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

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A B S T R A C T

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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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 rec reational 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 ex istence 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 sam ples collected during the warmer months, detectable con centrations 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 pro ducers 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) × 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 com pound, 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 per formed 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 spectropho tometry with a NanoDrop ND 8000 (Thermo Fisher Scientific Inc., MA, USA).

Taxonomic identification and phylogenetic analyses of filamentous cyanobacteria were carried out by the amplifica tion 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 vol ume 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 Sci entific), and 1 μl of DNA templates. Genomic DNA concentra tions 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 fol lowed 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 iden tification 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 pro tocols 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 Chro matogram 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*		μg L ^{1}		μg 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 synthe tase 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 iso lated 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 as sembly 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

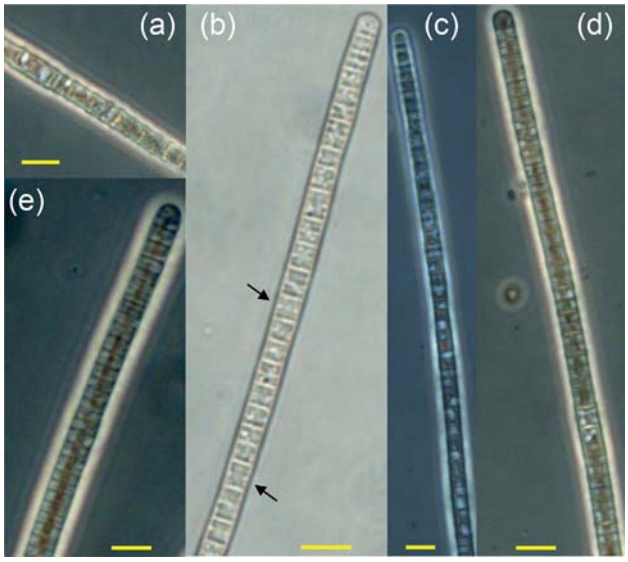

Fig. 1 – Micrographs of *Tychonema* isolated from samples collected in Lake Garda in (a) February, (b, c) March and (d, e) April. Scale bars 10 μm. From (a) to (e), filaments are 5.7, 5.8, 5.2, 5.3 and 6.8 μm wide, respectively. The arrows in (b) indicate the centripetal formation of the cross wall.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

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.

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

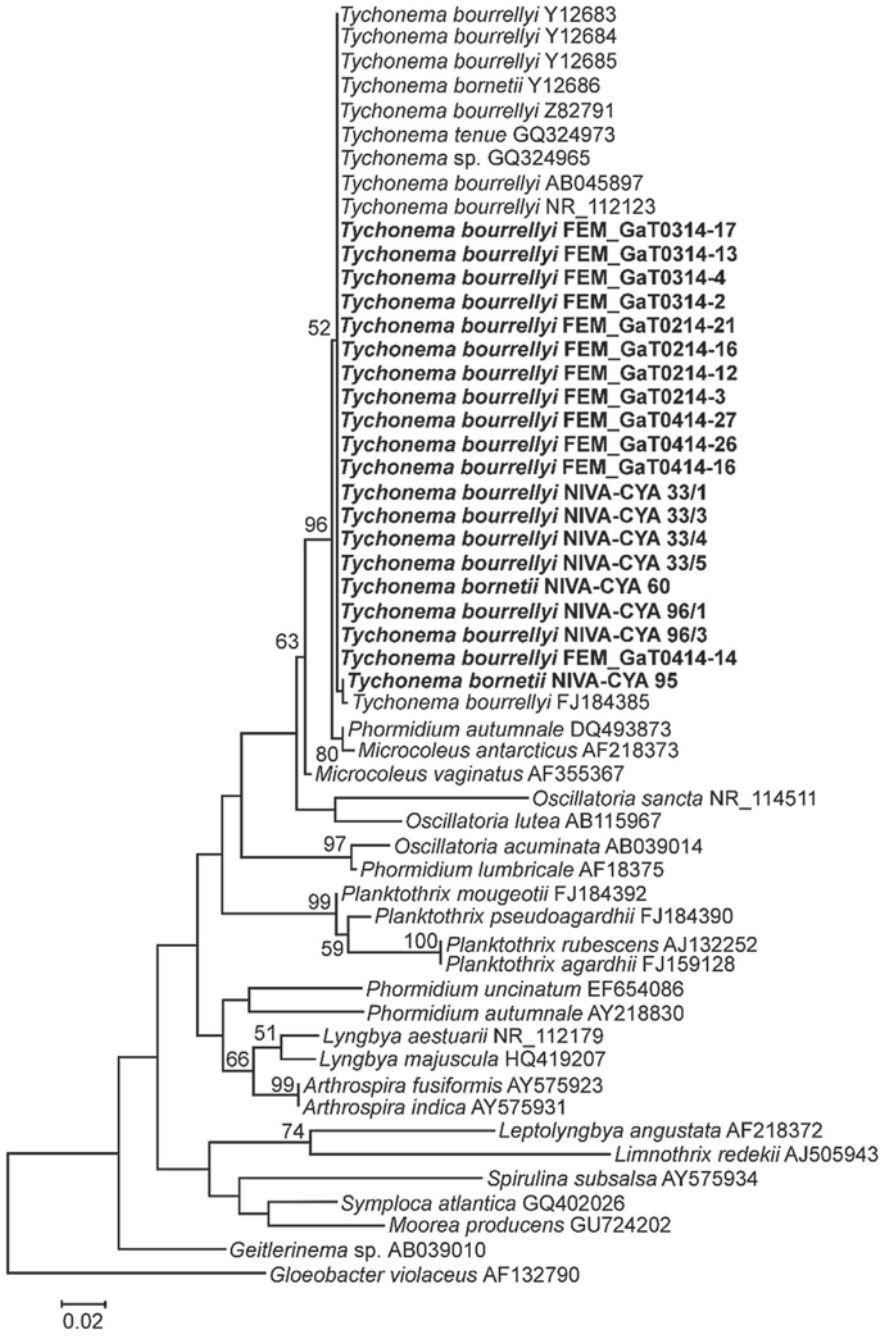

Fig. 2 – Maximum likelihood tree determined on the basis of partial 16S rRNA gene sequences of 53 Oscillatoriales strains. Outgroup *Gloeobacter violaceus* (AF132790). Strains from this study are marked in bold. Bootstrap values above 50 are included. The scale bar indicates 2% sequence divergence.

allowed amplifying the *anaC* 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 *anaC* PCR products were identified in all the non ATX producing strains, and in the 3 culture samples with very low concen trations of ATX (<0.1 μg L^{-1}).

A representative picture of the *anaC* 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.*

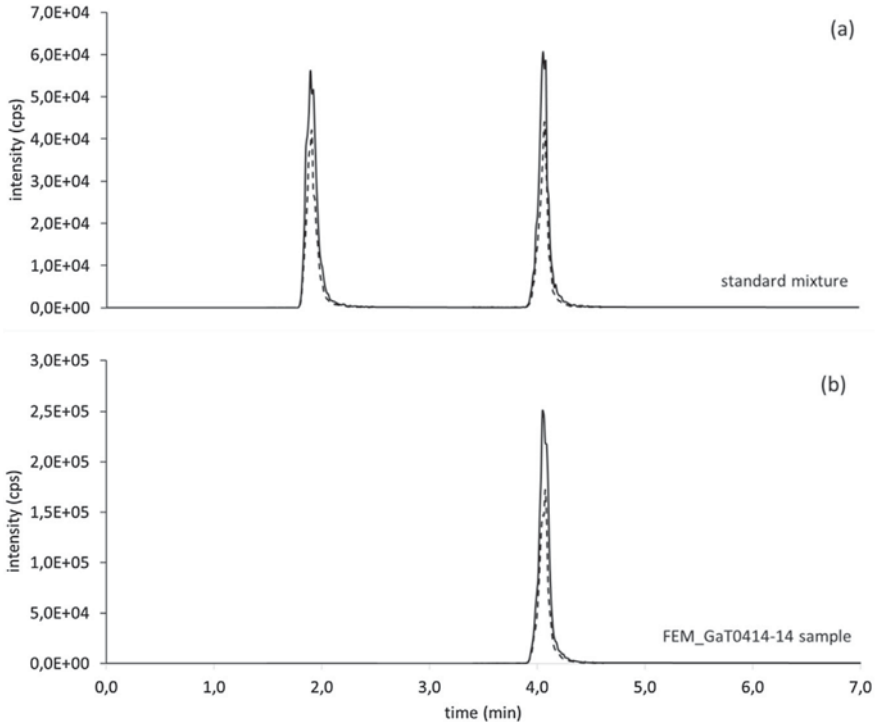

Fig. 3 – Liquid chromatography–mass spectrometry chromatograms resulting from the injections of a mixture of pure standards of cylindrospermopsin (50 ng/mL) and anatoxin-a (165 ng mL ^{1}) (panel a) and of an extract of a *Tychonema* culture (panel b). For each toxin, the traces of the two monitored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

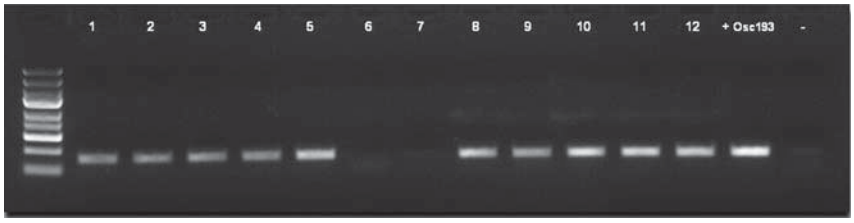

Fig. 4 – PCR products amplified with anac-osc primers (Rantala-Ylinen et al., 2011b). Samples from 1 to 12 are coded as in Table 4. "+" positive (Osc-193 UHCC strain) and " " negative controls. Size of the ladder (in base pairs): 100, 300, 500, 750, 1000, 1500, 2000, 3000, 5000. The amplicon products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

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