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A comparative study of the metabolic profiles of common nuisance cyanobacteria in southern perialpine lakes

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ABSTRACT

This work allowed the comparison of the metabolic profiles of the most important cyanobacteria species in southern perialpine lakes, namely *Aphanizomenon flos-aquae*, *Dolichospermum lemmermannii, Microcystis aeruginosa, Planktothrix rubescens*, and *Tychonema bourrellyi.* Monospecific cultures were obtained from samples of 3 different natural lakes (Garda, Idro, and Caldonazzo). LC-MS/MS analyses were conducted on strains. A first set of experiments was aimed at assessing the presence of the best known toxins (microcystins, nodularins, (homo)anatoxin-a, cylindrospermopsins, paralytic shellfish poisons) in the cultures. Results of this screening study revealed that *M. aeruginosa* and *P. rubescens* produced toxic peptides (microcystins), *T. bourrellyi* produced toxic alkaloids (anatoxin-a and possibly some paralytic shellfish toxins), *Aph. flos-aquae* and *D. lemmermannii* did not produce any of the analyzed toxins. *M. aeruginosa* and *P. rubescens* showed typical microcystin production with LR form dominant in the former, and RRdm form dominant in the latter. A second set of experiments was aimed at comparing the capability of the 5 cyanobacterial species to produce peptidic secondary metabolites. For this purpose, an untargeted peptidomic analysis was conducted on the strains. The analysis allowed revealing globally 328 metabolites, spanning in a mass range between 400 and 2000 Da. The majority of compounds with masses in the 500-1200 Da range (corresponding to the majority of peptidic secondary metabolites) resulted to be produced by *M. aeruginosa* and *P. rubescens* strains, thus indicating a higher ability of these species to produce non-ribosomal peptides compared to the others. 27 metabolites out of 328 could be putatively assigned to specific classes of compounds: microcystins, aeruginosins and anabaenopeptins were the most represented classes of compounds, and were mostly found in *M. aeruginosa* and *P. rubescens* strains.

Key words: Cyanobacteria; perialpine lakes; metabolic profiles; cyanotoxins; LC-MS.

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INTRODUCTION

Cyanobacteria are ubiquitous microorganisms, which cause environmental and health issues in lakes and reservoirs. The massive development of cyanobacteria alters the bio-chemical equilibrium in the water basin, possibly resulting in a deterioration of the water quality. Environmental alterations caused by climate changes, eutrophication and hydrological changes are considered the major factors favoring the dominance and the spreading of cyanobacteria in freshwaters (Schindler, 2006; Paerl and Huisman, 2009; Schopf, 2012; Hamilton *et al.*, 2016).

Many blooms-forming cyanobacteria have the potential of producing toxic secondary metabolites, which cause serious illnesses in case of human exposure (by ingestion, inhalation or skin contact). For this reason, many countries have issued specific regulations for preventing human exposure to cyanobacterial toxins both from drinking and recreational activities (Welker and von Döhren, 2006; IARC, 2010; Chorus, 2012).

Most of the bioactive metabolites produced by cyanobacteria can be classified in two major chemical classes: peptides and alkaloids. Microcystins (MCs) and Nodularins (NODs) belong to the former class with MCs representing the most common toxins. Anatoxins (ATXs), cylindrospermopsins (CYNs) and Paralytic Shellfish Poisons (PSPs) represent instead the most common toxic alkaloids found in cyanobacteria. The just cited compounds have attracted a lot of interest by the research community which has led to the development of efficient analytical procedures, and standardized extraction and analysis protocols are already available or on the way to be so (Codd, 1995; Zurawell *et al.*, 2005; van Apeldoorn *et al.*, 2007). Other bioactive compounds, sometimes produced by cyanobacteria in comparable amounts with the toxins reported above, have gained less interest and, consequently, there is a very limited knowledge about their occurrence. For example, anabaenopeptins, aeruginosins, microginins and microviridins are metabolites that are potentially toxic for mammals (Shin *et al.*, 1995; Neumann *et al.*, 1997;

Murakami *et al.*, 2000; Blom *et al.*, 2006; Bubik *et al.*, 2008; Ersmak *et al.*, 2008). Since they can contribute to the total toxicity of a given cyanobacteria population, the lack of information about their presence can potentially bias a correct risk assessment. Besides the health issue aspects, some of these metabolites have attracted interest for biotechnological applications, for example as source of new therapeutic agents (Sivonen and Borner, 2008; Anas and Harada, 2016).

Genetic diversity in cyanobacteria is reflected also in the secondary metabolites profiles. Different species exhibit different metabolic profiles, but also different strains of the same species can show substantial differences in the metabolites profile (Moore, 1996; Janse *et al.*, 2005; Kardinaal *et al.*, 2007; Yepremian *et al.*, 2007; Rohrlack *et al.*, 2008; Agha *et al.*, 2014). In addition, genetically identical organisms can exist as different chemotypes. This might be due to the high plasticity of the metabolic pathways, which can re-arrange with no or minimal genetic changes (Fischbach *et al*., 2008; Nikolouli and Mossialos, 2012).

Lakes in the southern perialpine region are experiencing a change in their cyanobacteria composition and structure. Five toxic species are commonly found in these lakes: *Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault, *Dolichospermum lemmermannii* (Richter) P.Wacklin, L.Hoffmann & J.Komárek, *Microcystis aeruginosa* (Kützing) Kützing, *Planktothrix rubescens* (De Candolle ex Gomont) Anagnostidis & Komárek, and *Tychonema bourrellyi* (J.W.G.Lund) Anagnostidis & Komárek. *T. bourellyi* has appeared in recent years and is replacing other species (Salmaso *et al.*, 2016). Changes in cyanobacteria populations lead to changes in toxic potential, as different species have a very different toxin diversity. These changes have therefore a great impact on the management of the risk connected with cyanobacterial blooms. *T. bourrellyi* for example is an ATX producer that is becoming dominant in lakes formerly dominated by MC producers (Salmaso *et al.*, 2016). In order to have a clear picture of the toxic potential of these five species, we have conducted a detailed investigation aimed at identification of the toxins produced by them. We used sensitive and selective LC-MS/MS techniques for the analysis of a broad spectrum of known toxins (MCs, NODs, ATXs, CYNs, and PSPs). In order to get more information on the secondary metabolites of these species and possibly identify additional bioactive ones, we analyzed the peptidomic profiles of the five species.

METHODS

Isolation of strains and culturing methods

Samples were collected from three lakes located in the Eastern Italian perialpine area: lakes Garda, Idro and Caldonazzo. Strains of *P. rubescens*, *T. bourrellyi*, and *D. lemmermannii* were isolated from Lake Garda in summer/early autumn of 2014. Strains of *Aph. flos-aquae* and *M. aeruginosa* were isolated, respectively, in April 2014 from Lake Idro and in August 2015 from Lake Caldonazzo. Water samples were collected in the deepest point of the basins, by vertical tows from 30 m to the surface with 25 cm diameter plankton net (80 µm mesh) and maintained at 20°C until processed (within 24 h). Single strains were isolated under a macroscope (WILD M420) using a microcapillary and identified according to Komárek and Anagnostidis (2005). After 3-4 washings in Z8 medium (Kotai, 1972), strains were grown, under non-axenic conditions, in microtiter plates filled with 3 mL Z8 medium, and then transferred to the final volume of 150 mL Z8 medium in cell culture flasks (CELLSTAR, Greiner Bio-One GmbH, Kremsmünster, Austria). *Aph. flos-aquae* was grown in Z8 medium without nitrogen (Kotai, 1972). All cultures were grown in climatic simulation chamber (ProClimatic, Imola, BO, Italy) at 20°C, using continuous light (25 µmol m^{-2} s^{-1}) in the case of *P. rubescens*, *T. bourrellyi*, and *Aph. flos-aquae*, and a 16:8 h light:dark photoperiod in the case of *D. lemmermannii* and *M. aeruginosa*. Culturing conditions were those allowing an optimal growth for each cyanobacterial species.

Metabolites extraction and LC-MS/MS analysis

All solvents and reagents used in the following procedures were of LC-MS grade and where all provided by Sigma-Aldrich (Milan, Italy), if not otherwise specified. LC-MS/MS analysis were performed using a Waters Acquity UPLC system, directly coupled to a SCIEX 4000 QTRAP hybrid mass spectrometer equipped with a turbo ion spray interface.

Biomass was harvested from the culture by filtration (between 200 and 250 mL) on 1.2 µm GF/C filters (Whatman-GE Healthcare Life Sciences, Little Chalfont, UK). Extraction of metabolites was achieved by extraction with 6 mL of acetonitrile/water mixture (60/40 v/v) containing 0.1% formic acid; the sample was firstly homogenized (Omni TH probe homogenizer, Omni-Inc., Kennesaw, GA, USA) for 5 min and then sonicated (Omniruptor4000 probe sonicator, Omni-Inc.) for 4 min, using 160W power in pulsed mode (50%). The solution was separated from the pellet by centrifugation (EBA 20, Hettich, Tuttlingen, Germany) for 6 min at 9850 G. The pellet was then treated with a second aliquot of extraction mixture (6 mL) and sonicated. After centrifugation, the two aliquots were put together and concentrated in a centrifugal evaporator (MivacDuo, Genevac Ltd., Ipswich, UK) down to approximately 2 mL. Acetonitrile was then added up to reach a 60/40 ratio between organic solvent and water. The solution was then filtered on 0.2 µm pore size RC syringe filters (Phenomenex, Castel Maggiore, BO, Italy), and analyzed by LC-MS/MS.

Targeted MCs and NODs analysis was performed using reverse phase chromatography, using a Phenomenex Kinetex XB-C18 column (1.7 µm particle size, 2.1×50 mm). The mass detector was operated in the positive Electro Spray mode (ESI+) using the Multiple Reaction Monitoring (MRM) scanning mode. The method was optimized for the detection of commercially available analytical standards MC-RR, [D-Asp^3]-RR, YR, LR, [D-Asp^3]-LR, WR, LA, LY, LW, LF, NOD-R (Sigma-Aldrich Co., St. Louis, MO, USA) and their variants (demethylated forms), and could potentially detect up to 40 MC congeners. A detailed description of the method is reported in Cerasino *et al.* (2016).

Targeted alkaloids analysis was performed by HILIC, using an Ascentis Express OH5 column (2.7 µm particle size, 50x2.1 mm, Sigma- Aldrich Co., USA). Elution was achieved by a binary gradient of eluents A (1% acetonitrile in water, containing 10 mM ammonium formate and 10 mM formic acid) and B (95% acetonitrile in water, containing 10 mM ammonium formate and 10 mM formic acid) according to the following scheme: t=0 (90% B), t=5 min (50% B), t=7 min (90% B), t=8 min (90% B). Flow rate was 0.3 mL min^{-1} and total run time was 8 min. Standard injection volume was 2 µL. Mass detector was operated in scheduled MRM mode using positive electrospray ionization (ESI+). General settings were as follows: ion spray voltage 5000 V, entrance potential 10 V, cell exit potential 10 V, and interface heater temperature 300°C. For toxin identification, two transitions were monitored for each analyte (Tab. 1). The method was set up using certified analytical standards: HomoATX (Novakits, France), ATX (Tocris, UK), CYN (Vinci Biochem, Italy), saxitoxin (STX), decarbamoyl STX (dcSTX), NeoSTX, gonyautoxin 1 (GTX 1), GTX4, GTX5, C1 and C2, (NRC-CNRC, Canada). A reference chromatogram is provided in Fig. 1. Retention times of analytes showed excellent stability over time. The method was suitable for the quantification of these toxins, at least in the working range between 0.2 and 200 µgL^{-1}. Nevertheless, only a semi-quantitative analysis was performed in this investigation owing to the heterogeneity of the cultures. A tentative detection of other PSP congeners not available as pure standards was performed, using transitions and MS-settings (cf. Tab. 1) taken from relevant literature (dell'Aversano *et al.*, 2005; Halme *et al.*, 2012).

Untargeted metabolic profiling experiments were con-

Tab. 1. Chromatographic and mass spectrometric parameters for alkaloids' targeted analysis. $[M+H]^+$ indicates the observed "quasi-molecular" ion for any given toxin. It usually corresponds to the precursor ion in MRM transitions. In some cases, however, other ions are used as precursor because the intensity of the $[M+H]^+$ adducts is too low (C1/2 and C3/4 toxins). For each compound two MRM transitions have been monitored. In the case of the pairs of isomeric compounds GTX1/4 and C1/2, the two transitions of the pairs coincide. Limits of quantification (LOQ) are also reported for each compound.

Toxin	RT (min)	$[M+H]^+$	Precursor ion (*m/z*)	Product ions (*m/z*)	DP (V)	CE (V)	LOQ (µgL^{-1})
HATX	0.82	180	180	145	60	23	2.0
				135	60	23	
ATX	0.98	166	166	149	60	21	0.5
				131	60	24	
CYN	2.50	416	416	194	80	53	2.0
				336	80	32	
GTX1/4	3.48 (GTX1)+3.72 (GTX4)	412	412	332	40	20	15.0
		412	332 ($[M+H-SO_3]^+$)	314	86	27	
C1/2	3.66 (C1)+3.93 (C2)	476	493 ($[M+H+NH_3]^+$)	396	40	13	10.0
		476	396 ($[M+H-SO_3]^+$)	298	80	27	
NeoSTX	4.08	316	316	298	70	25	30.0
				220	70	25	
GTX5	4.10	380	380	300	50	21	5.0
				282	50	25	
STX	4.10	300	300	204	80	35	10.0
				138	80	40	
DcSTX	4.17	257	257	239	80	25	20.0
				126	80	29	
DcNeoSTX*	-	273	273	255, 225, 179	70	25	-
GTX2/3*	-	396	396	378, 316	70	25	-
DcGTX2/3*	-	353	353	335, 273	70	25	-
GTX5*	-	380	380	300, 282	70	25	-
C3/4*	-	492	412 ($[M+H-SO_3]^+$)	332, 314, 138	70	25	-

*Compounds which are tentatively analyzed.

ducted using the same column and eluents of MC/NOD analysis. Differently from MC target analysis, a 15-min gradient was employed: the starting eluent was 20% B, increased to 90% B at 11 min, and finally restored at 20% B at 15 min. The flow rate was 0.25 mL min^{-1}. The mass detector was operated in the positive Electro Spray mode (ESI+) using the Information Depended Acquisition (IDA) mode: a full scan experiment (Enhanced Mass, EMS) in the range 400 - 1100 Da, was used as survey scan; the most intense peaks in EMS (with charge state between 1 and 3) were automatically selected and underwent a high-resolution experiment (Enhanced resolution, ER) and a fragmentation experiment (Enhanced Product Ion). IDA threshold was set at 500,000 cps. EPI spectra were acquired from 50 to 1000 Da with a scan speed of 1000 Da s^{-1} and a collision energy (CE) of 20 V and energy spread (CES) of 10 V. General settings were as follows: ion spray voltage 5000 V, entrance and cell exit potentials 10 V, and interface heater temperature 350°C. The detection of peptidic compounds was enhanced by enabling, in the acquisition software, the built-in algorithm for automatic optimization of CE for each putative peptide according to the specific m/z and charge values. Data acquisition and processing were accomplished using Analyst 1.5.2 and PeakView 2.2 softwares (AB Sciex). After acquisition, spectra were manually filtered to get rid of background organic contaminants (by comparison with a blank sample), and only compounds with high quality ER and EPI data were further considered (a threshold of 30% quality was normally used in PeakView).

Data analysis

Common and distinctive chemical compounds synthesized by the single species (irrespective of strains) and strains were represented and evaluated by using Venn's diagrams. Differences in mass values between species and strains were evaluated by 1-way ANOVA on log-transformed data (Sokal and Rohlf, 1995). The relationships among the single strains based on the putative assigned compounds found in the untargeted metabolic analysis were evaluated by Principal Coordinate Analysis (PCoA) applied to a dissimilarity matrix computed using the Bray & Curtis index. The same dissimilarity matrix was used to identify groups of strains by a cluster analysis (Ward's method) (Legendre and Legendre, 1998). Statistical analyses were carried out using the R statistical software (R Core Team, 2016).

RESULTS

Targeted toxin analysis

The strains were analyzed with LC-MS/MS methods specifically built and tested for detecting the most common

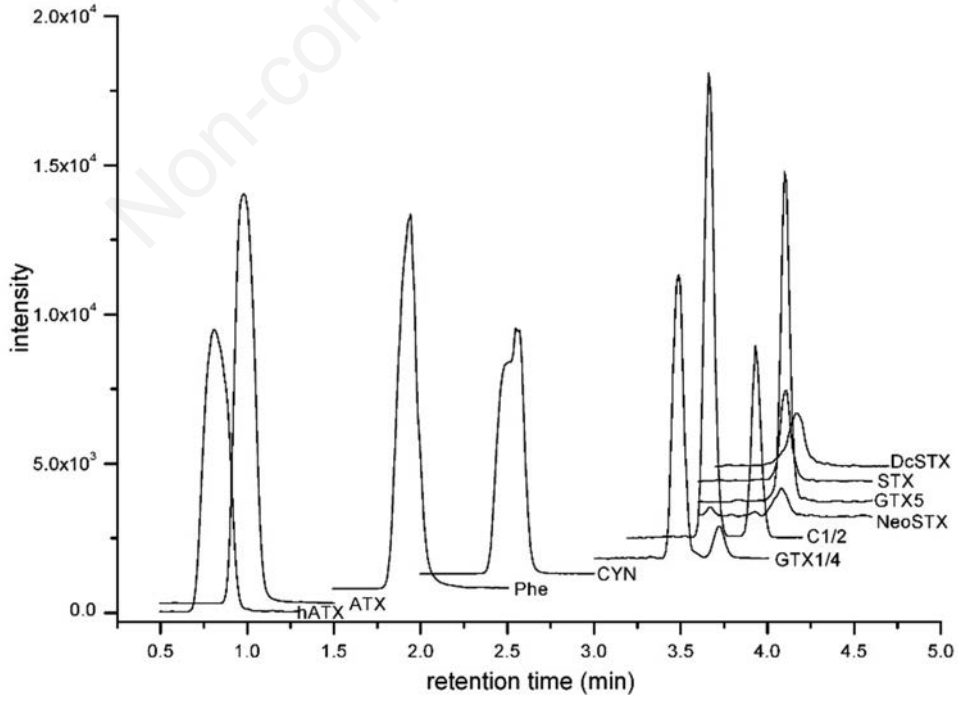

Fig. 1. Representative chromatogram of a standard mixture of alkaloids. Phe is the aminoacid Phenylalanine, which is not toxic but is isobaric with ATX and can be potentially confused with the toxin.

alkaloid and peptidic toxins with high specificity and sensitivity (Cerasino *et al.*, 2016). The results are reported in a schematic view in Tab. 2. Among the 14 strains, 2 out of 3 strains of *T. bourrellyi* tested positive for ATX: Tbour02 and Tbour05. The LC-MS chromatogram of Tbour05 strain is reported in Fig. 2. In the same cultures we could detect two adjacent peaks at about 4.2 min for the transition 412/138 (Fig. 2), which could suggest the presence of C3/4

Tab. 2. Comparative results of the toxin diversity found in the considered strains, obtained with targeted analysis for alkaloids and MCs. In the last column, the number of metabolites found with the untargeted analysis is reported. The presence of C3/4 in strains of *T. bourrellyi* is suspected but has not been confirmed (see text for details).

		Targeted analysis		Untargeted analysis
Species (Lake)	**Strain code**	**Alkaloids**	**MC (%)**	**Metabolites n.**
Aph. flos-aquae (Idro)	Aflos01			*19*
	Aflos03			*22*
	Aflos04			*26*
D. lemmermannii (Garda)	Dlemm14			*49*
	Dlemm16			*74*
	Dlemm21			*42*
M. aeruginosa (Caldonazzo)	MicroC1		*LR (96.6), LRdm (3.2), YR (0.2)*	*36*
	MicroC2		*LR (90.8), LRdm (9.1), YR (0.1)*	*46*
P. rubescens (Garda)	Prube11		*RRdm (83.1), LRdm (16.6), HtyrRdm (0.2), RR (0.1)*	*49*
	Prube17		*RRdm (89.4), LRdm (10.1), RR (0.3), HtyrRdm(0.1), LR (0.1)*	*63*
	Prube23		*RRdm (99.6), LRdm (0.3), RR (0.1)*	*39*
T. bourrellyi (Garda)	Tbour02	*ATX, C3/4(?)*		*27*
	Tbour04			*29*
	Tbour05	*ATX, C3/4(?)*		35

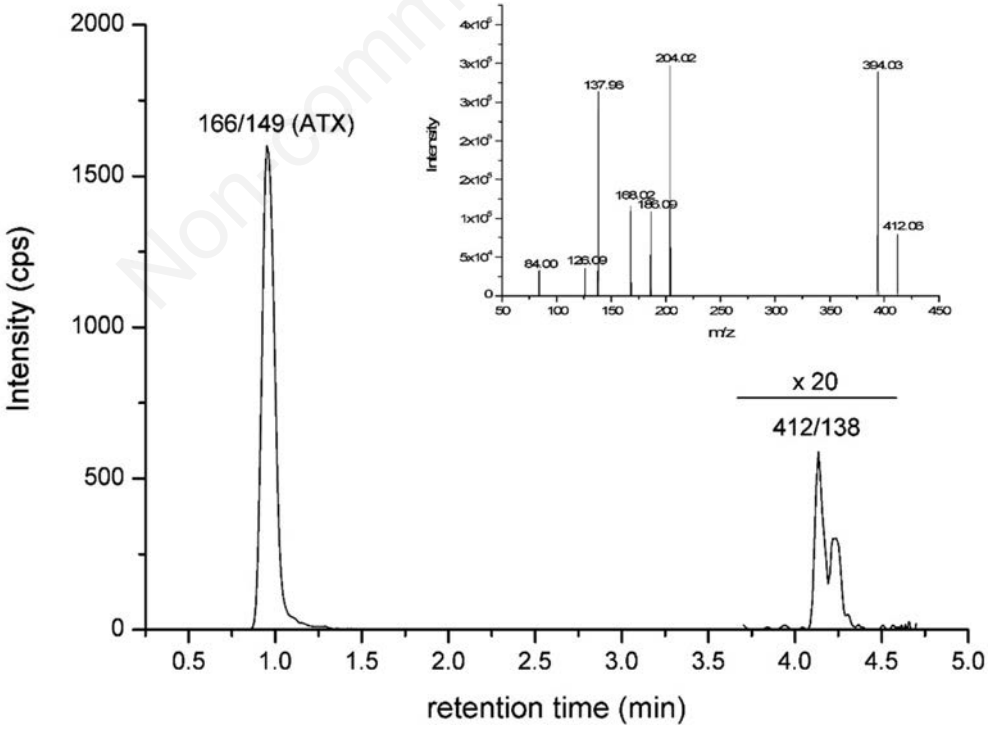

Fig. 2. Chromatogram of the *T. bourrellyi* strain Tbour05, analyzed with the alkaloids-targeted method. The most intense peak (RT 1.0 min) corresponds to ATX. The peaks at RT=4.2 min, are approx. 60 times less intense than ATX and have been tentatively attributed to the C3/4 toxins. The insert contains the EPI (Enhanced Product Ion) spectrum generated by the ion with mass of 412 Da.

toxins (Tab. 1). However, the lack of the other transitions typical of these toxins (412/332 and 412/314) makes this attribution uncertain. The lack of a specific analytical standard did not allow a certain attribution. Looking at data reported in literature about the most common PSP (for example Dell'Aversano *et al.*, 2005), the mass of 412 Da could indicate either the $[M+H]^+$ ion of GTX1/4 or the $[M-SO_3+H]^+$ ion of the C3/4 toxins (Tab. 1). Excluding the GTX1/4, because retention time and fragmentation did not match with those of the pure standard (Tab. 1), we are led to hypothesize that the compounds can correspond actually to the C3/4 toxins. The fragmentation pattern of the compounds (reported in the insert of Fig. 2), shows two peaks which are consistent with the attribution: 412 and 394 Da, respectively attributable to the $[M-SO_3+H]^+$ and $[M-SO_3-H_2O+H]^+$ ions of C3/4. The other intense peaks in the MS/MS spectrum (204, 186 and 138 Da) have been described also for other PSP (namely STX) and are therefore not diagnostic for a particular molecule, but, instead, can confirm the presence of a similar or identical chemical backbone. The absence of fragments corresponding to the loss of two SO_3 groups, as in the case of other di-sulfated PSP, is however in contrast with this attribution. All the other strains did not show any alkaloids.

Peptidic toxins were found exclusively in *P. rubescens* and *M. aeruginosa* strains (Tab. 2). These two species showed a typical MC diversity: RRdm variant was the most abundant in *P. rubescens* (over 83%), followed by LRdm, HtyrRdm, RR and LR. RRdm and LRdm variants together accounted for more than 95.5% of the total MC content. In one strain (Prube23), the two less abundant congeners (HtyrRdm and LR) were not detected. MC-LR, instead, was the prominent variant in *M. aeruginosa* strains (more than 90% of the total), with small amounts of two other congeners, namely LRdm and YR (Tab. 2).

Metabolic profiling

The untargeted analysis provided a list of metabolites (328 different compounds in total) characterized by a dyad of values: mass and RT (retention time). In the individual strains, we found a variable number of metabolites (Tab. 2), from a minimum of 19 to a maximum of 74. Most of the peptidic secondary metabolites produced by cyanobacteria were comprised in the considered mass range (400-1100 Da). Analyzing the distribution of masses, we noted substantial differences among strains (ANOVA, $F_{13,542}=13.0$, $P<0.001$; Fig. 3) and species

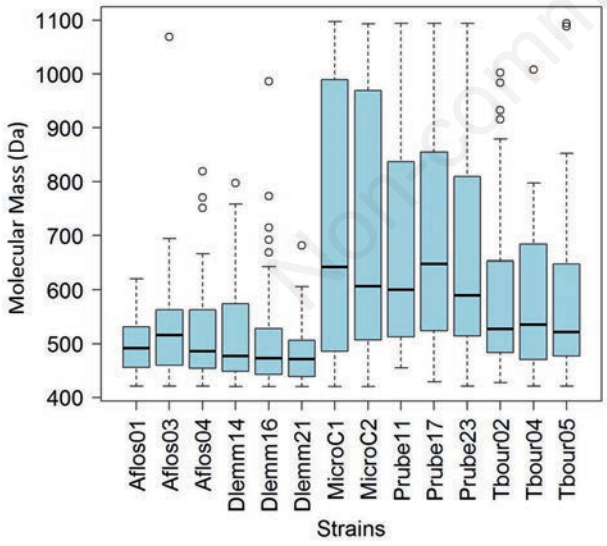

Fig. 3. Boxplot showing the distribution of molecular mass values coming from the untargeted metabolic analysis among the considered 14 strains. Boxes represent 25-75th percentiles (first and third quartiles) with median (line in the middle of the rectangle), whereas the top and lower hinges are versions of the first and third quartile computed as in R Core Team (2016), function boxplot.stats.

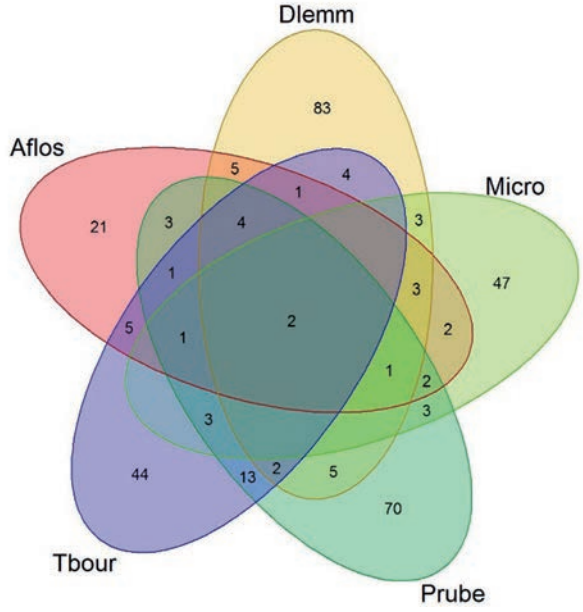

Fig. 4. Venn diagram showing the distribution of the compounds found in the untargeted metabolic analysis among the five different cyanobacterial species. The diagram is based on the couples of *m/z* (mass to charge ratio) and RT (retention time) values. Aflos, *Aphanizomenon flos-aquae*; Dlemm, *Dolichospermum lemmermannii*; Micro, *Microcystis aeruginosa*; Prube, *Planktothrix rubescens*; Tbour, *Tychonema bourrellyi*.

(groups of strains in Fig. 3; ANOVA, $F_{4,551}$=39.9, P < 0.001). In *M. aeruginosa* and *P. rubescens* we found compounds in a wide range of masses, meaning that they produce compounds with very different molecular weights. On the opposite, in *Aph. flos-aquae* and *D. lemmermanii*, we found that the majority of the compounds were in the lower part of the mass range, meaning they produce mainly low molecular weight compounds. In *T. bourrellyi,* finally, we found an intermediate situation.

When attributing the 328 compounds to the producing species (Fig. 4) and to the respective strains (Supplementary Fig. 1), we found only two compounds common to all species, one having mass 470.2 Da (RT 8.12 min), and the other mass 482.3 Da (RT 8.80 min). Most of the compounds (268 out of 328) were exclusively produced by a single species: *D. lemmermannii* and *P. rubescens* had the highest number of exclusive compounds (83 and 70, respectively); *M. aeruginosa* and *T. bourrellyi* had lower figures (47 and 44); *Aph. flos-aquae* had the lowest (21). Only 62 were the compounds produced by two or more species.

As a first attempt to identify some of the compounds, we compared the molecular weights of a subset of the 328 compounds (having molecular masses between 500 and 1200 Da; the list is reported in Supplementary Tab. 1) with those of known compounds. We used online resources (*e.g.,* Norine: http://bioinfo.lifl.fr/NRP/) (Flissi *et al.*, 2016), and literature (Czarnecki *et al.*, 2006; Welker *et al.*, 2006; Rounge *et al.*, 2007; Ersmark *et al.*, 2008; Rohrlack *et al.*, 2008; Briand *et al.*, 2016; Spoof *et al.*, 2016) for finding possible matches. We restricted the search in the mass range typical of cyanobacteria peptidic secondary metabolites: aeruginosins (600-700 Da), microginins (700-800 Da), anabaenopeptins (800 - 900 Da), microcystins and cyanopeptolins (900-1100 Da). Based on molecular weight equivalence and consistency of the MS/MS data (fragmentation characteristics and isotopic pattern), we were able to identify 7 potential peptides. For additional 20, an unambiguous attribution was not possible because, although compounds with the same molecular weight were found in databases, either they had not yet been characterized or multiple alternatives were possible; these compounds were generically indicated with the name of the class (aeruginosin, anabaenopeptin, cyanopeptolin, microcystin, etc.) or with "peptide" when the attribution to one class of peptides was not possible (6 compounds) (Tab. 3). The molecular weights of these 27 compounds were comprised between *approx.* 590 and 1180 Da. The most represented peptides were cyanopeptolins (7 compounds), followed by anabaenopeptins (6 compounds), and by aeruginosins and microcystins (6 compounds each). Interestingly, most of these compounds were found in *P. rubescens* (17) and *M. aeruginosa* (10); fewer compounds were found in *T. bourrellyi* (3) and *D. lemmermannii* (1), and none in *Aph. flos-aquae*.

The PCoA analysis of the distribution of these compounds found in the untargeted metabolic survey allowed us to determine a greater uniformity of metabolites in *M. aeruginosa* and *P. rubescens* compared to *D. lemmermannii* and *T. bourrellyi* (Fig. 5A). However, at a higher level of dissimilarity, the strains Tbour05 and Dlemm14 were not included in their respective species groups (Fig. 5 A,B).

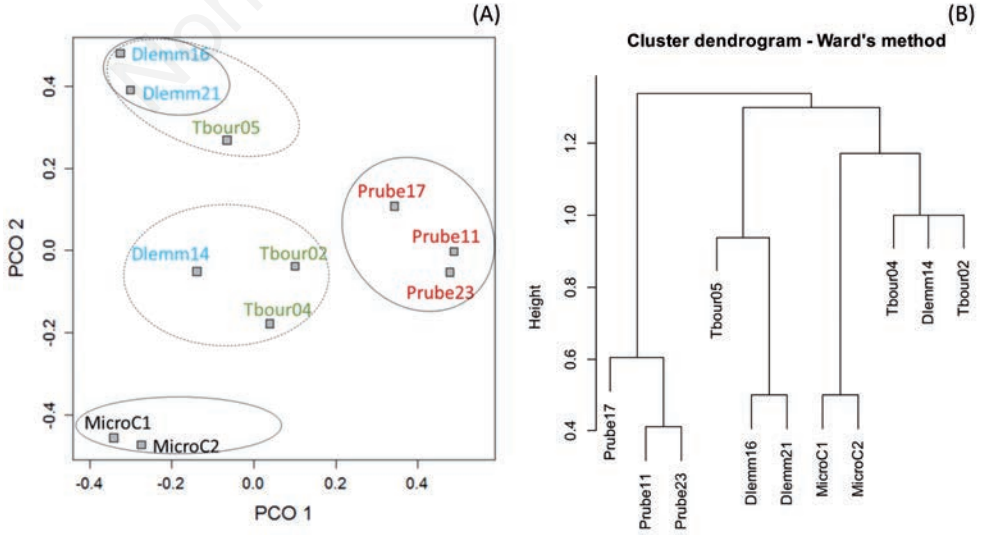

Fig. 5. A) Principal Coordinate Analysis performed on the distribution of putative secondary metabolites within the cyanobacteria strains. The first and second axes account for the 23% and 21% of the total variance, respectively. The continuous and dashed lines enclose together groups of strains at different level of dissimilarity based on the results of the (B) cluster analysis.

DISCUSSION

The five cyanobacterial species considered in this investigation are known to produce toxins. In particular, based on the analysis of isolated strains (Bernard *et al.*, 2016), *Aph. flos-aquae* and *T. bourrellyi* were reported as ATX producers (Sivonen *et al.*, 1989; Osswald *et al.*, 2009; Salmaso *et al.*, 2015; 2016; Shams *et al.*, 2015), whereas *M. aeruginosa* and *P. rubescens* were mainly reported as MC producers (Metcalf and Codd, 2012), and *D. lemmermannii* as MCs and anatoxin-a(S) producer (Sivonen *et al.*, 1992; Onodera *et al.*, 1997). Our targeted analysis confirmed this behavior for *M. aeruginosa* and *P. rubescens*, but not for *Aph. flos-aquae* and *D. lemmermanii.* This was not unexpected, considered that recent investigations had already shown that the populations isolated in the Italian district do not have the capability to produce ATX. *T. bourrellyi* was found to produce ATX, as reported also in recent papers (Salmaso *et al.*, 2015, 2016; Shams *et al.*, 2015). Moreover, based on evidences collected in this work, this species was possibly able to synthesize PSP toxins, possibly sulfated variants (like C3/4 toxins). *M. aeruginosa* and *P. rubescens* strains produced mixtures of different MCs (Tab. 2). The most abundant congeners were LR in *M. aeruginosa* and RRdm in *P. rubescens*. The relative abundances and identities of congeners were in accordance with previous observations (Cerasino and Salmaso, 2012; Salmaso *et al.*, 2014; Cerasino *et al.*, 2016), reporting demethylated MCs (either RR, LR or HtyR) as dominant in *Planktothrix* and LR in *Microcystis*.

The untargeted analysis revealed the presence in the strains of 328 compounds with molecular mass between

Tab. 3. List of putative compounds found in the analyzed strains. Compounds are ordered according to the molecular mass values. Most of the *m/z* values correspond to single charged [M+H] adducts; in some cases, marked with an asterisk, *m/z* values correspond to double charged [M+2H] adducts.

Molecular mass	Observed *m/z* and RT	Putative compound	Strain	Diagnostic fragmentation peaks[#]
592.3	593.3 at 1.37	Aeruginosin	Prube11, Prube17	140, 120,
642.4	643.4 at 4.89	aeruginosin 101	Dlemm16, Tbour05	309, 221, 86
650.4	651.3 at 1.92	aeruginosin 102	Prube11, Prube23	150, 140, 86,
698.3	699.2 at 0.83	anabaenopeptin	Tbour04, MicroC2, Prube11	120, 74
714.3	715.3 at 1.23	aeruginosin 126B	Prube17, Prube23	164, 150
816.3	817.3 at 4.82	anabaenopeptin	Prube11, Prube17, Prube23	120, 72
830.3	831.3 at 1.09	anabaenopeptin	MicroC1	243, 150, 120
836.4	837.4 at 1.25	anabaenopeptin B	Prube11	201, 175
850.3	851.3 at 1.57	anabaenopeptin F	Prube17, Prube23	201, 175
855.3	856.3 at 5.03	anabaenopetin	MicroC2	243, 120
983.4	984.4 at 4.06	cyanopeptolin	MicroC1, MicroC2	243, 150
987.4	988.4 at 7.54	Microcystin Asp3Dhb7-LY	Prube11	375, 213, 135, 107, 86
996.4	997.4 at 3.21	microcystin (L-MeAla7)LR	Prube17	375, 213
997.4	998.4 at 4.61	cyanopeptolin	MicroC1, MicroC2	243, 150, 120
1008.5	1009.5 at 5.17	Microcystin	MicroC1, MicroC2	375, 213, 135
1010.6	506.8 at 5.41*	cyanopeptolin	Prube17, MicroC2	243, 215, 150, 120
1011.4	1012.4 at 3.56	cyanopeptolin	Prube17	243, 150, 120
1023.5	1024.5 at 4.15	cyanopeptolin	MicroC1, MicroC2	150, 120
1030.5	1031.5 at 5.91	Microcystin	Prube11	375, 213, 135
1039.5	520.7 at 4.20*	cyanopeptolin	MicroC2	150
1093.5	1094.5 at 2.53	cyanopeptolin	Prube11, Prube17, Prube23, Tbour05	150, 107, 84
1107.6	554.8 at 3.60*	Peptide	Prube11, Prube17, Prube23	164, 107, 84
1121.7	561.8 at 4.18*	Peptide	Prube23, Prube11	339, 164, 107
1123.7	562.3 at 3.04*	Peptide	Prube17	150, 120, 84
1163.6	582.8 at 3.85*	Peptide	Prube17	164
1179.6	590.7 at 3.40*	Peptide	Prube11, Prube17	164
1182.7	592.3 at 0.88*	Peptide	MicroC2	120

[#]Diagnostic fragmentation peaks: 70 Pro-immonium, 72 Val-immonium, 74 Thr-immonium, 84 Lys-immonium, 86 Leu-immonium, 107 [CH_2PhOH], 120 Phe-immonium, 135 [$PhCH_2CH(OCH_3)$], 140 Choi immonium, 150 MeTyr, 164 MeHty, 175 [Arg+H], 201 [Arg-CO], 213 [Glu-MDha+H], 215 [Ahp-Phe - H_2O - CO], 221 [Leu-Choi], 243 [Ahp-Phe - H_2O], 309 [Choi-Arg - NH_2+H], 339 [MetO-MeHty+H], 375 [Adda-Glu-MeDha+H] (Choi: 2-carboxy-6-hydroxyoctahydroindole; MDha:N-methyldehydroalanine; Adda: 3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid); *double charged [M+2H] adducts.

400 and 2000 Da. Among them, we can have both primary and secondary metabolites. Many of these have fragmentation peaks typical of peptides (immonium ions fragments in the lower mass range) in their MS/MS spectra. These compounds could be either ribosomal or non-ribosomal. *Microcystis* and *Planktothrix* exhibit a greater ability to produce molecules having molecular weight above 550 Da, as can be clearly seen in Fig. 3. This net difference compared to the other species can be related to their remarkable ability to produce different classes of peptidic secondary metabolites (included toxic MCs), as demonstrated by the mass of data available in the literature (Czarnecki *et al.*, 2006; Welker *et al.*, 2006; Rounge *et al.*, 2007; Ersmark *et al.*, 2008; Rohrlack *et al.*, 2008; Briand *et al.*, 2016; Spoof *et al.*, 2016). This is confirmed by the fact that, among the 27 peptides reported in Tab. 3, the majority have been found in *Planktothrix* and *Microcystis* strains. *Aphanizomenon* and *Dolichospermum* produced much fewer peptides, as their secondary metabolism seems to be more oriented on the production of alkaloids (i.e. ATX and HATX). *Tychonema* constitutes an intermediate situation (Fig. 3) as it exhibits a higher ability in producing peptides compared to the *Aphanizomenon* and *Dolichospermum*. Finally, we need to consider that *P. rubescens, T. bourrellyi* and *Aph. flos-aquae* have been grown under different light conditions compared to *D. lemmermannii* and *M. aeruginosa*; therefore, the metabolism of these two groups of organisms could have been differently influenced by this variable.

CONCLUSIONS

The paper allowed the comparison of the toxic potential of five cyanobacterial species common in the lakes of the subalpine Italian district (namely *Aph. flos-aquae, D. lemmermannii, M. aeruginosa, P. rubescens*, and *T. bourrellyi*). *P. rubescens* and *M. aeruginosa* resulted to be MC producers with typical MC diversities. *Aph. flos-aquae* and *D. lemmermannii* resulted to be not toxic. Finally, *T. bourrellyi* resulted to produce ATX and possibly (at a minor extent) still not fully characterized PSP toxins. The comparison of the peptidomic profiles allowed us to classify *P. rubescens* and *M. aeruginosa* as extraordinary non-ribosomal peptides producers. A preliminary attempt aimed at identifying the peptidic compounds has revealed that anabaenopeptins and cyanopeptolins were the most represented, but many other peptides are still to be structurally determined. As demonstrated by recent reports (Svirčev *et al.*, 2016), the toxic potential of cyanobacteria can be only partially verified by current analytical techniques. Efforts to develop more comprehensive but specific methodologies are therefore still needed.

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