern European strains, and in the slight separation of the Lake Maggiore and north European strains from the other DSL strains in the *rpoB* ML tree. These observations, especially for the *rpoB* sequences, cannot be further investigated due to an insufficient number of strains in genetic sequence databases.

The first *Dolichospermum* populations in the DSL were identified due to the sudden appearance of extended water blooms that, until then, were never observed in the pelagic zones and along the shores (Salmaso et al., 1994; Salmaso, 2000). Though at least a few inocula were already present in the pelagic zone before the first identification of the blooms, it is undeniable that these episodes reflected a strong increase of populations in Lake Garda and in the DSL during and after the 1990s. Nevertheless, apart from these aspects, the question concerns the reason of the appearance and increased growth of *Dolichospermum* in the DSL. Many investigations have described the presence and ecology of this species in the northern European regions, including the Boreal latitudes (see Fig. S1A). For example, in a survey carried out in several lakes in the South of Norway during 1989–1991, Skulberg et al. (1994) showed that, among the *Anabaena* (*Dolichospermum*) species, *Dolichospermum lemmermannii* and *Dolichospermum flos-aquae* were the most abundant (frequency >80%), dominating, with toxic producing strains, in nearly 50% of the blooms. Investigating cyanobacteria in the Finnish boreal and arctic lakes, Lepistö and Holopainen (2008) showed that *D. lemmermannii* was the most frequently recorded species, with populations developing at low densities in large, oligotrophic clear water lakes. Similar results were found, investigating the Swedish forest lakes, by Willén (2003). Most of the successive records of this species were made after the 1990s, always between the 40th and 60th parallels (Fig. S1A). Therefore, the habitat of *D. lemmermannii* looks circumscribed to cold and temperate, oligo-mesotrophic environments. The spread toward south of this species is less straightforward to interpret compared with other warm-adapted Nostocales whose expansion toward north was explained by the recent widening of new suitable habitats caused by global warming (Sukenik et al., 2012).

The distribution of *Dolichospermum lemmermannii* in the 20° latitude range could be partly explained by the existence of different ecotypes characterized by specific ecological adaptations. By experimentally investigating the temperature and light optima of different *Dolichospermum* populations, Zapomělová et al. (2009) described two strains of *D. lemmermannii* with optimum ranges of temperature spanning from 13.5 °C to 18.5 °C, and from 18.5 °C to 25.5 °C. The same strains presented light optima for growth around 190–270 μmol m^{-2} s^{-1} and 120–300 μmol m^{-2} s^{-1}, respectively. These results suggested a wide adaptability of this species in temperate and subarctic regions, and in oligo-mesotrophic clear and non turbid lakes. At present, based on the scholarly databases (Thomson ISI and Scopus) this species was never found in warm environments. The southernmost site where this species was described is Lake Prespa (ca. 41° N), which is located at over 850 m a.s.l. in a mountain area. Water temperatures in this lake typically ranged between 1.8 °C and 26.6 °C (Tryfon et al., 1997), therefore with minimum values well below than those found in Lake Garda (Table 1) and in the other DSL (Salmaso et al., 2012).

The habitat template of *Dolichospermum lemmermannii* has been well characterized (Padisák et al., 2008; Reynolds et al., 2002). This species is considered typical of oligo-mesotrophic and deep stratifying lakes, or mesotrophic shallow lakes, with good light conditions. These peculiarities conform to the present development of *D. lemmermannii* in the DSL. In Lake Garda, it may be hypothesized that the increase of this species can be ascribed to the increase of nutrients, and to the shift of the lake from ultra-oligotrophy to oligotrophy and oligo-mesotrophy during the 1960s and 1970s (Milan et al., 2015). This hypothesis is supported by analyses made on sub-fossil akinetes conserved in the deep sediments, which allowed estimating the first appearance of populations of *D. lemmermannii* in Lake Garda during the 1960s (Salmaso et al., unpublished data). Nevertheless, eutrophic and hypertrophic conditions can favor the development of other cyanobacteria. For example, in Southern Sweden, in Lake Ringsjon, *D. lemmermannii* made its appearance in summer, after a long and slow increase in nutrient concentrations (Cronberg, 1999). After the lake had become eutrophic/hyper-eutrophic, *Dolichospermum*

Table 2
Zero inflated negative binomial (ZINB) coefficients. Relationships between the *Dolichospermum lemmermannii* abundances (0–20 m) and (A) water temperatures, and (B) water temperatures and solar radiation. Temp, water temperature (0–20 m); Rad, solar radiation measured during the three days before the sampling dates.

A

	Estimate	Std. error	*p*
Count model coefficients (negative binomial with log link)			
Intercept	7.78	0.93	***
Water temperature	0.15	0.05	**
log(theta)	−0.62	0.13	***
Zero-inflation model coefficients (binomial with logit link)			
Intercept	6.78	0.77	***
Water temperature	−0.43	0.05	***

B

	Estimate	Std. error	*p*
Count model coefficients (negative binomial with log link)			
Intercept	5.38	1.27	***
Water temperature	0.28	0.08	***
Solar radiation	0.31	0.13	*
Temp:Rad	−0.02	0.01	*
log(theta)	−0.58	0.13	***
Zero-inflation model coefficients (binomial with logit link)			
Intercept	3.75	1.50	*
Water temperature	−0.25	0.11	*
Solar radiation	0.37	0.16	*
Temp:Rad	−0.02	0.01	*

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

was replaced by *Microcystis* spp. After a nutrient reduction program, the lake returned back to eutrophy, and the *Microcystis* spp. were replaced by other *Dolichospermum* spp., and *Aphanizomenon* spp.

Until now, the contribution of *Dolichospermum* to the total biovolumes of phytoplankton and cyanobacteria in the trophogenic layers (0–20 m) was always very low. In the period of the maximum development of *Dolichospermum*, between June and October, the dominant species are represented by chlorophytes (*Mougeotia* sp. and Chlorococcales) and, among cyanobacteria, *Planktothrix rubescens* (D'Alelio et al., 2013). As for this last species, the depth and the trophic status of this clear lake represent optimal conditions for the development of summer metalimnetic populations.

The low abundances of *Dolichospermum* represent a challenge for the interpretation of the seasonal development of this species and, considering the high number of zeros, for the statistical evaluation of data. Nevertheless, some indications emerged, connecting the seasonal development of this species primarily to water temperature. At the lower temperatures, the excessive number of zeros (Fig. 6; Table 2A) were mostly due to repeatedly counting *Dolichospermum* during unsuitable environmental conditions for growth. Nevertheless, a fraction of zeros originated also from the difficulty to detect cells (when present, e.g. summer) at very low abundances. On the other side, the negative interaction between water temperature and solar radiation (Table 2B) appeared to suggest a lower development of this species during both persistent sunny and warm periods. These conditions could represent a stress factor increasing competition among species in persistent low-disturbance phases. We could hypothesize that, in summer, the development of *Dolichospermum* (and other phytoplankton taxa) in the DSL is hampered by constant low concentrations of phosphorus in the euphotic layers. Disturbance episodes decreasing water stability and favoring nutrient pulses therefore could support a delayed growth of species. Nevertheless, we did not find positive relationships between *Dolichospermum* and the total amount of rain during the 3 and 7 days before the sampling dates. The existence of such relationships were suggested for Lake Maggiore, where Callieri et al. (2014) indicated a connection between the *Dolichospermum* blooms and precipitation, lake water level fluctuations and P pulses. Conversely, owing to its N-fixing abilities, the low late-summer concentrations of N in lake Garda could have favored the development of *Dolichospermum* (see, e.g., Padisák et al., 2003). At present, however, we did not find any correlative support for this relationship in Lake Garda.

As it was described for this kind of gas-vacuolated species (Reynolds and Walsby, 1975), surface blooms are caused by a quick amassing of filaments already existing in the water column, providing a false idea of huge cyanobacterial abundance and growth in the surface waters. Considering that generally the maximum seasonal values of *Dolichospermum* cells in the first 20 m were between around 50 cell mL^{-1} and 200 cell mL^{-1} (Fig. 2C), a rapid accumulation of these dispersed filaments in thin layers of 2 cm localized at the surface of the lake would give a concentration around 50,000 and 200,000 cell mL^{-1}. These numbers are in line with the density estimates carried out on surface samples collected during the blooms in lakes Garda and Maggiore (Salmaso, 2000; Callieri et al., 2014). Moreover, the rapid accumulations were compatible with the upward velocity of the filaments of *Dolichospermum lemmermannii* (e.g. 0.7–0.9 m h^{-1}; Walsby et al., 1991). For these reasons, these episodes are referred to as "oligotrophic blooms" (Salmaso, 2000). The essential point to take into consideration is that the blooms are the result of a physical surface accumulation of filaments already present in the trophogenic layers, and not the effects of in situ growth processes. The environmental conditions triggering the blooms include the existence of a suitable number of gas-vacuolated filaments, suitable meteorological conditions, and a stable water column (Reynolds and Walsby, 1975). Though partially coincident, these environmental conditions, not considered in this work, should be distinguished from the conditions favoring the development of *Dolichospermum* in the trophogenic layers.

The strains of *Dolichospermum lemmermannii* isolated from the DSL tested negative for the production of MC, ATX, NOD and CYN. Similar results, with negative ATX and CYN detection, were found in strains isolated in Northern Spain (Cirés et al., 2014). Conversely, in Northern Europe, isolates of *D.* (*Anabaena*) *lemmermannii* were shown to produce MC (Sivonen et al., 1992; Savela et al., 2015). In Finland, Rapala et al. (2005) associated the presence of saxitoxin (STX) in cyanobacterial blooms to dominant populations of *D. lemmermannii*. However, recent analyses made on isolated strains did not show the capability of this species to synthesize STX, which was also confirmed by the absence of *sxtA*, *sxtG* and *sxtB* PCR amplicons in the cultures (Cirés et al., 2014; Savela et al., 2015). Though based on a very few and exclusive reports in Danish lakes (Henriksen et al., 1997; Onodera et al., 1997), anatoxin-a(S) (ATX-a(S)) in natural blooms and cultures of *D. lemmermannii* was identified as the cause of bird kills. In our work, STX and ATX-a(S) were not quantified because analytical protocols were still under evaluation. Based on these considerations, we cannot still exclude the possible presence of toxic strains testing positive for STX or ATX-a(S) in Lake Garda and in the DSL. In the end, the toxicity of the populations developing in these lakes should be evaluated also by toxicity tests (e.g. Marsálek and Bláha, 2004; Törökné et al., 2007). The low concentration of the cells in the water column seems to indicate that the potential toxicity of this population should not be high, except in the accumulations in scums. For certain, and as demonstrated from the analysis of isolated strains and environmental samples, the consistent concentrations of MC and ATX detected in Lake Garda were due to the development of *Planktothrix rubescens* and *Tychonema bourrellyi*, respectively (Salmaso et al., 2014; Shams et al., 2014, 2015).

Though developing in low numbers, owing to its ability to form surface water blooms, *D. lemmermannii* is considered as one of the most nuisance algae in the DSL. The appearance of the blooms in summer, when the tourist visits are at their seasonal maximum, undermines the attractiveness of lakes, considerably influencing their value. Water users are less likely to swim and to have sport activities in lakes during heavy algal blooms, and this can cause large potential economic impacts (Dodds et al., 2009). Actually, since the 1960s, the number of daily visits of tourists in Lake Garda has enormously increased until it reached, in the recent period, more than 20 millions every year (Comunità del Garda, 2013). The development of tourism in this lake district represents one of the pillars of the regional and national economy. The only strategy to avert the further proliferation of *Dolichospermum*, and frequency and intensity of water blooms, is to further reduce the nutrient loading to the lake.

5. Conclusions

Between the 1990s and 2000s, the largest lakes south of the Alps experienced a rapid settlement of *Dolichospermum lemmermannii*. In each lake, populations of this species appeared suddenly with extended surface water blooms in the pelagic zones and along the shores. In this work, phylogenetic analyses based on the 16S rRNA and *rpoB* genes allowed to clarify the taxonomic identity of the strains of *Dolichospermum* colonizing the southern subalpine district. The geographic areal of *D. lemmermannii* appeared quite delimited, between the 40th parallel and the Arctic Circle. The spread of this species in the DSL was in contrast with the prevalent south to north dispersion routes characterizing

other Nostocales (e.g. *Cylindrospermopsis* and *Aphanizomenon*), highlighting the impossibility to generalize ecological processes and characteristics even at the level of cyanobacterial orders. The seasonal development of *D. lemmermannii* in the largest of the DSL (Lake Garda) was narrowly limited to the warmest months (>15 °C). Although contributing very little to the total epilimnetic phytoplankton biovolume, *D. lemmermannii* is considered as one of the most nuisance algae in the DSL due to its ability to form rapid and extended surface water blooms during the tourist season. However, different strains isolated from the DSL tested negative for MC, ATX, NOD and CYN, and for the presence of *mcyE* and *anaC* amplicons. The toxicity of strains isolated from the DSL will be evaluated also testing other toxins that could be potentially produced by *D. lemmermannii*.

Acknowledgements

The first ideas of this paper were presented at the ASLO Aquatic Sciences Meeting, Granada (Spain), 22–27 February 2015. Investigations in Lake Garda and in the other DSL were made in the framework of the LTER (Long Term Ecological Research) Italian network, site "Southern Alpine lakes", IT08-000-A (http://www.lteritalia.it/). In particular, we want to thank our colleagues G. Morabito (CNR-ISE), F. Buzzi (ARPA Lombardia) and B. Leoni (University of Milan). The activity was supported by PhD fellowships to C.C. and S.S. from the E. Mach Foundation – Istituto Agrario di S. Michele all'Adige. We thank the European Cooperation in Science and Technology COST Action ES1105 CYANOCOST for networking and knowledge-transfer support, and K. Sivonen (University of Helsinki) for support to S.S. in a STSM COST (CYANOCOST) mission, and for providing *Anabaena* 37 (UHCC) as *anaC-gen* positive control. We are grateful to our colleagues in FEM, in particular A. Boscaini and A. Zampedri, for their support in the field and laboratory activities, and to the ARPA Veneto (G. Franzini) for logistic support in the field.[CG]

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2015.09.008.

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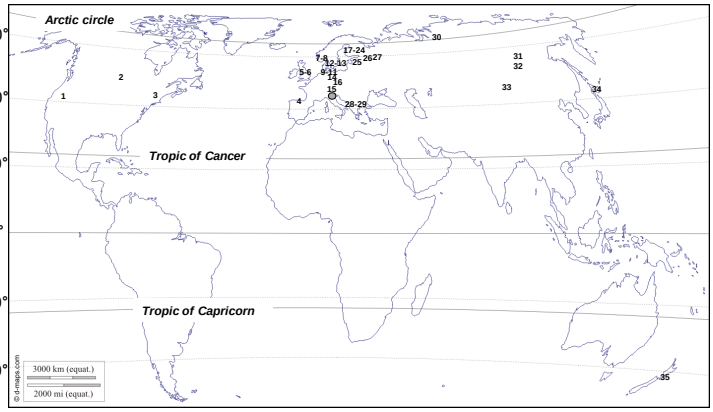
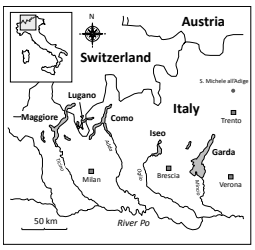
Supplementary data

Figure S1. (A) Reports of *Dolichospermum* (*Anabaena*) *lemmermannii*. (B) Map showing the location of the deep southern subalpine lakes (DSL). (C) Morphometrical characteristics and theoretical renewal time of the DSL. Huge surface blooms of *D. lemmermannii* were identified in lakes Garda, Iseo, Como and Maggiore.

(A)



(B)



(C)

	Altitude m	S km²	z_{max} m	V km³	τ_w yr
Garda	65	368	350	49.0	26.6
Iseo	186	62	251	7.6	4.1
Como	198	146	410	22.5	4.5
Lugano N	271	28	288	4.7	12.4
Maggiore	193	213	370	37.5	4.0

Figure S2. Surface blooms of *Dolichospermum lemmermannii* in Lake Garda. (A) NW basin, pelagic zone, 30 July 2002. (B) Harbour of Riva del Garda (northern shore), 7 October 2014.

(A) (B)

Table S1. (a) Accession numbers of the strains isolated in lakes Garda, Iseo, Como and Maggiore. The strains included in the phylogenetic analyses were indicated with an asterisk. (b) Accession numbers of additional taxa included in the phylogenetic analyses.

(A)				
Cyanobacterial taxon	16SrRNA	*rpoB*	Strain	Lake, sampling date
Dolichospermum lemmermannii *	LN871456	LN871470	FEM_GDL2	Garda, 5 Nov 2013
Dolichospermum lemmermannii	LN871457	LN871471	FEM_GDL9	Garda, 5 Nov 2013
Dolichospermum lemmermannii	LN871458	LN871472	FEM_GDL11	Garda, 5 Nov 2013
Dolichospermum lemmermannii	LN871459	LN871473	FEM_GDL8	Garda, 3 Dec 2013
Dolichospermum lemmermannii	LN871460	LN871474	FEM_GDL10	Garda, 3 Dec 2013
Dolichospermum lemmermannii	LN871461	LN871475	FEM_GDL1	Garda, 6 May 2014
Dolichospermum lemmermannii *	LN871462	LN871476	FEM_GDL5	Garda, 3 Jun 2014
Dolichospermum lemmermannii *	LN871463	LN871477	FEM_IDL5	Iseo, 24 Jun 2014
Dolichospermum lemmermannii	LN871464	LN871478	FEM_IDL7	Iseo, 24 Jun 2014
Dolichospermum lemmermannii		LN871479	FEM_IDL1	Iseo, 9 Sep 2014
Dolichospermum lemmermannii *	LN871465	LN871480	FEM_IDL2	Iseo, 9 Sep 2014
Dolichospermum lemmermannii		LN871481	FEM_IDL4	Iseo, 9 Sep 2014
Dolichospermum lemmermannii		LN871482	FEM_IDL13	Iseo, 9 Sep 2014
Dolichospermum lemmermannii		LN871483	FEM_IDL17	Iseo, 9 Sep 2014
Dolichospermum lemmermannii *	LN871466	LN871484	FEM_CDL1	Como, 20 Oct 2014
Dolichospermum lemmermannii *	LN871467	LN871485	FEM_CDL2	Como, 20 Oct 2014
Dolichospermum lemmermannii	LN871468	LN871486	FEM_CDL3	Como, 20 Oct 2014
Dolichospermum lemmermannii		LN871487	FEM_CDL4	Como, 20 Oct 2014
Dolichospermum lemmermannii *	LN871469	LN871488	FEM_MDL2	Maggiore, 11 Nov 2014

(B)			
Cyanobacterial taxon	16SrRNA	*rpoB*	Taxon code
Anabaena cf.*cylindrica*	AJ630414	AJ628074	Ana_cf.cylindrica_XP6B
Anabaena oscillarioides	AJ630426	AJ628086	Ana_oscillarioides_BECID22
Anabaena oscillarioides	AJ630427	AJ628087	Ana_oscillarioides_BECID32
Anabaena sp.	AJ133156	AJ628124	Ana_sp._90
Aphanizomenon flos-aquae	AJ630441	AJ628101	Aph_flos-aquae_tu29s19
Aphanizomenon flos-aquae	AJ630443	AJ628103	Aph_flos-aquae_1tu26s2
Aphanizomenon gracile	AJ630444	AJ628104	Aph_gracile_HEANEY_1986_Camb140_1_1
Aphanizomenon gracile	AJ630445	AJ628105	Aph_gracile_tu26s16
Cuspidothrix issatschenkoi	AJ630446	AJ628106	Cus_issatschenkoi_0tu37s7
Desmonostoc muscorum	AJ630451	AJ628111	Des_muscorum_I
Desmonostoc muscorum	AJ630452	AJ628112	Des_muscorum_II
Dolichospermum cf.*crassum*	AJ630413	AJ628073	Dol_cf.crassum_1tu27s7
Dolichospermum circinale	AJ630416	AJ628076	Dol_circinale_tu30s11
Dolichospermum circinale	AJ630417	AJ628077	Dol_circinale_tu33s12
Dolichospermum compactum	AJ630418	AJ628078	Dol_compactum_ANACOM_KOR
Dolichospermum flos-aquae	AJ630422	AJ628082	Dol_flos-aquae_1tu30s4
Dolichospermum flos-aquae	AJ630423	AJ628083	Dol_flos-aquae_tu35s12
Dolichospermum cf.*lemmermannii*	AJ133157	AJ628126	Dol_cf.lemmermannii_66A
Dolichospermum lemmermannii	AJ630424	AJ628084	Dol_lemmermannii_tu32s11
Dolichospermum mucosum	AJ630425	AJ628085	Dol_mucosum_1tu35s5
Dolichospermum planctonicum	AJ630430	AJ628090	Dol_planctonicum_1tu28s8
Dolichospermum planctonicum	AJ630431	AJ628091	Dol_planctonicum_tu30s13
Dolichospermum sigmoideum	AJ630434	AJ628094	Dol_sigmoideum_0tu36s7
Dolichospermum sigmoideum	AJ630435	AJ628095	Dol_sigmoideum_0tu38s4
Dolichospermum smithii	AJ630436	AJ628096	Dol_smithii_1tu39s8
Dolichospermum spiroides	AJ630440	AJ628100	Dol_spiroides_tu39s17
Nodularia harveyana	AJ781143	AJ783325	Nod_harveyana_Bo53
Nodularia harveyana	AJ781148	AJ783330	Nod_harveyana_UTEXB2093
Nodularia sphaerocarpa	AJ781147	AJ783323	Nod_sphaerocarpa_BECID_36
Nodularia sphaerocarpa	AJ781149	AJ783332	Nod_sphaerocarpa_BECID_35
Nodularia spumigena	AJ781134	AJ628132	Nod_spumigena_HEM
Nodularia spumigena	AJ781131	AJ783307	Nod_spumigena_PCC_9350
Nostoc calcicola	AJ630447	AJ628107	Nos_calcicola_III
Nostoc calcicola	AJ630448	AJ628108	Nos_calcicola_VI
Nostoc edaphicum	AJ630449	AJ628109	Nos_edaphicum_X
Nostoc ellipsosporum	AJ630450	AJ628110	Nos_ellipsosporum_V
Nostoc sp.	AJ630453	AJ628113	Nos_sp._1tu14s8
Microcystis aeruginosa	KF286992	EU151907	Mic_aeruginosa_PCC_7806

IV.

Advances in Oceanography and Limnology, 2015; 6(1/2): 33-45
DOI: 10.4081/aiol.2015.5456

ORIGINAL ARTICLE

Historical colonisation patterns of *Dolichospermum lemmermannii* (Cyanobacteria) in a deep lake south of the Alps

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ABSTRACT

Since the beginning of the 1990s, Lake Garda showed the appearance of extended surface water blooms of *Dolichospermum lemmermannii* (Nostocales, Cyanobacteria). Between the 1990s and the 2000s, the blooms appeared also in the other large lakes south of the Alps (Iseo, Como and Maggiore). Despite the sudden appearance of the blooms, the correct identification of the establishment time of the populations of *Dolichospermum* in the southern subalpine lake district remained unclear. In this work, the establishment of the populations of *D. lemmermannii* in Lake Garda has been evaluated by the direct counting of sub-fossil akinetes extracted from sediment cores, and by estimating the abundance of filaments germinated from sub-fossil viable akinetes. The two techniques provided comparable results, allowing locating the beginning of the establishment of *Dolichospermum* around the middle of the 1960s. Four strains of *Dolichospermum* germinated from akinetes isolated from the core sediments between around the 1989 and 2012 did not show any mutation or recombination signal in the *rpoB* gene sequences, suggesting a strong founder effect. The establishment of *Dolichospermum* coincided with the beginning of the rapid increase of total phosphorus as inferred from the distribution of sub-fossil diatoms in the sediment core. These results supported the hypothesis of a strong link between the shift of Lake Garda from ultraoligotrophy/oligotrophy to oligo-mesotrophy and the development of *Dolichospermum*. This colonisation pattern was possibly reinforced by the increase in the water temperatures in the subalpine lake district during the last 3 decades. In warmer lakes, gas-vacuolated Nostocales are favoured by high replication rates and, in particular, by their ability to control vertical movements in stratified water columns. This allows these species to exploit the gradients of light and nutrients, giving them a competitive advantage compared to other species. From a management point of view, the control and decrease of *Dolichospermum* should be obtained through the reduction and control of nutrient loads to the lake.

Key words: Cyanobacteria; Dolichospermum; invasion; Lake Garda; akinetes; resurrection ecology.

Received: July 2015. Accepted: November 2015.

INTRODUCTION

Cyanobacteria are the most primitive and ancient photo-oxygenic organisms on earth. With a variety of morphologically different types, they occur in aquatic and terrestrial habitats, as well as in extreme environments such as ultraoligotrophic oceans (Seymour, 2014), soil crusts in semi-arid areas (Metcalf *et al.*, 2012), and geothermal hot springs (Ward and Castenholz, 2012). In freshwater ecosystems, they can dominate the phytoplankton assemblages forming blooms in the water column or at the surface of lakes and rivers. This ability to colonise and dominate virtually every habitat on earth is due to their long evolutionary history (Tomitani *et al.*, 2006) and to a number of adaptations that render them competitively superior to many eukaryotic phytoplankton groups (Dokulil and Teubner, 2000). In this regard, cyanobacteria are one of the major eubacterial lineages. The diversity within this group includes morphological characters (from single cells to branching filaments), the formation of specialized cells (akinetes and heterocytes) and cell structures (aerotopes or gas-vesicles), and physiology (nitrogen fixation, heterotrophy, motility) (Graham *et al.*, 2009).

The growth and dominance of cyanobacteria have important consequences on the characteristics of water bodies. Along with effects in common with other eukaryotic algae (decrease of water transparency, increase of pH, alteration of biogeochemical cycles), cyanobacteria have further critical impacts, which include changes in the biomass size spectrum and edibility by zooplankton, and the production of an impressive range of secondary toxic metabolites (Sukenik *et al.*, 2015), including hepatotoxins and neurotoxins (Metcalf and Codd, 2012). The massive development of this algal group is considered as one of the principal problems for the utilisation of water resources, with possible impacts on human health (Chorus and Bartram, 1999; Ibelings *et al.*, 2014).

The cyanobacterial species belonging to the Nostocales have the ability to develop specialized cells with distinctive morphological and physiological adaptations for

the fixation of atmospheric nitrogen (heterocytes) and survival in harsh environmental conditions while quiescent in bottom sediments (akinetes). Mature akinetes have a normal cell wall surrounded by a thick three layered coat and a conspicuous granulation due to high concentrations of glycogen and cyanophycin, a nitrogen-storage polymer (Lee, 2008; Meeks *et al.*, 2002). The loss of gas-vesicles and the increase in cytoplasmic density favour the sinking of overwintering akinetes to the bottom sediments. Functionally, akinetes act as spores. They differentiate from vegetative cells by increasing in size and thickening the walls, and by drastically reducing photosynthetic and respiratory capabilities. For example, studies carried out on *Aphanizomenon ovalisporum* Forti showed that mature akinetes isolated from cultures maintained only residual photosynthetic activity, as indicated by very low values of maximal photosynthetic quantum yields (Sukenik *et al.*, 2007). The rearrangement of the photosynthetic apparatus with the maturation of akinetes is essential to enter the dormant period, assuring, at the same time, a quick recovery of photosynthesis after germination. The environmental factors activating the formation of akinetes differ between species and even strains. After the examination of several case studies, Kaplan-Levy *et al.* (2010) identified light intensity as the major factor triggering the formation of akinetes, though other factors, such as light quality, temperature, limitation of phosphorus and other nutrients and micronutrients, were considered important in many other instances. On the other hand, the germination of akinetes is controlled by various environmental factors, including temperature, nutrients, day length, penetration of light to the sediments and intensity of turbulence inducing resuspension near the bottom sediments (Kaplan-Levy *et al.*, 2010).

In general, akinete formation takes place at the end of the growth season. Reynolds (1972) observed akinetes in water columns after a dense *Anabaena* population had decreased. Kim *et al.* (2005) found a gradual increase in akinete cell densities in the surface sediments after a drop of *Anabaena flos-aquae* Bréb. ex Born. & Flah. densities. Nevertheless, studying the seasonal dynamics of *Anabaena flos-aquae*, Kravchuk *et al.* (2006) identified two types of akinetes. While in early summer akinetes germinated in water column soon after differentiation, contributing to the vegetative reproduction of populations, in late summer akinetes were deposited to bottom sediments, acting as resting stages.

Along with other algal remains, the study of akinetes in core sediments may provide more comprehensive knowledge of past lake phytoplankton assemblages and ecological conditions (Livingstone, 1984). Combined with other paleolimnological proxies, sub-fossil akinetes may contribute to the assessment of the effects of eutrophication and climate change on lake biota. Akinetes are very resistant to degradation. Fossil akinetes have been identified in 1.6 to 2.1 billion years old cherts in different regions of the world (Tomitani *et al.*, 2006).

At the beginning of the 1990s, Lake Garda showed a quick and unexpected appearance of surface blooms of a pelagic Nostocales, *Dolichospermum lemmermannii* (P.G. Richter) P. Wacklin, L. Hoffmann & J. Komárek. Since their first records, the blooms raised major concerns because of the potential toxigenic effects and the impacts on the tourism economy. The exact timing of the colonisation, however, remained uncertain because it was based almost exclusively on the detection of the surface water blooms and on a few scattered phytoplankton analyses available before the 1990s. Moreover, since the distribution of *Dolichospermum* is clearly localised in the temperate and boreal regions (Salmaso *et al.*, 2015a), the north to south colonisation pattern of this species contrasted with the prevalent south to north invasion routes of tropical species to the European continent (Sukenik *et al.*, 2012). On the other hand, many studies demonstrated that the spread of cyanobacteria can be favoured, besides by eutrophication, by the increase of water temperatures caused by global warming (Paerl and Huisman, 2009; Mehnert *et al.*, 2010). In a recent work, (Salmaso *et al.*, 2015a) hypothesised that the increase of *Dolichospermum* in Lake Garda could be ascribed to the increase of nutrients observed during the last 40 years. This hypothesis will be further explored in this study, by comparing the distribution of sub-fossil akinetes of *D. lemmermannii* in the deep sediments of the lake with the secular trend of phosphorus recently inferred from sub-fossil diatoms by Milan *et al.* (2015).

Specific objectives of this work include: i) the reconstruction of the historical colonisation patterns of *Dolichospermum lemmermannii* in Lake Garda based on the analyses of sub-fossil akinetes and its interpretation based on the secular changes in P-availability and long-term changes in water temperatures; ii) the assessment of the comparability of results obtained from the direct counting of sub-fossil akinetes extracted from sediments and the estimation of the abundances of strains germinated from sediments; iii) the phylogenetic confirmation of the taxonomic identity of recent and ancient populations of *D. lemmermannii* isolated from environmental samples and originated from the germination of akinetes from the sediment layers, respectively.

METHODS

Study site

Lake Garda is the largest Italian lake. It has a volume of 49×10^9 m^3, a surface of 368 km^2, and a maximum depth of 350 m. Thanks to attractive landscape and water quality,

the lake is an important resource for recreation and tourism. Moreover, its waters are also used for agriculture, industry, fishery and drinking. Owing to its climatic location, Lake Garda should be classified as warm monomictic. In this category, lakes circulate completely once a year in the winter at or above 4°C, showing stable stratification for the remainder of the year. Nevertheless, owing to its great depth, complete mixing can occur only irregularly, after cold winters (oligomixis). Since the beginning of the 1990s, Lake Garda has been regularly investigated with monthly samplings in a station located at the deepest point of the basin (45.69 N, 10.72 E). The Lake Garda sampling station was included in the Italian Network for the Long Term Ecological Research in 2007 (LTER, www.lteritalia.it).

Climatic, environmental, and phytoplankton data

Mean monthly homogenised air temperatures were obtained from the long-term instrumental climate data recorded in the Alpine region in the framework of the project HISTALP (Auer *et al.*, 2007). In this work, we have considered the temperatures recorded at Torbole - Riva del Garda, which is the nearest HISTALP meteorological station to the lake sampling point (ca. 20 km north). Since a preliminary interpolation by loess of the long-term temporal development of the air temperatures showed the presence of abrupt changes, the data were analysed by piecewise regressions. The selection of the break-point was guided by computing the residual standard error of several two-segment piecewise models and by the results of the loess interpolation (R Core Team, 2015).

The collection of samples and measurements in the water layers 0-2, 9-10 and 19-21 m were made at monthly intervals since 1991 in the LTER station. Samples for the analysis of phytoplankton abundance and nutrients were collected since 1993 and 1995, respectively. Vertical profiles of water temperature were measured with multi-parameter probes (Idronaut Ocean Seven 401 and 316Plus, and Seabird SBE 19-03) (Sharma *et al.*, 2015). The significance of the linear long-term trend of annual mean water temperatures in the layer 0-50 m was tested with the Mann-Kendall (MK) test, whereas the trend coefficients were estimated with the non-parametric Theil-Sen estimate of slope following the procedures described in Salmaso and Mosello (2010). Statistical analyses were carried out with R 3.2.2 (R Core Team, 2015). Nitrate nitrogen (NO_3-N) and total phosphorus (TP) were analysed using standard methods (Cerasino and Salmaso, 2012). Phytoplankton counting was carried out using the Utermöhl method as described in Salmaso (2010).

Sediment sampling, and akinetes analyses

A sediment core was collected with a gravity Kajak corer (UWITEC, Mondsee, Austria) at the LTER station of Lake Garda on 11 March 2014. The core was vertically extruded and sliced in the laboratory at 0.5 cm intervals from 0 to 30 cm and at 1 cm intervals from 31 cm down to the core bottom (45 cm). Hereafter the layers will be indicated by their mid-depth (*e.g.*, 1-1.5 cm: 1.25 cm). Water content was determined after drying ~2 g of wet sediment at 105°C for 24 h.

The direct microscopic analysis of akinetes is disturbed by the presence of sediments and organic material (including siliceous diatoms). This is especially true in low productivity, nutrient-poor lakes, where the abundance of akinetes is low. In such environments, a reliable counting could require a pre-treatment of the samples aimed at removing carbonate and silicate crystalline material, and diatom carpets. Before observation, the sediment samples were therefore processed adapting standard laboratory methods used for pollen analysis (Faegri and Iverson, 1989). Examples of applications of these methods in the analyses of sub-fossil akinetes and cyanobacteria are reported in Platt Bradbury *et al.* (2004), Danielsen (2009), Menozzi *et al.* (2010) and Miras *et al.* (2015). For each sediment layer, subsamples of ca. 2 g of fresh sediment were put in 15 mL plastic test tubes. The sediment was initially processed with 10% hydrochloric acid (HCl) for the removal of carbonates. For the removal of organic matter, 10 mL 10% KOH were added to the test tubes placed in a boiling water bath for 10 min, stirring slowly. The siliceous material was removed by adding hydrofluoric acid (HF). Compared with standard methods, the concentrations of HF were reduced to 10% to reduce the risk in laboratory procedures. The fluorosilicates gels formed during the reaction with HF were removed by adding 10 mL hot 10% HCl for 20 min. After the treatment with HCl, KOH and HF, the samples were centrifuged at 3000× g for 3-4 minutes, pouring away the supernatant. After the last treatment with HF, the final residue was diluted in 100 mL water and fixed with Lugol's solution. The samples, diluted 1:20, were transferred into10 mL sedimentation chambers and analysed by an inverted microscope at 400×.

The enumeration of akinetes was carried out analysing the whole bottom chamber of two replicates (Supplementary Fig. 1). The application of this method allowed obtaining clean samples, rich in akinetes. Furthermore, after this treatment the akinetes were not hidden by siliceous frustules, therefore reducing the risk of misidentification, which is particularly high in lakes rich in diatoms, such as Lake Garda (Salmaso, 2010). During this research, the reliability of the method was tested with a parallel counting of akinetes on treated (T, n=4) and non treated (NT, n=8) fresh sediment samples (without step 2 in Supplementary Fig. 1) extracted from a core rich in akinetes collected in Lake Balaton in November 2010 within the framework of the project EULAKES (www.eulakes.eu). The concentrations (average

±SE) of akinetes in the T and NT samples were equivalent (83±9 and 88±6 akinetes mg^{-1} fresh sample, respectively; ANOVA, $F_{1,10}$=0.23, P>0.60).

The germination of filaments from sub-fossil akinetes was obtained using ca. 4 g of fresh sediment inoculated in 100 mL conical flasks. A final volume of 60 mL was obtained by adding ASM-1 culture media (Carmichael and Gorham, 1974). The cultures were added with actidione (cycloheximide, 250 mg L^{-1}) as an inhibitor of eukaryotic growth (Livingstone and Jaworski, 1980). The flasks were incubated in a growth cabinet at 20°C under continuous irradiance with day light florescent lamps at 85 µmol m^{-2} s^{-1}. The germination of akinetes was observed up to 16-21 days. Later on, the culture media with germinated vegetative cells were harvested and preserved in 100 mL bottles fixed with Lugol's solution. After dilution 1:40, the subsamples were transferred into 10 mL sedimentation chambers. The enumeration of the germinated cells was carried out with an inverted microscope by analysing the whole bottom chamber of 4-6 replicates (Supplementary Fig. 2). Owing to the successive vegetative replication after the initial germination, the final densities of the cells recorded in the flasks were only roughly proportional to the initial number of akinetes in the sediments. On the other hand, one of the advantages of this method is to allow resurrection of ancient akinete-forming populations.

Core dating and analysis

The 2014 sediment core was dated by parallelisation (Thompson *et al.*, 2012) with a core collected at the same sampling point in October 2009. The 2014 and the 2009 cores showed a well resolved and comparable macroscopic layering, and very similar depth profiles of water content (r=0.94, P<0.01). The core collected in 2009, and used for the parallelization, was dated analysing radionuclide activity of ^{210}Pb, ^{226}Ra, ^{137}Cs and ^{241}Am in subsamples of individual sections, as described in Milan *et al.* (2015). The radionuclides allowed dating the core until the end of 1800s (26-27 cm). The dating of the deeper layers was estimated based on the ^{14}C analysis made on a vegetable remain isolated at 48.5 cm, which was dated back to 1388-1448 AD (Milan *et al.*, 2015). In order to assign an age to each sediment layer of the core collected in 2014, a LOESS interpolation of radiometrically determined dates for non-contiguous sediment layers was performed using R 3.2.2 (R Core Team, 2015).

In this work, we will also report additional results regarding the bacterial photosynthetic pigments and secular lake TP concentrations reconstructed from sub-fossil diatoms obtained from the analyses of the 2009 core (Milan *et al.*, 2015). Photosynthetic pigments were determined after sonication of sediments, extraction with acetone and analysis by light spectrometry and HPLC. Sub-fossil diatom frustules were cleaned following standard procedures (Battarbee *et al.*, 2001). Diatom-inferred total phosphorus concentrations (DI-TP) were reconstructed using a weighted-average regression model with inverse deshrinking that was calibrated against the Northwest European training set (NWEu-TP) (Bennion *et al.*, 1996). The detailed description of these procedures was reported in Milan *et al.* (2015).

Isolation of *Dolichospermum* strains and genetic analyses

Single filaments of germinated *Dolichospermum* were isolated from the cultures (stage 2 in Supplementary Fig. 2) obtained from the layers 1.25, 3.25, 4.25 and 6.25, corresponding to the years 2012, 2005, 2000, and 1989, respectively. Compared with filaments isolated from environmental samples, the individuals obtained from the germinated akinetes were more difficult to grow. After washing 3-4 times, the single filaments were grown in Z8 medium at 20°C under 16:8 h light:dark photoperiod and at 25 µmol m^{-2} s^{-1}. DNA extraction of the cultures containing the single strains was carried out following Shams *et al.* (2014). Phylogenetic analyses were carried out by the amplification of the *rpoB* gene encoding the β subunit of RNA polymerase. *rpoB* was amplified with the primers rpoBanaF and rpoBanaR, following a modified protocol described in Rajaniemi *et al.* (2005). The reaction mix (20 µL), contained 1X Optimized DyNAzyme PCR Buffer, 0.25 mM dNTPs mix, 0.4 U of DyNAzyme II DNA Polymerase, 0.5 µM of the two primers, and 5-20 ng DNA templates. PCR amplification involved an initial denaturation for 5 min at 94°C, 30 cycles of amplification: 1 min at 94°C, 1.5 min at 59°C and 2 min at 68°C, and a final elongation for 7 min at 68°C. PCR products were separated by 1% agarose gel electrophoresis stained with ethidium bromide. The *rpoB* gene was sequenced with the same primers used in the PCR following the methods described in Shams *et al.* (2014). The sequences (564-576 bp long) were deposited to the European Nucleotide Archive (ENA) with accession numbers LN871489- LN871492.

Phylogenetic analysis

The *rpoB* sequences of the germinated *Dolichospermum* strains were compared with other 2 sequences of *D. lemmermannii* isolated from recent pelagic samples collected in Lake Garda in 2013 and 2014 (accession numbers LN871471 and LN871475, respectively; Salmaso *et al.*, 2015a) and other *Dolichospermum* species from GenBank. *Microcystis aeruginosa* (KÜTZING) KÜTZING (EU151907) was included as outgroup. All the steps in the phylogenetic analysis were performed with the free statistical software R 3.2.2 (R Core Team, 2015). Sequences were aligned with MUSCLE (Edgar, 2004), and

poorly aligned positions and divergent regions were eliminated using Aliscore (Misof and Misof, 2009). Phylogenetic trees were computed by Maximum Likelihood (ML) with 1000 bootstrap (BS) replicates using the package phangorn. The analysis of DNA substitution models indicated in the K80+G the best-fitting evolutionary model for the *rpoB* gene.

RESULTS

Long term development of air and water temperatures

The long-term development of the mean annual air temperatures recorded at the northern border of the lake showed an overall tendency to increase (Fig. 1A). The interpolation by the loess function suggested the existence of at least two different linear relationships in the data, with an abrupt change of slopes around the first half of the 1960s. The piecewise regression (R^2=0.60, P<0.001) indicated an increase in air temperature around 0.07°C per decade between the end of the 1800s and 1965, and 0.45°C per decade in the successive years. Overall, since 1965, the mean annual air temperatures increased from around 12.5°C to over 14°C.

The long term change of the lake water temperatures was evaluated in the mixolimnetic layer, between 0 and 50 m (Fig. 1B). Since 1991, this layer underwent complete cooling every year, thus representing the part of the lake most affected by the winter climate. In this layer, the annual mean water temperatures showed a significant increase (Kendall's tau 0.34, P<0.05), at a rate of 0.24°C per decade (P<0.05).

Nutrients

In the first half of the 1970s, total phosphorus concentrations measured in the whole water column (0-350 m) were around 5-10 µg P L^{-1} (Fig. 2A). In the following 30 years, TP increased continuously, until reaching concentrations between 20 and 25 µg P L^{-1} around 2000. More recently (*i.e.*, after 2005), TP showed a slow tendency to decrease. Present concentrations are around 18 µg P L^{-1} (Fig. 2A). During the coldest months, in the epilimnetic layers TP reached homogeneous concentrations from the surface to the bottom only during complete overturn. During summer stratification, epilimnetic TP concentrations were generally between 5 and 10 µg P L^{-1} (Salmaso, 2011).

The pattern characterizing the long-term temporal changes of TP evaluated on the basis of chemical analyses (Fig. 2A) was confirmed by the TP values inferred from the composition of sub-fossil diatoms analysed in the core collected in 2009 (Fig. 3). The reconstruction of diatom-

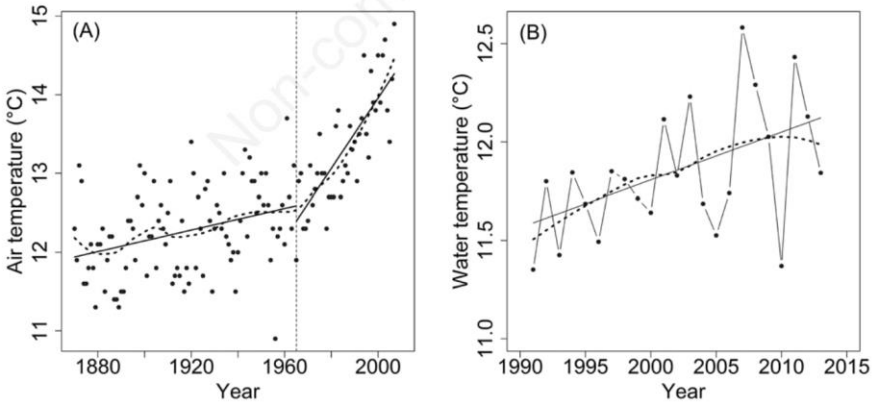

Fig. 1. Long-term temporal development of the mean annual values of (A) air temperatures recorded at the HISTALP meteorological station of Torbole - Riva del Garda, around 20 km north the sampling station; the interpolation by the 2-segmented line was obtained by a piece-wise regression. (B) Annual mean water temperatures in the layer between 0 and 50 m recorded in the LTER station of Lake Garda; the continuous line shows the linear trend obtained by the Theil-Sen estimate of slope. In (A) and (B) the series have been smoothed using the LOESS procedure (dashed lines).

inferred TP concentrations showed that, at least since the last period of the Middle Ages and until the beginning of the 1960s, Lake Garda was characterised by near ultra-oligotrophic conditions (DI-TP <10 µg P L^{-1}). The increase of DI-TP began only during the second half of the 1960s, in line with the results obtained by the modern limnological analyses (Mosello and Giussani, 1997; Fig. 2A and Fig. 3). Though less distinct, this pattern was confirmed also by the TP values inferred by pigment analyses (Car-TP; Fig. 3).

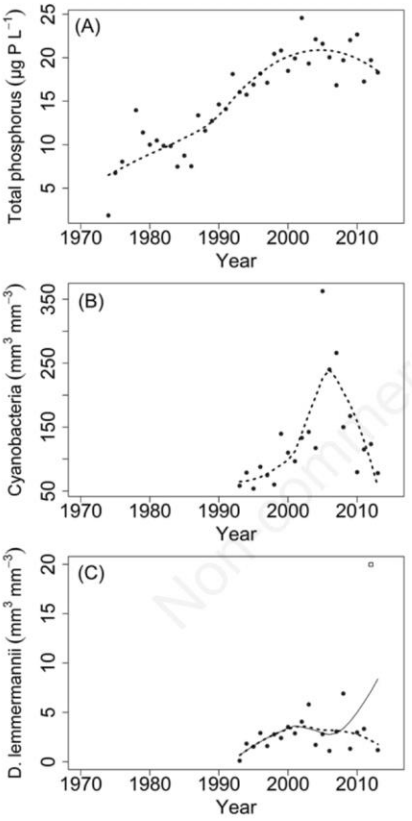

Fig. 2. Long-term temporal development of the mean annual values of (A) total phosphorus in the whole water column (0-350 m), and (B) cyanobacteria and (C) *Dolichospermum lemmermannii* in the trophogenic layers (0-20 m). In (A) the data before 1995 were redrawn from Salmaso and Mosello (2010). The series have been smoothed using the LOESS procedure (dashed lines). In (C), the continuous line reports the LOESS smoothing computed including the outlier.

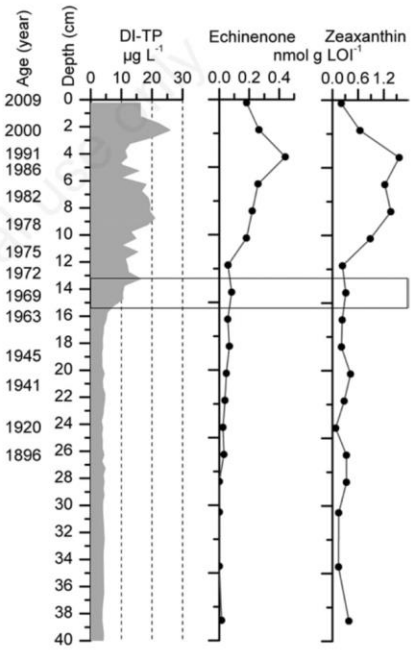

Fig. 3. Depth profiles of reconstructed phosphorus and sub-fossil cyanobacterial pigments in the sediment core collected from the deepest point of Lake Garda in 2009. DI-TP are TP concentrations reconstructed from sediment diatom abundance data using an inverted weighted averaging procedure based on the North West Europe training set (Milan *et al.*, 2015). Echinenone is a xanthophyll synthesised exclusively by some cyanobacteria. While zeaxanthin is a major pigment in cyanobacteria, it can be present in low quantities in most other eukaryotic phytoplankton species (Takaichi, 2011; Milan *et al.*, 2015). LOI, loss-on-ignition; the box highlights the first establishment of *Dolichospermum lemmermannii*.

In the whole water column, the average annual concentrations of nitrates (the prevalent N-compound) between 1996 and 2013 ranged between 290 and 400 µg L^{-1}. In the trophogenic layers (0-20 m), annual averages of NO_3-N generally varied from 200 to 260 µg N L^{-1}, without showing any apparent trend in the analysed period (P»0.1).

Cyanobacteria

Since the beginning of phytoplankton analyses, the annual averages of the cyanobacteria biovolumes showed a gradual increase from 50-100 mm^3 m^{-3} until 250-350 mm^3 m^{-3} in the middle of 2000s (Fig. 2B). In the successive period, cyanobacteria showed a dramatic decrease, until reaching biovolumes similar to those measured at the beginning of the 1990s.

The analyses of phytoplankton allowed inferring the response of cyanobacteria to the changes of phosphorus in the last 20 years. On a multi-decadal scale, zeaxanthin and echinenone, which are carotenoids typically produced by cyanobacteria, showed a sudden increase in the second half of the 1970s (Fig. 3). In the previous period, both pigments were measured with very low or undetectable values. The increase of cyanobacteria as inferred from these two proxies was shifted forward by around 10 years compared with the increase of DI-TP (Fig. 3). It is worth considering that both zeaxanthin and echinenone did show a tendency to decrease during and after the first half of 2000s, therefore confirming the recent tendency of cyanobacterial decrease estimated from the algal counts.

***Dolichospermum lemmermannii*: long-term temporal development and water blooms**

The annual means of *Dolichospermum lemmermannii* biovolume were over one order of magnitude lower than those of total cyanobacteria (Fig. 2C). Excluding the higher biovolume value computed in 2012, the long-term temporal pattern of *D. lemmermannii* biovolume was comparable to that of total cyanobacteria.

The very low annual average biovolumes of *D. lemmermannii* at the beginning of the 1990s were consistent with the timing in the appearance of the first visible water bloom. The surface accumulation of filaments of *D. lemmermannii* was observed for the first time in the shallower and wind sheltered E-basin (Salmaso *et al.*, 1994). In the successive years, and quite rapidly, the water blooms expanded over the whole lake, including the northernmost and deeper zone. The blooms, which were observed both in the central zones and along the lake shores, were usually accumulated by the wind in sheltered bays and harbours. The episodes were always observed between early summer and autumn. A careful investigation among the scientists studying the lake since the 1950s and the fisherman allowed confirming the absence of the water blooms of *Dolichospermum* before the 1990s (Salmaso *et al.*, 1994). Between the second half of the 1990s and 2000s, *D. lemmermannii* appeared also in the other largest lakes south of the Alps, *i.e.,* lakes Iseo, Como and Maggiore (Garibaldi *et al.*, 2003; Mosello *et al.*, 2010; Salmaso, 2010; Callieri *et al.*, 2014).

Historical distribution of akinetes and resurrected populations of *Dolichospermum*

The last measurable quantity of sub-fossil akinetes in the core collected in 2014 was found at 16.75 cm (1965; Fig. 4A, Supplementary Fig. 3). Repeated counts made on layers corresponding to the previous period, between 1935 and 1961, were negative. Since 1965, the number of akinetes remained practically constant until the end of the 1970s. Since then, akinetes abundance suddenly and fastly increased until 2007, then began to decrease. The morphology and morphometry of akinetes in the sediments were the same as those found in the pelagic samples, with length and width around 14-20 µm and 6.5-8.5 µm, respectively (Salmaso *et al.*, 2015a).

The germination of akinetes, with the corresponding growth of resurrected populations of *Dolichospermum*, was observed between the surface and the core depth of 14.25 cm (1971; Fig. 4B). Similarly to what was observed with the direct counting of akinetes (Fig. 4A), the new filaments developed with constant and low abundances until 1985. In the following periods, densities steadily increased till the end of 2000s. The counts corresponding to the uppermost analysed layer (dating 2012) showed a decrease in the cell abundances (Fig. 4B). The vegetative cells in the filaments presented characteristics fully compatible with the physiographic characteristics of *D. lemmermannii*. Nevertheless, compared with the natural populations, the formation of akinetes and heterocytes was usually not observed in the sediment cultures.

The phylogenetic analyses allowed clearly confirming the taxonomic nature of the sub-fossil populations of *Dolichospermum* germinated from the sediments. The four *rpoB* sequences of the resurrected strains corresponding to the years 1989, 2000, 2005 and 2012 were identical. These four strains formed a compact clade along with the strains of *D. lemmermannii* isolated in the pelagic samples of Lake Garda and other two strains isolated in N-Europe (Fig. 5). The other *Dolichospermum* species formed different separated clades.

DISCUSSION

The analyses of the distribution of the sub-fossil akinetes preserved in a sediment core collected in 2014 allowed to reconstruct the colonisation pattern of *Dolichospermum lemmermannii* in Lake Garda. The clear

identification of the beginning of the colonisation is a necessary requirement to interpret the causes that favoured the introduction and the successive rapid spread of this Nostocales in Lake Garda and in the whole southern subalpine lake district. The two laboratory procedures used to estimate the quantitative presence of *Dolichospermum* in the sediment layers provided consistent and reproducible results. The direct determination of akinetes in cleaned sediments and the counting of the cells germinated from viable akinetes indicated the beginning of the colonisation in the middle of the 1960s and in 1971, respectively. Though the discrepancy between the two methods, around 5 years, was very limited we cannot exclude the possible presence of a few other akinetes also in the older sediment layers. Nevertheless, both the consistency of the two methods in detecting the presence of akinetes, and the very rapid increase of akinetes after the 1970s, strongly suggest that the populations of *Dolichospermum* increased their importance during the 1980s and 1990s (Fig. 4), *i.e.,* during the period coinciding with the appearance of the first surface blooms (Salmaso *et al.*, 1994). Adopting the terminology by Williamson and Fitter (1996), the methods used in this work allowed to identify the period of *establishment* of self-sustaining and naturalized populations, after a first *importing* and *introduction* of the species.

The analysis of the records of sub-fossil akinetes preserved in deep layers of lake sediments represents a powerful and efficient tool to reconstruct the long-term temporal development of potentially toxic Nostocales in water bodies at secular timescales. Akinetes can be pre-

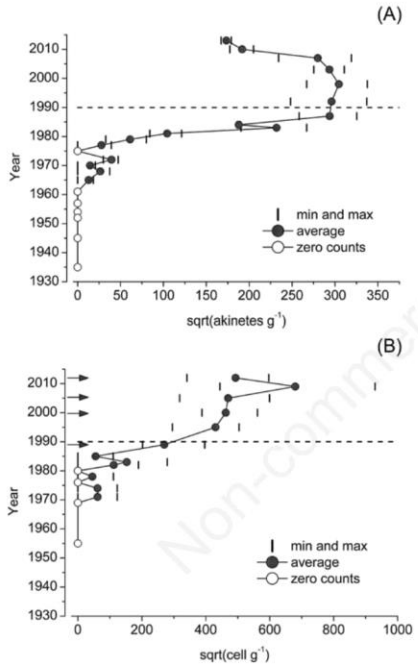

Fig. 4. Vertical distribution of (A) sub-fossil akinetes and (B) cells developed and reproduced from the germination of viable akinetes of *Dolichospermum lemmermannii* preserved in the sediment layers. The densities have been transformed by square root. The horizontal dashed lines indicate the period of first appearance of the blooms. In (B), the isolation and resurrection in culture conditions of single ancient *Dolichospermum* strains was obtained from the layers indicated with the arrows, which correspond to the years 2012, 2005, 2000, and 1989. The counting of akinetes in the sediments and the counting of the cells originated from the germination of akinetes indicated the beginning of the colonisation of *Dolichospermum* in the middle of the 1960s and in 1971, respectively.

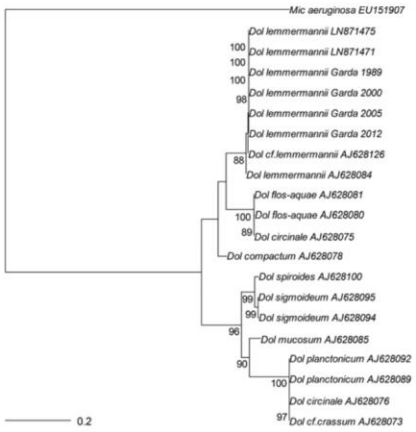

Fig. 5. Maximum likelihood (ML) rooted topology of the four resurrected *Dolichospermum lemmermannii* strains isolated from the sediments of Lake Garda (1989-2012) and other cyanobacteria (identified by names and accession numbers) based on alignment of the *rpoB* gene. The *D. lemmermannii* strains isolated from the pelagic samples collected in 2013 and 2014 in Lake Garda have accession numbers LN871471 and LN871475, respectively. Bootstrap values <70 were not shown.

served for very long times in the sediments. van Geel *et al.* (1994) found that, since 1000 AD on, sub-fossil akinetes of *Aphanizomenon* and *Anabaena* were present in enormous quantities in the deep sediment layers of Lake Gosciaz, in Poland. The increase of Nostocales was interpreted as the effect of the intensification of farming and land fertilization, which triggered and intensified the eutrophication of the lake. Similarly, peaks of akinetes of *Anabaena* (*Dolichospermum*) in the ancient sediments of Lake Aydat, were found since the 5th century AD (Miras *et al.*, 2015). In Lake Kirmanjärvi, Finland, Kauppila *et al.* (2012) utilised the mutual changes in the abundance of the akinetes produced by *Anabaena* and *Aphanizomenon* to infer the long-term trophic status changes since the mid 16th century.

Akinetes are markedly more resistant than vegetative cells to a wide range of extreme physical, chemical and biotic disturbances (Fay, 1988). Nevertheless, the resistance against specific stressors can be different between species. For example, while the akinetes of *Anabaena cylindrica* Lemmermann were resistant to desiccation, hot temperatures and sunlight (Hori *et al.*, 2003), the akinetes of *Anabaena circinalis* Rabenhorst ex Bornet & Flahault were susceptible as vegetative cells to desiccation or ultraviolet radiation (Fay, 1988).

When buried in the sediments, the akinetes can retain their viability for decades. In this study, we use the term viability as capability to germinate and to produce new filaments and populations once given the appropriate conditions. In Lake Okaro, germination experiments demonstrated that akinetes of *Aphanizomenon issatschenkoi* (Usačev) Proshkina-Lavrenko retained their ability to germinate in core sediments dating 120 BP (Wood *et al.*, 2009). Livingstone and Jaworski (1980) demonstrated that akinetes of *Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault and *Dolichospermum* (*Anabaena*) *lemmermannii* isolated from 18 and 64 years old sediments were able to germinate, giving rise to viable populations. In the case of *Aphan. flos-aquae*, the inability to germinate was observed in spite of a large abundance of akinetes in layers older than 18 years. On the one hand, that study demonstrated that akinetes not only have overwintering functions, but are capable of contributing to the long-term survival of Nostocales in lakes. On the other hand, it showed a different ability of akinetes produced by different species to germinate giving rise to mature populations. The implications are clear, suggesting that the germination of akinetes cannot be used to compare the long-term changes in different species of Nostocales. In Lake Garda, the concordance in the temporal distribution of sub-fossil akinetes and germinated cells indicates that the akinetes of *D. lemmermannii* retain the ability to germinate in sediments of at least 35 years old. To date, it is not possible to evaluate how much this approximation is underestimated, because the deepest layer of germinability (Fig. 4B) roughly coincides with the limit of akinete presence (Fig. 4A).

Since akinetes can be resurrected, the genetic structure of sub-fossil populations can be compared with that of modern strains, allowing a more robust assessment of historical fluctuation patterns. In principle, the use of methods of resurrection ecology might allow to reconstruct evolution of organisms by comparing the strains originated from resistance forms with their present descendants (Kerfoot and Lawrence, 2004). The comparison of strains along temporal gradients could help reveal whether direct effects of climate change or other anthropogenic stressors have caused micro-evolutionary processes (Angeler, 2007). The results obtained from sequencing the *rpoB* gene in populations of *Dolichospermum* revitalized in the lake sediments spanning 23 years (1989-2012) and isolated from recent pelagic samples did not show any mutation or recombination signal. Actually, previous analyses of other cyanobacteria species (*Planktothrix rubescens* (de Candolle ex Gomont) K. Anagnostidis & J. Komárek) in the large lakes south of the Alps proved that local clonal expansion and recombination processes were influential in affecting among-lake diversity (D'Alelio *et al.*, 2013). Present investigations made on the 16S rRNA and *rpoB* genes of several *D. lemmermannii* isolated from environmental samples are showing the existence of different local clonal populations living in the perialpine lakes (Salmaso *et al.*, 2015a) and in Europe (Capelli *et al.*, unpublished data). Overall, though based on a very limited number of strains, these results could allow to hypothesise a strong founder effect following the beginning of the establishment of *D. lemmermannii* (between the half of the 1960s and the end of 1970s) and the successive development of the species in Lake Garda. Possibly, these characteristics could allow identifying the geographical path of colonisation by comparing the local clones with those isolated in other European water bodies.

It is widely recognised that the environmental factors mostly affecting the development of cyanobacteria in lakes are eutrophication and high water temperatures (Istvánovics, 2009; O'Neil *et al.*, 2012; Paerl and Paul, 2012). The increase of cyanobacteria following nutrient enrichment (P and N) has been described in a wide number of studies (see Dokulil and Teubner, 2000), and confirmed by experimental works (Schindler, 2012). The response is strongly dependent from the autecology of the single species, and the distinction is particularly apparent when considering the ability to fix nitrogen (Levine and Schindler, 2011). Nevertheless, more than eutrophication, a number of recent studies highlighted the role of global warming in favouring the recent spread of Nostocales (*e.g.*, *Cylindrospermopsis* and *Aphanizomenon*) from tropical to subtropical and temperate regions (Sukenik *et al.*, 2012).

In warmer environments this group, along with Chroococcales, is favoured by its high replication rates and by the ability to control the vertical positioning in well stratified water columns induced by light availability and nutritional conditions (*e.g.*, carbohydrates content) (Oliver and Walsby, 1984; Reynolds, 2006). The control of the vertical position allows exploiting the full gradients of light and nutrients, giving to Nostocales and other gas-vacuolated species a chief competitive advantage compared to eukaryotic taxa. With the exception of a few localized regions, lake warming was clearly observed and documented all over the world (Sharma *et al.*, 2015). Besides Lake Garda (Fig. 1B), the increase in water temperatures was confirmed also in other lakes of the southern perialpine area. Long-term measurements carried out since the 1970s at spring overturn in lakes Garda, Iseo, Como and Maggiore demonstrated an increase in water temperatures ranging from 0.11 to 0.21°C decade^{-1} (Salmaso and Mosello, 2010). These data are confirmed also by models simulating the effects of a climate change scenario in Lake Iseo (Valerio *et al.*, 2015). Along with the rapid increase of air temperatures recorded during the 1960s, these data could suggest an important role played by climate warming in the establishment and growth of *Dolichospermum* in Lake Garda. Nevertheless, compared to many other Nostocales (Sukenik *et al.*, 2015), the geographical area of *D. lemmermannii* was shown to be circumscribed to the temperate and northernmost countries (Komárková, 1988), whereas this species was never found in tropical regions (Komárek, 2013). Conversely, the establishment of this Nostocales in the 1960s does appear rather linked to the increase of phosphorus and to the shift of the lake from a state between ultra-oligotrophy and oligotrophy to oligo-mesotrophy. The ecological requirements of *D. lemmermannii* have been well described (Cronberg, 1999; Padisák *et al.*, 2008; Reynolds *et al.*, 2002; Salmaso *et al.*, 2015b), conforming completely to this assumption. In fact, this species is well adapted to clear, deep stratifying oligo-mesotrophic lakes, or mesotrophic, non turbid shallow lakes. Therefore, we could hypothesise that the establishment of this species in Lake Garda might be favoured primarily by the increase of anthropogenic pressure and changes in trophic status. The successive development of this species could have been reinforced by the climate warming in the subalpine area and by the consequent increase in water temperatures. The absence of any consistent change in the long term-availability of NO_3-N since the 1990s suggests a possible minor role played by nitrogen. Nevertheless, as highlighted in previous works (Salmaso, 2010), owing to the N-fixing abilities of heterocytous species, the low summer concentrations of N that are usually found in the upper surface layers in lake Garda could be a positive factor for the growth of *Dolichospermum* in the warmer months.

As demonstrated by the analysis of the long-term phytoplankton data (Salmaso *et al.*, 2015a), the biovolume values of *D. lemmermannii* in the trophogenic layers (0-20 m) of Lake Garda were always low (<50 mm^3 m^{-3}), contributing for a very small fraction to the total annual phytoplankton and cyanobacterial biovolumes (less than 1% and 5%, respectively; see also Fig. 2 B,C). The dominant cyanobacterial species in Lake Garda and in the other large lakes south of the Alps is in fact *Planktothrix rubescens* (Salmaso *et al.*, 2012; Leoni *et al.*, 2014). On the other hand, since the blooms are caused by a rapid vertical movement of filaments present in the euphotic layers, these accumulations provide a false idea of massive cyanobacterial development and growth in the surface waters. These considerations confirm that *D. lemmermannii* should be considered a well *established* species rather than an *invasive* or *pest* species (Williamson and Fitter, 1996; Keller *et al.*, 2011).

We could anticipate that, if the downward trend of P in Lake Garda will be confirmed, then we could expect a further decrease of cyanobacteria and Nostocales, and a decrease in the frequency of summer water blooms of *Dolichospermum*. Overall, this should favour the recovery of the lake to near-pristine conditions. Conversely, a further increase in the trophic status, especially if associated with an additional increase in summer water temperatures, could increase cyanobacteria abundances and surface water blooms. However, it is worth to highlight that an excessive increase in nutrients is a deterrent for the development of *D. lemmermannii*, which would be replaced by other cyanobacteria better adapted to mesotrophic and eutrophic conditions, such as different species of *Dolichospermum*, as well as *Aphanizomenon*, *Microcystis* and, provided a sufficient amount of light is present in the metalimnion, *P. rubescens*. These considerations could be confirmed by the absence of consistent populations and blooms of *D. lemmermannii* in Lake Lugano. The reasons are due to the lower water temperatures and higher mixing that distinguish the epilimnion of this lake (Salmaso *et al.*, 2012) and to the higher trophic status of the trophogenic layers. In fact, the dominant Nostocales in Lake Lugano is *Aphan. flos-aquae*, a species that is not only well adapted to develop under eutrophic conditions (Reynolds *et al.*, 2002; Salmaso *et al.*, 2015b), but that it is also well fitted to grow in mixed surface layers (Kangro *et al.*, 2007).

CONCLUSIONS

In this work, we investigated the timing of establishment of the populations of *D. lemmermannii* in Lake Garda by the direct counting of sub-fossil akinetes and by the estimation of the abundances of strains germinated from viable akinetes conserved in the sediments. The two

techniques provided comparable results, allowing locating the beginning of the establishment of *Dolichospermum* between the middle of the 1960s and the early 1970s, respectively. The sequencing of the *rpoB* genes in populations of *Dolichospermum* revitalized in the lake sediments between around 1989 and 2012 and isolated from recent pelagic samples did not show any mutation or recombination, suggesting a strong founder effect following the successive development of the species. The establishment of *Dolichospermum* in Lake Garda has been linked to the rapid increase of TP in the lake since the 1960s, as inferred from the study of the distribution of sub-fossil diatoms in the core sediment layers. The significant increase in the air and water temperatures observed in the last decades did reinforce and probably increased the effects triggered by anthropogenic eutrophication. If the downward trend of phosphorus that was observed in Lake Garda since the half of the 2000s will continue, then the biomass of cyanobacteria and Nostocales and the frequency of summer water blooms of *Dolichospermum* should show a further and consistent decrease.

ACKNOWLEDGMENTS

Investigations in Lake Garda and in the other large lakes south of the Alps were made in the framework of the LTER (Long Term Ecological Research) Italian network, site "Southern Alpine lakes", IT08-000-A (http://www.lteritalia.it/). The activity was supported by PhD fellowships to C.C. from the E. Mach Foundation - Istituto Agrario di S. Michele all'Adige. We thank the European Cooperation in Science and Technology COST Action ES1105 CYANOCOST for networking and knowledge-transfer support. The core sediment in Lake Balaton was collected in the framework of the EU Central Europe project EULAKES (2CE243P3). We are grateful to our colleagues in FEM for their support in the field and laboratory activities, and to the ARPA Veneto for logistic support in the field.

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Supplementary data

		AKINETES COUNTING PROCEDURE Laboratory operations	Weight of fresh sediment after laboratory operation
#1		2 g of fresh sediment sample were put in a 15 mL plastic tube (the exact weight of the sediment is measured with an analytical balance)	2 g
#2		The sample was exposed to cold and hot acid and basic treatment (10% HCl, 10% KOH, 10% HF)	2 g
#3		The resulting sample was diluted to 100 mL and fixed with Lugol's solution. The sample was stored in the dark at 4°C	2 g
#4		5 mL of fixed sample (3) were diluted in 95 mL of tap water	0.1 g
#5		10 mL of sample (4) were transferred to Utermohl counting chambers	0.01 g
#6		The whole bottom of a counting chamber was observed under an inverted microscope at 40x (Zeiss Axiovert 135)	0.01 g

The final counting provides the number of akinetes in 0.01 g wet weight (ww). The number of cells N is expressed in cells/grams ww: N = cells/0.01 = cells x 100.*
This value is transformed in no. of akinetes/grams dry weight (dw) multiplying the result by a coefficient obtained from % H_2O in the respective sediment layer.

*The computation will take into consideration the exact weight of the fresh sediment measured with an analytical balance.

Supplementary Fig. 1. Laboratory procedure used to estimate the density of akinetes in the core sediment layers. The scheme reports the principal laboratory steps performed to obtain samples of akinetes free of most of the organic and inorganic sediment.

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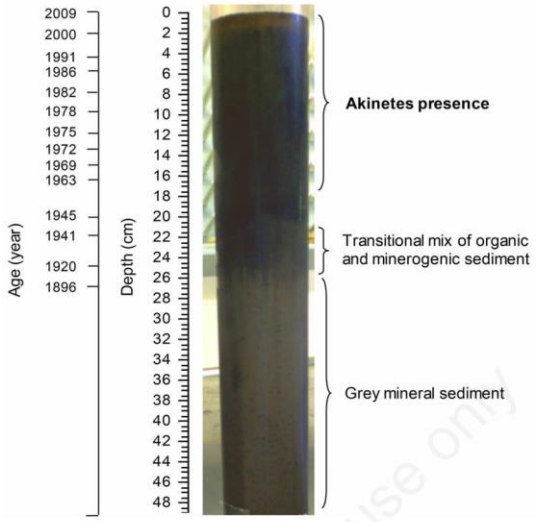
		AKINETES GERMINATION PROCEDURE **Laboratory operations**	**Weight of fresh sediment after laboratory operations**
#1		4 g of fresh sediment sample was diluted in ASM-1 medium (the exact weight of the sediment is measured with an analytical balance)	4 g
#2		A total volume of 60 mL was made by adding culture media ASM-1 containing actidione (cycloheximide, 250 mg L^{-1}) as an inhibitor of eukaryotic growth. The flasks were incubated at 20°C under continuous irradiance with day light florescent lamps at 85 µmol m^{-2} s^{-1}.	4 g
#3		After 16 or 21 days, half of the total volume (30 mL, with the germinated filaments of Nostocales) was diluted to 100 mL and fixed with Lugol's solution. Samples were stored in the dark at 4°C	2 g
#4		2.5 mL of 100 mL fixed sample (3) were diluted in 97.5 mL of tap water	0.05 g
#5		10 mL of this 100 mL solution (4) were transferred to Utermohl counting chambers	0.005 g
#6		The whole bottom of a counting chamber was observed under an inverted microscope at 40x (Zeiss Axiovert 135)	0.005 g
		The final counting provides the number of cells in 0.005 g wet weight (ww). The number of cells N is expressed in cells/grams ww: N= cells/0.005 = cells x 200.* This value is transformed in no. of cells/grams dry weight (dw) multiplying the result by a coefficient obtained from % H_2O in the respective sediment layer.	

*The computation will take into consideration the exact weight of the fresh sediment measured with an analytical balance.

Supplementary Fig. 2. Laboratory procedure used to estimate the density of cells germinated from viable akinetes isolated from the core sediment layers. The procedure allows to obtain living populations of *Dolichospermum*.



Supplementary Fig. 3. The core sediment collected in 2009. The 2014 core (where the sub-fossil akinetes were determined) and the 2009 core showed a well resolved and comparable pattern of water content. Excluding the 5 years gap between the two cores, the figure shows the approximate extent of the sediment layer where akinetes were found. In the last ~50 years (akinetes presence), the organic content approximately doubled due to the combined increase in lake productivity and decreasing input of minerogenic materials in relation to the construction of hydropower plants within the lake watershed (Milan *et al*., 2015).

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V.

Biogeography of bloom-forming toxic and non-toxic populations of *Dolichospermum lemmermannii* (Cyanobacteria)

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Summary

In the last 50 years, the cyanobacterium *Dolichospermum lemmermannii* has increasingly spread from northern temperate and boreal regions towards Southern Europe, raising serious concerns due to its ability to produce cyanotoxins. The increase of its geographic distribution and the observation of strains showing high optimum temperature underline its ecological heterogeneity, suggesting the existence of different ecotypes. To investigate its biogeography, new isolates from different European water bodies, together with strains maintained by the Norwegian Institute for Water Research Culture Collection of Algae, were genetically characterised for the 16s rRNA gene and compared with strains obtained from public repositories. Geographic distance highly influenced the differentiation of genotypes, further suggesting the concurrent role of geographic isolation, physical barriers and environmental factors in promoting the establishment of phylogenetic lineages adapted to specific habitats. Differences among populations were also examined by morphological analysis and evaluating the toxic potential of single strains, which revealed the exclusive ability of North European strains to produce microcystins, whereas the populations in Southern Europe tested negative for a wide range of cyanotoxins. The high dispersion ability and the existence of toxic genotypes indicate the possible spread of harmful blooms in other temperate regions.

Introduction

The genus *Dolichospermum* (Nostocales) includes species sharing at least 92% of 16S rRNA gene similarity (Wacklin et al., 2009). *Dolichospermum lemmermannii* (Richter) P.Wacklin, L.Hoffmann and J.Komárek, first described in 1903 in Northern Germany (e.g. Grosser Plöner See; Forti, 1907), is typical for oligo-mesotrophic and deep, stratifying lakes or mesotrophic shallow lakes, with good light conditions (Reynolds et al. 2002; Padisák et al. 2009). This species was reported in the whole temperate zone and at boreal latitudes (Komárek and Zapomělová, 2007; Lepistö and Holopainen 2008), mainly between the 40th and 60th parallels (Salmaso et al. 2015a), and was commonly observed in cold environments of Northern Europe (Skulberg et al. 1994; Willén 2003). Conversely the only observation recorded in tropical areas refers to the Riogrande II Reservoir, at 2200 m a.s.l. (Central Andes, Colombia), where water temperature typically ranged between 16 to 24°C (Palacio, 2015). Zapomělová et al. (2010) defined the optimal growth temperature for *D. lemmermannii* between 13 to 18° C, whereas specific strains showed high temperature optima (i.e. between 18°C and 25°C), demonstrating its high variability to temperature adaptation.

In Southern Europe, extended surface blooms of *D. lemmermannii* progressively appeared during summer stratified conditions in the large and deep lakes south of the Alps (i.e. Lake Garda, Iseo, Como, and Maggiore) since the 1990s (Salmaso et al., 2012). The spread in the subalpine region followed an altitudinal gradient, likely in relation to the warming of these lakes (Callieri et al. 2014; Salmaso et al. 2015a). Global climate change significantly affects the development of

invasive cyanobacteria, especially Nostocales (Sukenik et al., 2012; Cirés et al., 2014), enhancing their growth rates, dominance and geographic distributions (Paerl and Huisman, 2009). Through the study of sub-fossil akinetes, the establishment of *D. lemmermannii* in Lake Garda was identified between the 1960s and the 1970s (Salmaso et al. 2015b), when the post-war economic growth led to a booming of tourism industry and intensive agriculture in the subalpine region (Milan et al., 2015). Global warming and nutrient enrichment likely were the major factors supporting the development of this species and the increase of its geographic distribution.

Phytoplankton species have the ability to disperse and resettle in new environments (Naselli-Flores and Padisák, 2015). Passive dispersal mechanisms exerted by wind and aquatic birds (Incagnone et al. 2015), as well as human activities, commonly occur in cyanobacteria (Kristiansen, 1996) and tend to homogenize species distribution. However, despite these dispersal abilities, and similarly to larger species (Avise, 2006), the distribution of microscopic organisms is affected by geographical barriers, which results in biogeographical patterns (Fontaneto 2011, 2014) characterised by founder effects and localized genetic differentiation (De Meester et al., 2002). The presence of spatial patterns was investigated in several cyanobacteria species (Moreira et al., 2013). While on a global scale a phylogeographic structure is absent in *Microcystis aeruginosa* (van Gremberghe et al., 2011), revealing its cosmopolitan distribution, in *Cylindrospermopsis raciborskii* different clusters with a high degree of genetic divergence were identified (Gugger et al., 2005; Haande et al., 2008; Moreira et al., 2011; Wood et al. 2014). Godhe et al. (2016) described the influence of geographic distance and environmental selective forces in the genetic differentiation of algal blooms. Observing the propagation of a Baltic Sea spring bloom, the authors identified the succession of different genotypes significantly structured by oceanographic connectivity and distance, enhanced by salinity gradient. Likewise, the presence of *D. lemmermannii* at lower latitudes was supposed to be due to the existence of different ecotypes characterised by specific ecological adaptations (Salmaso et al. 2015a). Both geographic isolation and differential adaptation are indeed considered as factors acting upon variation within and among cyanobacteria populations (Papke et al., 2003).

The ability of *D. lemmermannii* to produce hepatotoxic microcystins (Sivonen et al., 1992) and the neurotoxic anatoxin-a(s) (Henriksen et al., 1997; Onodera et al., 1997), which was demonstrated in North European strains, implies a potential hazard for human health and freshwater ecosystems. Besides, the isolation of non-toxic genotypes (Henriksen et al. 1997; Rajaniemi et al. 2005; Cirés et al. 2014; Salmaso et al. 2015a), could imply the existence of different phylogenetic lineages adapted to specific ecological niches. The geographical segregation of neurotoxin production among strains was observed in Anabaena circinalis (Beltran and Neilan, 2000), whereas Kurmayer et al. (2015) showed that, in *Planktothrix*, microcystins distribution depended on phylogeny, ecophysiological adaptation and geographic distance.

The general objective of this study is to investigate the phylogenetic relationships between strains of *D. lemmermannii* with the purpose to describe its biogeography and to test if the distribution patterns are congruent with a geographical segregation or a cosmopolitan pattern. Additionally, the potential toxicity will be assessed to identify any possible geographical segregation of cyanotoxin producing strains. The contrasting distribution patterns (segregation or panmixia) will be evaluated by analysing the genetic diversity among strains isolated from distinct geographic locations. Our hypothesis is that geographic factors (e.g. geographic distance) can significantly influence the spatial genetic structure of populations. Investigations will include *D. lemmermannii* strains isolated from different European water bodies, together with strains maintained by the Norwegian Institute for Water Research Culture Collection of Algae (NIVA-CCA), and strains obtained by public archives (INSDC, International Nucleotide Sequence Database Collaboration). The evolutionary pattern of cyanotoxin producing strains will be evaluated by plotting the distribution of alternative states on the

phylogenetic trees obtained by the analysis of the 16s rRNA gene.

Results

Morphological characterization

Thirteen strains were isolated from different water bodies in southern and northern side of the Alps (Table 1) and were attributed to *D. lemmermannii* on the basis of the diacritical features described by Komárek and Zapomělová (2007). Morphometrical and morphological traits of vegetative cells, heterocytes and akinetes showed no significant differences among strains. The average length of vegetative cells, akinetes and heterocytes was measured in cultures from lakes Iseo (8.9 µm, 12.4 µm, 9.8 µm), Como (8.0 µm, 18.1 µm, 10.3 µm), Maggiore (8.6 µm, 14.1 µm, 9.0 µm), Lugano (8.8 µm, 20.0, 10.4 µm), Caldonazzo (8.7 µm, 15.1 µm, 10.0 µm), and Starnberger (7.8 µm, 17.7 µm, 9.1 µm). The morphometrical features of the *D. lemmermannii* population in Lake Garda, which were previously described in Salmaso et al. (2015a), were within the ranges reported above.

The ultrastructural analysis of the strains from lakes Garda and Como, showed that the vegetative cells had quite dilated thylakoids, distributed in the whole cell volume (Figures 1a-c). Polyhedral bodies were normally located more peripherally (Fig. 1a, arrow). The gas vesicles were widely distributed in the vegetative cells of the filaments and they were concentrated at the free pole in the apical cell of the filament (Fig. 1d, arrows). The terminal cell of the filament had a restriction at the level of the cell wall separating it from the neighbour cell (Fig. 1d). In a number of cells, a wide mainly central, quite electron dense area, containing strongly electron dense granules was observed (Figures 1b, 1c). In a few of these cells, some electron-transparent spheres appeared within this area, surrounded by electron dense granules (Fig. 1b, arrows). This central electron dense area was the last to be subdivided during the cell division (Fig. 1e). The akinetes appeared roundish-oval in shape and with less numerous thylakoids with respect to the vegetative cells (Figures 1g-i). Electron dense granules (cyanophycin) increased in number (Fig. 1g, arrows). Cyanophycin granules are indicated by arrows in Fig. 1i. The external envelope of the akinete was a layer of medium electron density placed on a more internal electron transparent layer (Fig. 1h, arrow). In the analysed *D. lemmermannii* strains, no real honeycomb structure was observed in heterocytes, where also carboxysomes (asterisks) were observed, but few thylakoids (Fig. 1f).

Identification of cyanotoxin producers

The 12 *D. lemmermannii* strains isolated in lakes Maggiore, Lugano, Caldonazzo, and Starnberger See were unable to produce microcystins (MCs), anatoxins (ATX), nodularin (NOD), and cylindrospermopsin (CYN). No toxin analysis was carried out on the strain isolated in Ammersee (FEM_ AMDL11) (Table 1). As a preliminary analysis, STX production was tested on four *D. lemmermannii* strains isolated in Lake Garda in October and November 2014, showing negative results. The amplification of the *mcyE* and *anaF* genes was not observed in any of these 13 analysed strains (Table 1). These results were consistent with the analyses carried out on several isolates from the group of large Italian lakes (Salmaso et al. 2015a).

All 12 *D. lemmermannii* strains maintained by NIVA-CCA tested negative for the amplification of the *anaF* gene, whereas the presence of the *mcyE* gene was observed in 5 strains isolated between 1981 and 2000 from Norwegian lakes (Table 1). All the strains carrying the *mcyE* gene produced MCs, as demonstrated by the positivity of ELISA tests.

Partial *mcyE* sequences (418 bp) from NIVA-CCA strains (Table 1) shared a 100% similarity to each other. A Blast (NCBI) homology search on these *mcyE* sequences showed a 100% similarity to *D. lemmermannii*, a 99% similarity to *Dolichospermum* and *Anabaena*, and a 75% to 81% similarity to *Microcystis*, *Oscillatoria*, and *Planktothrix*.

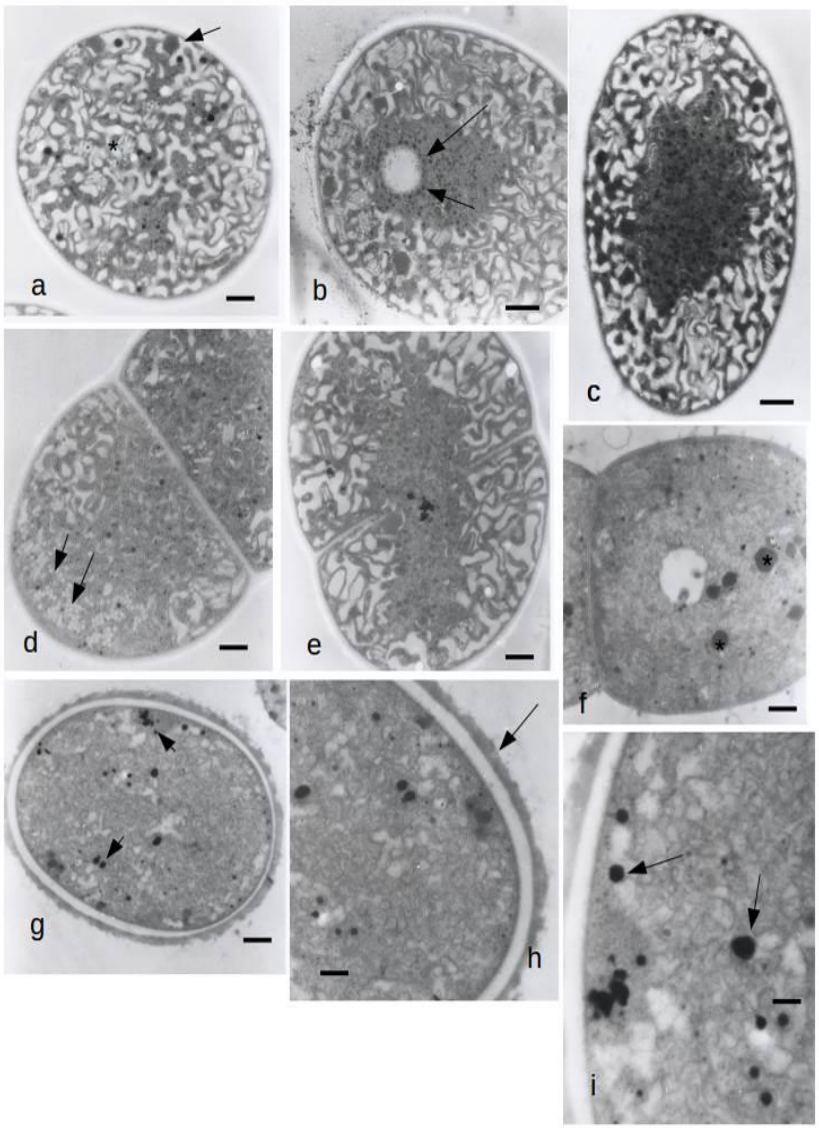

Figure 1. Transmission electron microscopy (TEM) of *D. lemmermannii* strains isolated in lakes Garda (a-b; d-e) and Como (c; f-i). a: vegetative cell. Dilated thylakoids and gas vesicles (asterisk) are evident. Polyhedral bodies are located more peripherally (arrow). Bar = 1 µm. b: vegetative cell. A wide central, quite electron dense area, containing strongly electron dense granules is observed. Within this area, an electron-transparent sphere, surrounded by electron dense granules can be observed (arrows). Bar = 1 µm. c: vegetative cell. Central area, more electron dense than that of fig. 1b. Bar = 1 µm. d: terminal vegetative cell of a filament. The gas vesicles are concentrated at the free pole in the apical cell of the filament (arrows). Bar = 1 µm. e: vegetative cells in division. The central electron dense area still crosses the forming wall. Bar = 1 µm. f: heterocytes. Carboxysomes (asterisks) can be observed. Thylakoids are rare. Bar = 1 µm. g: akinete. The thylakoids are less numerous with respect to the vegetative cells. Electron dense granules (cyanophycin) are common (arrows). Bar = 1 µm. h: akinete. The external envelope appears as a layer of medium electron density (arrow) placed on a more internal electron transparent layer. Bar = 0.5 µm. i: akinete. Detail of cyanophycin granules (arrows). Bar = 250 nm.

Table 1. Strains of *D. lemmermannii* isolated and cultured from new samples collected in 2015 or obtained from available cultures (isolations carried out between 1981 and 2008). MCs and ATX columns indicate the ability of the isolates to produce microcystins and anatoxin-a, respectively; *mcyE* and *anaF* indicate the results of PCR amplification of MCs and ATX biosynthesis encoding genes, respectively. + detected; - not detected; n.a. not analysed. a LC-MS; b ELISA. (*) strains included in phylogenetic analyses.

						MC		ATX	
Isolation source	**Strain code**	**Water Body**	**Country**	**Country code**	**Year**	**MCs**	***mcyE***	**ATX**	***anaF***
new sampling	FEM_MDL1	Lake Maggiore	Italy		2015	– [a]	–	– [a]	–
new sampling	FEM_MDL5	Lake Maggiore	Italy		2015	– [a]	–	– [a]	–
new sampling	FEM_MDL6	Lake Maggiore	Italy		2015	– [a]	–	– [a]	–
new sampling	FEM_MDL7	Lake Maggiore	Italy		2015	– [a]	–	– [a]	–
new sampling	FEM_LDL8*	Lake Lugano	Italy	IT_L	2015	– [a]	–	– [a]	–
new sampling	FEM_LDL10	Lake Lugano	Italy		2015	– [a]	–	– [a]	–
new sampling	FEM_CADL2	Lake Caldonazzo	Italy		2015	– [a]	–	– [a]	–
new sampling	FEM_CADL9*	Lake Caldonazzo	Italy	IT_Ca	2015	– [a]	–	– [a]	–
new sampling	FEM_STDL9	Stamberger See	Germany		2015	– [a]	–	– [a]	–
new sampling	FEM_STDL13*	Stamberger See	Germany	DE_St	2015	– [a]	–	– [a]	–
new sampling	FEM_STDL16	Stamberger See	Germany		2015	– [a]	–	– [a]	–
new sampling	FEM_STDL17	Stamberger See	Germany		2015	– [a]	–	– [a]	–
new sampling	FEM_AMDL11*	Ammersee	Germany	DE_Am	2015	n.a.	–	n.a.	–
culture	NIVA_CYA696*	Nehmitzsee, Brandenburg	Germany	DE_Ne1	2008	– [b]	–	n.a.	–
culture	NIVA_CYA697*	Nehmitzsee, Brandenburg	Germany	DE_Ne2	2008	– [b]	–	n.a.	–
culture	NIVA_CYA698*	Nehmitzsee, Brandenburg	Germany	DE_Ne3	2008	– [b]	–	n.a.	–
culture	NIVA_CYA83-2*	Lake Edlandsvatnet, Rogaland	Norway	NO_Ed	1981	+ [b]	+	n.a.	–
culture	NIVA_CYA266-1*	Lake Bergesvatnet, Hordaland	Norway	NO_Be	1990	+ [b]	+	n.a.	–
culture	NIVA_CYA270-2*	Lake Arefjordvatnet, Hordaland	Norway	NO_Ar	1990	+ [b]	+	n.a.	–
culture	NIVA_CYA281-1*	Lake Storavatn, Lindås, Hordaland	Norway	NO_StL0	1990	– [b]	–	n.a.	–
culture	NIVA_CYA298*	Lake Storavatn, Lindås, Hordaland	Norway	NO_StL1	1991	+ [b]	+	n.a.	–
culture	NIVA_CYA335*	Lake Hallevatnet,Vestfold	Norway	NO_Ha	1993	– [b]	–	n.a.	–
culture	NIVA_CYA426*	Lake Storvatnet, Smøla, Møre and Romsdal	Norway	NO_StS	2000	– [b]	–	n.a.	–
culture	NIVA_CYA438*	Lake Steinsfjorden, Buskerud	Norway	NO_Ste	2000	+ [b]	+	n.a.	–
culture	NIVA_CYA462-2*	Lake Søndre Heggelivatnet, Akershus	Norway	NO_So	2003	– [b]	–	n.a.	–

Phylogenetic characterization

The phylogenetic relationship of *D. lemmermannii* strains listed in Table 1 were determined on the 16s rRNA gene; besides these newly sequenced strains, further sequences of Nostocales obtained from INSDC archives (Table S2) were included in the analysis (1001 bp alignment; Fig. 2). The new strains isolated in this study and the NIVA-CCA strains grouped together in cluster I, with most of the INSDC sequences of *D. lemmermannii*. Some *D. lemmermannii* strains formed a cluster together with *Dolichospermum flos-aquae* sequences (cluster II). Except for this cluster, overall *D. lemmermannii* formed a distinct clade, separated from other *Dolichospermum* spp. by high branch-support value. The strains from the Italian subalpine lakes formed a unique homogeneous sub-cluster, in which only the strain isolated in Lake Maggiore showed slight genetic variation.

The 16s rRNA gene tree (Fig. 2) showed that a few of the MCs-producing strains were grouped in the same sub-cluster, whereas some of them clustered with non-MCs producing strains. However, information about toxicity was not available for several of *D. lemmermannii* strains retrieved from INSDC, allowing therefore only a partial description of the toxic potential of the species. The geographical location of the 29 strains included in cluster I, is represented in Fig. 3. While non-MCs producing strains were distributed from northern to southern Europe, MCs-producers were confined in Northern Europe (Norway and Finland).

Phylogeography of *D. lemmermannii*

The *D. lemmermannii* strains included in cluster I of the 16S rRNA gene tree (Fig. 2) were used for a more detailed phylogenetic analysis of the species (the metadata for the strains obtained from INSDC are in Table S3). The phylogenetic tree (1070 bp alignment of the 16S rRNA gene) showed 4 distinct subclusters with evident genetic differences, far from a central group in which the strains were closely related (Fig. 4). Each subcluster included strains isolated from the Italian subalpine lakes (except Lake Maggiore), New Zealand, United Kingdom, and Northern Norway, respectively. The main group contained strains from Germany, Czech

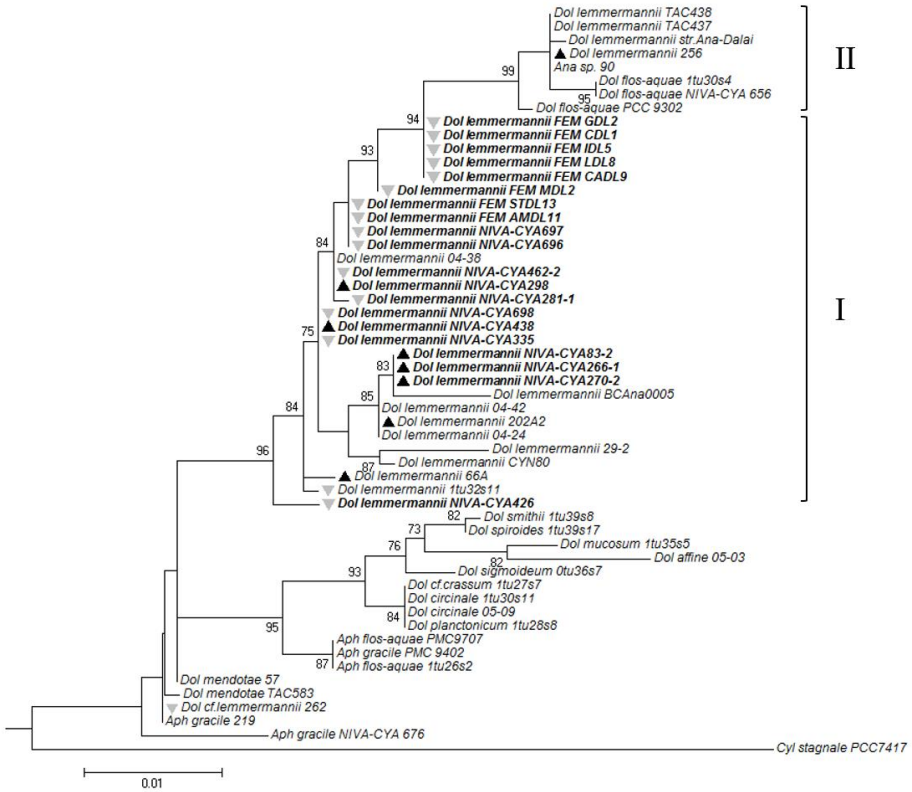

Figure 2. Maximum likelihood tree based on the 16s rRNA gene of 54 Nostocales strains. The tree was rooted on the outgroup *Cylindrospermum stagnale* (NR_102462). Strains from this study are marked in bold. The list of accession numbers is reported in Tables 1 and S2. SH-aLRT > 60 are included. Information on cyanotoxins production of strains from INSDC archives was retrieved from references (Henriksen et al. 1997; Sivonen et al. 1992; Rajaniemi et al. 2005) and reported in Table S4. ▲MCs producers; ▼non-MCs producers

Figure 3. Location of the water bodies where *D. lemmermannii* strains included in cluster I (Fig. 2) were isolated. Detailed information on latitude/longitude and cyanotoxins production are listed in Tables 1, S1 and S3. (a) Europe; (b) Oceania. ▲MCs producers; ▼non-MCs producers; ○ unknown toxicity

Republic, Norway, and Finland. The principal coordinate analysis (PCoA) based on pairwise genetic distances between strains (Fig. 5) resulted in a similar clustering pattern as the 16S rRNA gene phylogenetic tree. Strains from the Italian subalpine lakes, New Zealand, United Kingdom, and Northern Norway were genetically different from each other and from those isolated in Central and Northern Europe. Both the phylogenetic and PCoA analysis approaches, revealed that the strain isolated in Lake Maggiore was outside the subalpine lake subcluster, and showed a closer relation to the central-north European group.

The geographic isolation hypothesis and the effect of dispersal barrier in the differentiation of *D. lemmermannii* strains were examined by applying a Mantel test. The results showed a significant correlation between the geographic and genetic distances, based on both Pearson's and Spearman's correlations ($R = 0.65$, $P = 0.003$; $R = 0.49$, $P = 0.0001$). To check if New Zealand strains affected the strength of the analysis, a Mantel test was undertaken exclusively on European strains, confirming a significant correlation between the geographic and genetic distances (Pearson's $R = 0.33$, $P = 0.0001$; Spearman's $R= 0.33$, $P = 0.0002$).

Discussion

This study highlighted the existence of genetic differences and capability to synthesize microcystins among populations of *Dolichospermum lemmermannii* isolated from different geographical regions. The morphometric characteristics of vegetative and specialized cells were slightly variable in all the examined populations and reflected the typical range of this species. *Dolichospermum* is characterised by a large degree in morphological features, especially in vegetative cells and the loosening of the trichome coiling and morphological changes commonly occur in long-term cultivation (Komárek and Zapomělová, 2007). Although the position of the akinetes at both sides of the heterocytes is stable, being a good criterion for the accurate characterization of *D. lemmermannii*, this character can be lost in cultured strains, which can, in general, lose the ability to form heterocytes, akinetes and gas vesicles (Komárek and Zapomělová, 2007; Zapomělová et al. 2011).

The thylakoidal patterns within vegetative cells did not contrast with clusters derived from molecular sequencing. This character can be used to further evaluate phylogenetic relations based on the 16S rRNA gene in cyanobacterial classifications (Komárek and Kaštovský, 2003). The Nostocales have a generally uniform thylakoidal arrangement, with coiled (wavy) thylakoids usually more abundant at the periphery of the cells, extending towards the centre. Their distribution and aspect depend on the light intensity and quality (Komárek and Kaštovský, 2003). In line with the basic fine structures of Nostocales, the *D. lemmermannii* strains analysed by TEM (lakes Garda and Como) showed wavy dilated thylakoids distributed in the whole vegetative cell volume. Compared with related species, particular structural characteristics were revealed, as the barely evident restriction at the terminal cell, which was more remarkable in *Anabaena cylindrica* (de Vasconcelos and Fay, 1974). The akinetes, and their external envelope, were quite similar to that described by Cmiech et al. (1986) in *Anabaena solitaria*. The most striking feature observed in *D. lemmermannii* was a large area of medium electron density present in many vegetative cells containing electron dense granules. In the heterocytes no "honeycomb" structure was observed. This structure was also absent in Anabaena sp. strain CVST4, a mutant deficient of gene *FraH*, whose function is considered to be related to the maintenance of the filament compactness (Merino-Puerto et al., 2011). As a matter of fact, cells of the cultured *D. lemmermannii* strains were often found separated from the filaments, and the reason of that may refer to a fragility of the cell-cell connection due to a low efficiency of the proteins connecting one cell to the other. Such arrangement may facilitate propagation in this species. The unusual presence of carboxysomes in the heterocytes may be related to the presence in the Z8 medium of a nitrate sources, which may reduce the activity of heterocytes themselves and even block the development of this cell type, as observed in many cultured cyanobacteria strains (Komárek and Zapomělová, 2007). The same presence of

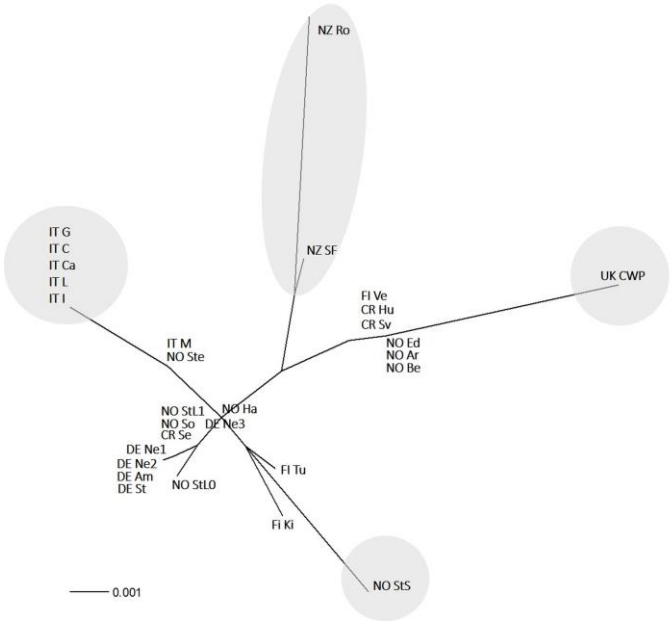

Figure 4. Maximum likelihood tree based on the 16s rRNA gene of the *D. lemmermannii* strains included in cluster I (Fig. 2). Strain codes based on Country are listed in Tables 1 and S3.

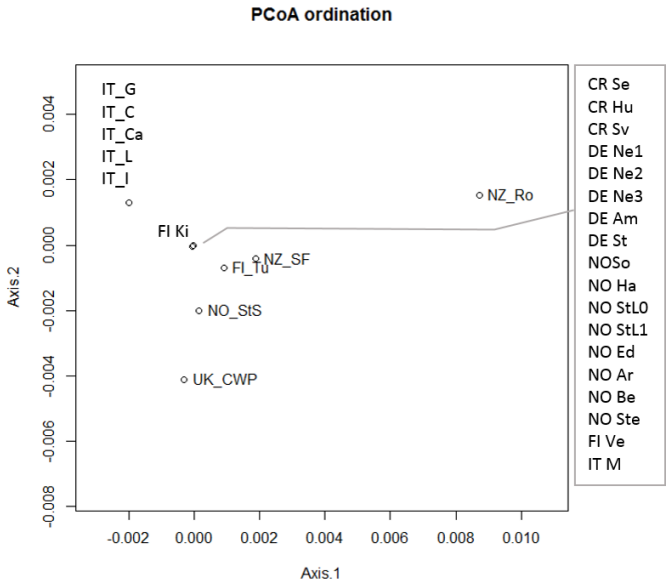

Figure 5. Principal coordinate analysis (PCoA) plot based on pairwise genetic distances between 16S rRNA gene of *D. lemmermannii* strains included in cluster I (Fig. 2). Strain codes based on Country are listed in Tables 1 and S3.

carboxysomes in the heterocytes (together with a reduced presence of the honeycomb structure) was also observed in heterocytes of *Anabaena* sp. strain CSAV141 (cox2 cox3) with inactivated cox genes (Valladares et al., 2007: Fig. 1c). These genes encode heterocytes-specific terminal respiratory oxidases related to the organization of the honeycomb structure (Valladares et al., 2007). In this strain nitrogenase activity was impaired and we may suppose that the similar situation in *D. lemmermannii* appears when grown in presence of nitrate supply in the medium.

Five Norwegian strains isolated between 1981 and 2000 and maintained in long-term culturing at NIVA were MCs producers. Toxigenic strains were confirmed by the identification of the *mcyE* gene of the microcystin synthetase gene cluster and by ELISA. This gene encodes the glutamate-activating adenylation domain (Tillett et al., 2000) and is a sensitive marker for the evaluation of toxic potential of cyanobacteria (Rantala et al., 2006; Mankiewicz-Boczek, 2012). The *D. lemmermannii* populations located in the Italian and German lakes were non-MCs producers, confirming earlier findings (Salmaso et al. 2015a). Comparing these results with data reported in literature (Sivonen et al. 1992; Rajaniemi et al. 2005; Salmaso et al. 2015a), MCs production emerged as an exclusive ability of North European strains (Norway and Finland), suggesting a geographical segregation. Metabolic differences between strains of the same species commonly occur in cyanobacteria (Cerasino et al. submitted), and are the effect of differentiation processes influenced by ecophysiological adaption, phylogeny and geographic factors (Kurmayer et al., 2015). In Northern Europe, toxic populations coexisted along with non-toxic populations in lakes of the same regions and even in a single lake (Lake Storavatn, Lindås, Hordaland, Norway). Kurmayer et al. (2015) identified evidences of *mcy* gene loss in *Planktothrix* by evaluating the presence of the full *mcy* gene cluster and of the *mcy*T gene; the latter occurred in toxic strains and as a remainder in nontoxic strains. Similar losses can be also speculated in non-toxic *D. lemmermannii* strains as well. As observed for *Cylindrospermopsis* (Cirés et al., 2014), these findings suggest a complex evolutionary history of cyanotoxin biosynthesis genes, which were affected by mutations events and horizontal gene transfer. In our work, the partial *mcyE* sequences amplified from MC-producing *D. lemmermannii* strains were identical to each other and resulted well conserved in the genera *Dolichospermum* and *Anabaena*, whereas a higher degree of diversity was observed within the Oscillatoriales and Chrococcales, confirming earlier observation on the correlation between phylogeny and *mcy* genotypes (Kurmayer et al., 2015).

Preliminary analysis of STXs on a few *D. lemmermannii* strains isolated from Lake Garda showed negative results. On the other hand, in literature the production of these toxins by *D. lemmermannii* was only suspected, because it was based on the collection of environmental samples (Bernard et al, 2016). Conversely, the neurotoxin ATX-a(s) production was observed in a few *D. lemmermannii* strains from Danish lakes and was associated with birds poisoning during blooms events (Henriksen et al. 1997; Onodera et al. 1997). ATX-a(s) was identified by mouse bioassay and acetylcholinesterase inhibition (Ellman et al., 1961) and the method for determination by LC-MS is still under progress. Therefore, ATX-a(s) production in the strains analysed in the present study cannot be excluded.

The 16S rRNA gene proved to be suitable in the study of the phylogenetic relationship of the *Dolichospermum* genus and among *D. lemmermannii* populations. The phylogenetic analysis of the sequences obtained in the present study together with Nostocales sequences retrieved from INSDC, showed a clear separation between *D. lemmermannii* and other *Dolichospermum* species. The strict relationship between some *D. lemmermannii* and *D. flos-aquae* strains can be referred to misidentification due to the wide variability of morphological features in natural populations and morphological changes in cultured strains (Komárek and Zapomělová, 2007) or to a different phylogenetic lineage. This mixed cluster (II) included the hepatotoxic *D. lemmermannii* strain 256 (Lake Knud, Denmark) (Henriksen et al., 1997) and three strains from East Asia (Japan and

Mongolia). The strains TAC437 and TAC438 (Lake Akan, Hokkaido, Japan) (Tuji and Niiyama, 2010) were otherwise reported as *Dolichospermum mendotae* by Li and Watanabe (2004). The phylogenetic identity of this cluster should be clarified, therefore could not be used to finely describe the phylogenetic structure of *D. lemmermannii*.

The *D. lemmermannii* strains included in cluster I showed high sequences homology, and this group contained both MCs producers and non-toxic strains. Whether the non-toxic strains conserve remnants of *mcy* gene cluster should be verified. Though most of the hepatotoxic strains were part of the same subcluster, the dependence of *mcy* distribution on phylogeny was hampered by the lack of data about several strains from INSDC.

Compared with the 16s rRNA, the *rpoB* gene is poorly represented in the INSDC archives, thus not allowing to obtain a representative biogeographical analysis. Moreover, based on the available sequences, this gene has a weaker phylogenetic resolution. As observed in other housekeeping genes (Rudi et al., 1998; Gugger et al., 2002) this variability may be due to lateral gene transfer between related species, which was estimated to affect the 60% of cyanobacterial gene families (Dagan et al., 2013).

The phylogenetic analysis of the 16s rRNA of the *D. lemmermannii* strains comprised in cluster I in Fig. 2 confirmed the genetic stability of the populations settled in the deep subalpine lakes. Compared with observations in other genera (Bittencourt-Oliveira et al. 2001; Kurmayer and Gumpenberger 2006), in this lake district there was no genetic variation within a population in a single water body and among the lakes. The population of Lake Maggiore was slightly different from this group demonstrating a closer relation with Norwegian strains. In summer, the deep subalpine lakes showed strong differences in the mean epilimnetic temperatures, which decreased from Lake Garda to Lake Lugano according to the altitudinal gradient. In lakes Maggiore and Lugano temperatures were 2 to 3 °C lower than other lakes (Salmaso et al., 2012). Zapomělová et al. (2010) underlined the high variability to temperature adaptation of different groups of *D. lemmermannii* strains, including the existence of isolates with high temperature optima (between 18°C and 25°C). Salmaso et al. (2015a) observed that the growth of this species in Lake Garda was favoured by higher water temperatures and thermal stability. Hence, the genetic differences between populations from Lake Maggiore and the other subalpine lakes can be the result of the environmental selection of ecotypes with different temperature optima. Moreover, *D. lemmermanni* blooms have never been reported in Lake Lugano (IST-SUPSI, 2015). The inability to achieve high biomass growth rate in Lake Lugano can be explained by water temperature lower than the growth optima of this ecotypes and higher mixing in the epilimnion (Salmaso et al., 2012). Increasing temperature due to climate change had a key role in the spread of other Nostocales, *Cylindrospermopsis* and *Aphanizomenon*, which reached subtropical and temperate regions following south to north dispersion routes (Sukenik et al., 2012).

Statistical analyses supported the role of geographic distance and segregation in within-species genetic variation. Similarly to the Italian group, a distinct clustering was observed for strains from New Zealand, United Kingdom and Northern Norway, whereas strains from central and northern Europe are more closely related. This spatial pattern can be explained by founder effects for the establishment of this species in the deep subalpine lakes, United Kingdom, Northern Norway and New Zealand, likely stabilised over the years by geographic isolation reinforced by physical barriers (e.g. Alps, Sea, ice cover). In Central Europe (Germany, Czech Republic) and in the southern part of Norway and Finland, these effects may have been mitigated by multiple colonisation events. The probable historical spread among neighbouring regions resulted in a dynamic process of subsequent integration of different genotypes which have led to a homogenization of these populations. Overall, in this process, environmental selective factors were influential in the successful establishment of genotypes adapted to particular geographical niches. Whether the current distribution pattern of *D. lemmermannii* has ancient or recent origin still needs to be disclosed, albeit the low degree of genetic diversity among

strains suggests a quite recent spread. In Lake Garda, the study of subfossil akinetes conserved in deep sediments demonstrated the establishment of *D. lemmermannii* during the past 50 years, however this evidence couldn't exclude its presence as barely perceptible biovolume or previous failed introduction events throughout history (Salmaso et al. 2015b). On the contrary, *D. lemmermannii* was already reported in New Zealand at the beginning of the 1900s (Forti, 1907). Interestingly, it was first observed in Chatham Islands (Lake Huro), which were inhabited by European sealers and whalers starting from 1791 (Chisholm, 1910) and later reached by German Lutheran missionaries from Berlin in 1842 (Holmes, 1993). Therefore, human activities as dispersal mechanism driving the distribution of *D. lemmermannii* can be speculated, as well as water birds which followed migratory flyways across Europe. Akinetes ensure the long term survival through the ability to resist at low temperature and desiccation, and can give an ecological advantage serving as dispersal units (Kaplan-Levy and Hadas, 2010; Murik et al. 2016). The possible role of akinetes in the spread in new environments was hypothesized for several invasive Nostocales species (Padisák, 1998; Stüken et al., 2006).

In conclusion, the present study deepens the phylogeography of *D. lemmermannii* showing its spreading potential over dispersal barriers, thanks to adaptability to temperature variation. Nevertheless, the spatial pattern suggests a high incidence of geographic distance and physical barriers on the differentiation of genotypes. After colonisation events, a coexistence of geographic isolation and environmental factors may have promoted the establishment of ecotypes adapted to specific ecological niches. The spread of this species is the cause of several concerns. For example, the deep subalpine lakes are renowned tourist destinations, and highly used for agriculture, industry, and drinking water supply (Salmaso et al., 2012), hence their economic value may be negatively affected by the appearance of invasive species. The *D. lemmermannii* populations settled in this lake district tested negative for the production of a wide range of cyanotoxins. However the ability to form huge surface blooms has many implications for water management, tourist attractiveness and economic value (Dodds et al., 2009). Furthermore, the identification of MCs producers *D. lemmermannii* throughout Northern Europe and the dispersion ability of this species raise an issue on the appearance of harmful algal blooms in other temperate areas.

Experimental procedures

Isolation and morphological characterization of *Dolichospermum* strains

The new strains analysed in this work included individuals isolated from Lake Maggiore and Lugano, which are the largest lakes south of the Alps together with lakes Garda, Iseo, and Como (Salmaso and Mosello, 2010) (Table 1). Further strains were isolated in the alpine Lake Caldonazzo in Northern Italy, and in two neighbouring pre-alpine hardwater lakes in Southern Germany, i.e. Ammersee and Starnberger See, where *D. lemmermannii* was formerly observed in the early 2000s (Teubner et al., 2003) (Table 1). In these lakes, water samples were collected in 2015 in summer and early autumn (from July to mid-November) in the deepest point of the basins, by vertical tows from 30 m to the surface with 25 cm diameter 80 µm mesh plankton nets. Samples were stored at 20°C and processed within 24 h. Single trichomes of *D. lemmermannii* were isolated under a macroscope (WILD M420) using a microcapillary. The filaments were washed 3-4 times in Z8 medium (Kotai, 1972) and put in wells on microtiter plates filled with 3 mL Z8 medium. The successfully grown strains were then transferred 2 times and maintained in 300 mL CELLSTAR (Greiner Bio-One GmbH) cell culture flasks containing 150 mL Z8 medium, at 20 °C under 16:8 h light:dark photoperiod (25 µmol m-2 s-1). Species were determined on the basis of morphological traits according to Komárek and Zapomělová (2007). The dimensions of a minimum of 25 vegetative cells, heterocytes and akinetes in *D. lemmermannii* cultures were measured for each lakes, using a LEICA DM2500 light microscope.

Besides the above new 13 strains, further 12 *D. lemmermannii* strains maintained by the NIVA-Culture Collection of Algae were

analysed. This group included individuals isolated between 1981 and 2008 in Lake Nehmitzsee (Northern Germany) and in 8 lakes located in different Norwegian regions (Table 1).

The ultrastructural morphological characterisation was carried out in two *D. lemmermannii* strains isolated in lakes Garda and Como in October 2014 by TEM (Transmission Electron Microscopy). The samples of *D. lemmermannii* were collected together with some of the growth medium and fixed overnight in 1.25 % glutaraldehyde at 4° C in filtered sea water, then post-fixed in 1% OsO4 in 0.1 M phosphate buffer (pH 6.8) for 1 hr. After dehydration in an ethanol series and a propylene oxide step, the samples were embedded in Spurr's epoxy resin (Spurr, 1969). At each step the cells were sedimented with a 5 minutes centrifuge step at 1500 g and only the sediment was used for the following step, in order to substitute safely the various solvents with micropipettes without losing cells. Transverse sections approximately 80 nm thick were cut with a diamond knife and a Reichert-Jung ULTRACUT ultramicrotome. The sections were then stained with uranyl acetate, lead citrate, and then examined with a Philips EM300 TEM at 80 kV.

Cyanotoxins analysis

For the determination of intracellular concentration of cyanobacterial toxins, 100 mL of each growing cultures were filtered through 1.2 µm GF/C filters (Whatman - GE Healthcare Life Science) and frozen at -20°C. Cyanotoxins were extracted by water:methanol 30:70 v/v - 0.1% formic acid solution, according to Cerasino et al. (2016). The analysis of toxins extracted from filtered cultures was performed by liquid chromatography-mass spectrometry (LC-MS), using a Waters Acquity UPLC system coupled to a SCIEX 4000 QTRAP mass spectrometer equipped with a turbo ion spray interface. The analysis was performed for microcystins (MCs), anatoxin-a (ATX), nodularin (NOD), cylindrospermopsins (CYN), and saxitoxins (STX).

The LC-MS protocols for MC, NOD, ATX, CYN are described in details in Cerasino and Salmaso (2012) and Cerasino et al. (2016). The protocol for STX was adapted from Dell'Aversano et al. (2005) and was conducted using an Ascentis Express OH5 (2.7 µm particle size, 50 x 2.1 mm) column (kept at 20 °C) (Cerasino et al. submitted). Quantification was performed using calibration curves obtained with commercially analytical standards MC (RR, [D-Asp3]-RR, YR, LR, [D-Asp3]-LR, WR, LA, LY, LW, LF), ATX (Tocris Cookson Ltd), NOD-R, CYN (Vinci Biochem), GTX1/4, C1/2, NeoSTX, GTX5, STX and dcSTX (NRC-CNRC, Canada). The limits of quantitation (LOQ) were between 0.5 and 10.0 ng mL-1 (MC congeners), 3.0 ng mL-1 (NOD-R), 0.5 ng mL-1 (ATX), 4.0 ng mL-1 (CYN), between 5.0 and 30.0 ng mL-1 (STX congeners).

Genomic DNA extraction, PCR amplification and sequencing

Fresh culture material from each strain (50 mL) was collected as described in Salmaso et al. (2015a) and stored at -20°C. Genomic DNA was extracted from the cell pellet using Glass beads-acid washed (212-300 µm; 425-600 µm) (Sigma-Aldrich CO., MO, USA) and the E.Z.N.A. SP Plant DNA Kit (Omega Bio-Tek Inc., GA, USA).

PCRs were executed on an Eppendorf Mastercycler ep (Eppendorf AG, Germany) using the DyNazyme II DNA Polymerase enzyme (Thermo Scientific). The amplification of the 16s rRNA gene (ca. 1700 bp) was performed with the primers pA (Edwards et al., 1989) and 23S30R (Taton et al., 2003) as previously described (Gkelis et al., 2005). The *rpoB* gene (ca. 580 bp) was amplified with the primers rpoBanaF and rpoBanaR (Rajaniemi et al., 2005), following PCR conditions and protocol reported in Salmaso et al. (2015a).

Amplified products of 16s rRNA and *rpoB* genes were purified by Exonuclease plus Shrimp Alcaline Phosphatase (ExoSAP) and sequenced using a BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Germany), according to the manufacturer's instructions. The 16S rRNA gene was sequenced with the internal primers 16S544R, 16S1092R and 16S979F (Rajaniemi-Wacklin et al., 2006), whereas the sequencing of the *rpoB* gene was performed with the same

primers used in the PCR. The purified products (Agencourt CleanSEQ® Kit, Beckman) were run on an Automated Capillary Electrophoresis Sequencer 3730XL DNA Analyzer (Applied Biosystems, Germany). The sequences were analysed by Chromatogram Explorer 3.3.0 (Heracle Biosoft) and forward and reverse strands assembled using the Contig Assembly Program (CAP) in BioEdit 7.2.5 (Hall, 1999). After formatting with Sequin 15.10 (NCBI), final sequences were deposited to the European Nucleotide Archive (ENA) under the accession numbers listed in Table 1.

The isolated strains were also checked by PCR for the potential production of MCs and ATX. The presence of the *mcyE* gene, which is part of the microcystin synthetase gene cluster, was determined using the primer pairs mcyE-F2/R4 (Rantala et al., 2004), as described previously (Shams et al. 2015). The presence of the polyketide synthase (*anaF*) gene of the ATX cluster was screened using the atxoaf and atxoar primers (Ballot et al., 2010, 2014) and Anabaena 37 (UHCC) as positive control (Rantala-Ylinen et al., 2011).

Analysis of NIVA-CCA strains

Fresh culture material was collected and genomic DNA was extracted according to Ballot et al. (2014). The 16s rRNA gene was amplified with the primer described in Ballot et al. 2010. PCR conditions and sequencing are described in Ballot et al. (2010). The *rpoB* gene was amplified and sequenced as reported above.

The same strains were tested for the presence of *mcyE* and *anaF* genes by PCR, using primers and protocols described above. In the strains that showed positive amplification, the *mcyE* gene was sequenced using the same primers as for PCR. All the amplified genes were sequenced on an ABI 3100 Avant Genetic Analyzer using the BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany). Sequence data were submitted to ENA under the accession numbers reported in Table 1.

All the 12 strains were analysed for MCs content using the Abraxis Microcystins/Nodularins (ADDA) ELISA Kit.

Phylogenetic analysis

The phylogeny and taxonomic position of the *D. lemmermannii* strains were analysed using the 16S rRNA gene (Fig. 2). Overall, among the 13 strains isolated in this study and the 4 strains described before by Salmaso et al. (2015a), only one for each lake was chosen for the analysis. Conversely, all the 12 NIVA-CCA strains were included (Table 1). 34 additional Nostocales sequences (Table S1) from INSDC were included in the phylogenetic analysis of the 16s rRNA and *Cylindrospermum stagnale* PCC 7417 (NR_102462) was used as outgroup. The analysis was entirely realized by R software (R Core Team 2013). Sequences were first aligned using MUSCLE (Edgar, 2004), whereas poorly aligned positions and divergent regions were removed by Aliscore (Misof and Misof, 2009); finally, sequences were trimmed to equal length (1001 positions alignment). Phylogenetic trees were computed by Maximum Likelihood (ML) using PhyML 3.1 (Guindon et al., 2010) and approximate likelihood-ratio test based branch support (aLRT, SH-like; Anisimova and Gascuel 2006). The best-fitting evolutionary models, evaluated by the phymltest in the R package ape (Guindon et al. 2010; Paradis et al. 2004), indicated HKY85+I+G as the best substitution models for the 16S rRNA alignment.

Additionally, the phylogeny of *D. lemmermannii* was investigated by aligning the 16S rRNA gene sequences (1070 bp) of 29 *D. lemmermannii* strains included in the previous tree, which sequences showed high homology (Tables 1, S3; Fig. 4). The unrooted tree was computed by ML as described above.

Statistical analysis

The selected 29 strains were used to explore the biogeography of *D. lemmermannii* by statistical analysis (R software) (Figures 4, 5). A data set with 16S rRNA of 1070 positions was used to compute a matrix of pairwise distances from DNA sequences by applying the K80 evolutionary model, and a principal coordinate analysis (PCoA) was performed (package ape; Paradis et al. 2004). The spatial location of each strain was visualized on a

map, plotting longitude and latitude variables by the package ggmap (Kahle and Wickham, 2013). Dissimilarity matrices of the geographic distances and genetic distances between strains were computed by, respectively, packages gdistance (van Etten, 2015) and ape (K80 model; Paradis et al. 2004). A Mantel statistic, based both on Pearson's and Spearman's correlations, was applied to evaluate the significance of the correlation between the two dissimilarity matrices, using the package vegan (Oksanen et al., 2015).

Acknowledgements

The activity was supported by PhD fellowship to C. Capelli from the E. Mach Foundation – Istituto Agrario di S. Michele all'Adige, and by STSM COST (ES1105 CYANOCOST) grant. Investigations in the deep subalpine lakes were made in the framework of the LTER (Long Term Ecological Research) Italian network, site "Southern Alpine lakes", IT08-000-A (http://www.lteritalia.it/). In particular, we want to thank our colleagues G. Morabito (CNR-ISE), F. Buzzi (ARPA Lombardia) and B. Leoni (University of Milan). We thank the European Cooperation in Science and Technology COST Action ES1105 CYANOCOST for networking and knowledge-transfer support. We are grateful to our colleagues in FEM, for their support in the field and laboratory activities, and to the ARPA Veneto (G. Franzini) for logistic support in the field.

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Supplementary data

Table S1. Main characteristics of the lakes where strains of *D. lemmermannii* were isolated.

Water Body	Country	Latitude	Longitude	Altitude (m)	Max. depth (m)	Size (Km2)	Trophic status	References
Lake Garda	Italy	45.66	10.69	65	350	368	Oligo-mesotrophic	Salmaso and Mosello (2010)
Lake Iseo	Italy	45.72	10.07	186	251	62	Meso-eutrophic	Salmaso and Mosello (2010
Lake Como	Italy	46.04	9.28	198	410	146	Oligo-mesotrophic	Salmaso and Mosello (2010
Lake Maggiore	Italy	45.98	8.67	193	370	213	Oligotrohic	Salmaso and Mosello (2010
Lake Lugano	Italy	46.01	9.02	271	288	28	Eutrophic	Salmaso and Mosello (2010
Lake Caldonazzo	Italy	46.02	11.24	449	49	5.6	Eutrophic	Gaggino and Cappelletti (1984)
Starnberger See, Bayern	Germany	47.91	11.31	584	127,8	56.4	Mesotrophic	Ernst *et al*. (2009)
Ammersee, Bayern	Germany	48.01	11.13	533	81,1	46.6	Mesotrophic	Ernst *et al*. (2001, 2009); Teubner *et al*. (2003)
Nehmitzsee, Brandenburg	Germany	53.13	12.99	60	18.6	1.71	Mesotrophic	Nixdorf *et al*. (2004)
Lake Edlandsvatnet, Rogaland	Norway	58.76	5.87	104	37	2.11	Oligo-mesotrophic	Molversmyr and Bergan (2010)
Lake Bergesvatnet, Hordaland	Norway	59.64	5.21	11	20	0.6	Oligo-mesotrophic	Oredalen (2002); Vannmiljø (2016)
Lake Arefjordvatnet, Hordaland	Norway	60.36	5.15	20	-	0.11	-	No reference available
Lake Storavatn, Lindås, Hordaland	Norway	60.65	5.27	20	-	1.96	Oligo-mesotrophic	Johnsen (2012)
Lake Hallevatnet, Vestfold	Norway	59.03	9.91	48	57	3.58	Oligo-mesotrophic	Skulberg (1990)
Lake Storvatnet, Smøla, Møre & Romsdal	Norway	63.62	10.20	20	5	0.38	Mesotrophic	Skulberg (1995)
Lake Steinsfjorden, Buskerud	Norway	60.10	10.32	63	24	13.9	Mesotrophic	Halstvedt *et al*. (2007)
Lake Søndre Heggelivatnet, Buskerud	Norway	60.06	10.49	488	34	1.5	Oligotrophic	Brabrand (2007); Holtan and Skulberg (1964); Hagman (2016)

Table S2. GenBank accession numbers of the 16s rRNA gene of Nostocales strains included in phylogenetic analysis (Fig. 2)

Taxon	Strain	16s rRNA
Dolichospermum lemmermannii	FEM_GDL2	LN871456
Dolichospermum lemmermannii	FEM_IDL5	LN871463
Dolichospermum lemmermannii	FEM_CDL1	LN871466
Dolichospermum lemmermannii	FEM_MDL2	LN871469
Dolichospermum lemmermannii	BCAna0005	DQ023199
Dolichospermum lemmermannii	TAC438	AB551444
Dolichospermum lemmermannii	TAC437	AB551443
Dolichospermum lemmermannii	str. Ana-Dalai	AY701571
Dolichospermum lemmermannii	04-42	FM242086
Dolichospermum lemmermannii	256	AJ293113
Dolichospermum lemmermannii	1tu32s11	AJ630424
Dolichospermum lemmermannii	202A2	AJ293104
Dolichospermum cf. *lemmermannii*	262	AJ293114
Dolichospermum lemmermannii	29-2	EF634475
Dolichospermum lemmermannii	CYN80	JQ687338
Dolichospermum lemmermannii	04-38	FN691917
Dolichospermum lemmermannii	04-24	FN691916
Dolichospermum lemmermannii	66A	AJ133157
Anabaena sp.	90	AJ133156
Dolichospermum flos-aquae	1tu30s4	AJ630422
Dolichospermum flos-aquae	NIVA-CYA_656	HG970731
Dolichospermum flos-aquae	PCC 9302	AY038032
Aphanizomenon flos-aquae	1tu26s2	AJ630443
Aphanizomenon flos-aquae	PMC9707	AJ293130
Dolichospermum cf. *crassum*	1tu27s7	AJ630413
Dolichospermum circinale	1tu30s11	AJ630416
Dolichospermum circinale	05-09	KC297501
Dolichospermum mucosum	1tu35s5	AJ630425
Dolichospermum planctonicum	1tu28s8	AJ630430
Dolichospermum sigmoideum	0tu36s7	AJ630434
Dolichospermum smithii	1tu39s8	AJ630436
Dolichospermum spiroides	1tu39s17	AJ630440
Dolichospermum affine	05-03	FN691906
Aphanizomenon gracile	NIVA-CYA_676	HG917861
Aphanizomenon gracile	219	AJ293124
Aphanizomenon gracile	PMC9402	AJ293127
Dolichospermum mendotae	57	AJ293107
Dolichospermum mendotae	TAC583	AB551481

Table S3. Geographic origin (see Fig. 3), year of isolation, toxicity, and GenBank accession number of the 16S rRNA gene of *D. lemmermannii* strains included in the phylogenetic analysis of Fig. 4.

Taxon	Strain	16s rRNA	Water Body	Country	Country code	Latitude	Longitude	Year	MCs
D. lemmermannii	FEM_GDL2	LN871456	Lake Garda	Italy	IT_G	45.66	10.69	2013	–
D. lemmermannii	FEM_IDL5	LN871463	Lake Iseo	Italy	IT_I	45.72	10.07	2014	–
D. lemmermannii	FEM_CDL1	LN871466	Lake Como	Italy	IT_C	46.04	9.28	2014	–
D. lemmermannii	FEM_MDL2	LN871469	Lake Maggiore	Italy	IT_M	45.98	8.67	2014	–
D. lemmermannii	BCAna0005	DQ023199	Cotswold Water Park	United Kingdom	UK_CWP	-42.83	172.31		NA
D. lemmermannii	04-42	FM242086	Svet fishpond	Czech Republic	CR_Sv	48.99	14.75	2004	NA
D. lemmermannii	04-38	FN691917	Senecky fishpond	Czech Republic	CR_Se	49.78	13.39	2004	NA
D. lemmermannii	04-24	FN691916	Husinec reservoir	Czech Republic	CR_Hu	60.88	24.33	2004	NA
D. lemmermannii	1tu32s11	AJ630424	Lake Tuusulanjarvi	Finland	FI_Tu	-38.02	176.53	2001	–
D. lemmermannii	202A2	AJ293104	Lake Vesijarvi	Finland	FI_Ve	51.65	-1.97	1987	+
D. lemmermannii	66A	AJ133157	Lake Kiikkara	Finland	FI_Ki	61.03	25.59	1986	+
D. lemmermannii	29-2	EF634475	Lake Rotoehu	New Zealand	NZ_Ro	49.04	13.98	2006	NA
D. lemmermannii	CYN80	JQ687338	Sylvia flats-Alpine Lake	New Zealand	NZ_SF	60.44	25.05	2010	NA

+ detected; - not detected; NA not available

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ACKNOWLEDGEMENTS

I am grateful to my supervisors Dr. Nico Salmaso and Prof. Alessio Papini, my colleagues in FEM, and my co-author Dr. Andreas Ballot for their support in my PhD research. I thank the colleagues of ARPA Veneto for the logistic support on the field.
I gratefully acknowledge the financial support of Fondazione E. Mach – Istituto Agrario di S. Michele all'Adige, for PhD fellowship, and COST action (ES1105 CYANOCOST) and IRSAE for funding my working travels.