Re-evaluation of paralytic shellfish toxin profiles in cyanobacteria using hydrophilic interaction liquid chromatography-tandem mass spectrometry

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Highlights:

- Thirty-five paralytic shellfish toxin variants were detected in five cyanobacterial strains and one environmental sample
- Twenty of these variants had not been detected in these strains previously
- *Dolichospermum circinale* (AWQC131C) produced 23 variants
- M-toxins were detected of the first time in cyanobacteria

Abstract: To date Paralytic shellfish toxin (PST) variants in cyanobacteria have primarily been characterized using high performance liquid chromatography coupled with fluorescence detection. In this study we re-evaluated the PST profiles of five cyanobacterial cultures (*Dolichospermum circinale* AWQC131C, *Aphanizomenon* sp. NH-5, *Raphidiopsis raciborskii* T3, *Scytonema* cf. *crispum* CAWBG524 and CAWBG72) and one environmental sample (*Microseria wollei*) using hydrophilic interaction liquid chromatography coupled with electrospray ionization tandem mass spectrometry. A total of 35 different PST variants were detected. *D. circinale* contained the highest number of variants (23), followed by *S.* cf. *crispum* CAWBG72 (21). Many of the variants detected in the cultures/environmental sample had not been reported from these strains previously: *D. circinale* (14 variants), *S.* cf. *crispum* CAWBG72 (16), *S.* cf. *crispum* CAWBG524 (9), *Aphanizomenon* sp. (9), *R. raciborskii* (7), and *M. wollei* (7). Of particular interest was the detection of M-toxins (*Aphanizomenon* sp., *R. raciborskii, D. circinale*). These have previously only been identified from shellfish where they were thought to be metabolites. Well-characterized PST variant profiles are essential for research investigating the genetic basis of PST production, and given that the toxicity of each variants differs, it will assist in refining risk assessments.

Keywords saxitoxin; paralytic shellfish toxins; hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS); cyanobacteria; saxitoxin profiles

1. Introduction

Paralytic shellfish toxins (PSTs) are a group of naturally occurring neurotoxic alkaloids produced by freshwater cyanobacteria and marine dinoflagellates (Llewellyn, 2006; Wiese et al., 2010). Ingestion of water or organisms containing PSTs can result in paralytic shellfish poisoning (PSP) syndrome, which has been responsible for several recent human intoxication events (McLaughlin et al., 2011; Turnbull et al., 2013). PSTs produced by cyanobacteria have caused numerous animal mortalities (Negri et al., 1995; Steffensen et al., 1999) and in many parts of the world pose a serious threat to humans *via* contact with contaminated water used for recreational activities or drinking water supply (Steffensen, 2008). PSTs also have a number of beneficial uses; they have been vital for the study of voltage-gated sodium channels, and certain analogues show great pharmaceutical potential as local anesthetics (Epstein-Barash et al., 2009; Stevens et al., 2011).

PSTs have a characteristic tricyclic perhydropurine backbone and structural variation is achieved by differing functional groups at six locations, R_1-R_6 (Figure 1; Wiese et al., 2010). They can be categorized into three generic groups based on their net charge. Analogues that have both C11-hydroxysulfate and *N*-sulfocarbamoyl groups have an overall charge state of 0 and are typically referred to as C-toxins (C1-C4). Analogues that have either C11-hydroxysulfate or *N*-sulfocarbamoyl have a charge state of +1, and are commonly known as gonyautoxins (GTX1-6, dcGTX1-4), but also include several other analogues such as the M-toxins (M1, M3, and M5). Analogues that are non-sulfated have a charge state of +2 and include; saxitoxin (STX), neosaxitoxin (NEO), and their respective decarbamoyl analogues. Additionally, a group of PSTs with an acetoxy function for R_4 (Figure 1) have been identified that are produced solely by the

cyanobacterium *Microseira wollei* (basionym *Lynbya wollei;* McGregor and Sendall (2015)) and are referred to as *L. wollei* toxins 1-6 (LWTX1-6; Carmichael et al. (1997)).

The toxicity of each structural variant can vary greatly. *N*-sulfocarbamoyl analogues such as GTX5, and the C-toxins, have been reported to have much lower toxicity than non-sulfated analogues (Oshima, 1995). Toxicity differences are also observed in the structural orientation of the C11-hydroxysulfated epimer pairs, such as GTX1 and GTX4. Analogues that possess a single alcohol group at the C12 position (LWTX-1, LWTX-4, LWTX-6, 12α/β-do-doSTX and 12α/β-do-dcSTX) have been reported to have significantly lower toxicity than respective hydrated ketone analogues (Koehn et al., 1981; Onodera et al., 1997).

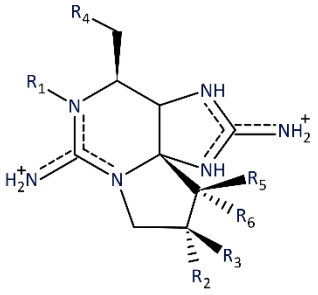
Cyanobacterial species known to produce PSTs include; *Dolichospermum circinale* AWQC131C (basionym *Anabaena circinalis*) in Australia (Llewellyn et al., 2001), *Scytonema* cf. *crispum* in New Zealand (Smith et al., 2012), *Raphidiopsis raciborskii* (basionym *Cylindrospermopsis raciborskii*; Aguilera et al. (2018)) and *Raphidiopsis brookii* in Brazil (Lagos et al., 1999; Stucken et al., 2010), *M. wollei* in North America, *Planktothrix* sp. in Italy (Pomati et al., 2000) and *Aphanizomenon* spp. in USA, Europe and China (Ballot et al., 2010; Carmichael et al., 1997; Dias et al., 2002; Li et al., 2000; Li et al., 2003; Liu et al., 2006; Mahmood and Carmichael, 1986; Pereira et al., 2004). Recently, the PST biosynthetic (*sxt*) gene cluster of six species has been sequenced; *R. raciborskii* T3 (Kellmann et al., 2008), *D. circinalie* AWQC131C, *Aphanizomenon* sp. NH-5 (Mihali et al., 2009), *R. brookii* D9 (Stucken et al., 2010), *M. wollei* (Mihali et al., 2011) and *S.* cf. *crispum* (Cullen et al., 2018). Identification of the *sxt* gene cluster led Kellmann et al. (2008) to propose a new PST biosynthetic pathway, which has since been revised by Soto-Liebe et al. (2010) and D'Agostino et al. (2014a). While several proposed biosynthetic intermediates have been identified, it is important to note that with the exception of SxtA, SxtN, SxtDIOX, SxtH and SxtT, *sxt* protein function is based solely on sequence homology predictions (Chun et al., 2018; Cullen et al., 2018; Kellmann et al., 2008; Lukowski et al., 2018; Tsuchiya et al., 2014). The *sxt* clusters span from a minimal size of 25.7 kb in *R. brookii* D9 (Stucken et al., 2010) to maximum of 53.3 kb in *S.* cf. *crispum* CAWBG524 (Cullen et al., 2018). Each cluster encodes a 'core' set of enzymes putatively responsible for biosynthesis of STX and additional 'auxiliary' enzymes thought to be involved in biosynthesis of PST analogues. Comparisons of species-specific toxin profiles with their corresponding genetic profile have been used to infer the function of several *sxt* encoded proteins. For example, SxtX has been proposed to catalyze N1-hydroxylation based on the presence in all identified *sxt* clusters with the exception of *D. circinale* AWQC131C and *R. brookii* D9, both of which have only been reported to produce non-N1 hydroxylated PSTs.

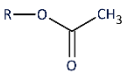
A variety of methods have been developed to detect and quantify PSTs. These have primarily been established for monitoring shellfish. Traditionally, the mouse bioassay mouse (AOAC 959.08) was the most common testing method (LeDoux and Hall, 2000). However, ethical issues, low sensitivity, poor reproducibility and interference from sample matrices have prompted the development of alternative chemical methods. Currently, the most common analytical method includes either pre-column (AOAC 2005.06; (Lawrence et al., 2005)) or post-column oxidation (AOAC 2011.02; van de Riet et al. (2011)) high performance liquid chromatography coupled with fluorescence detection (HPLC-FLD). To date, the profiles of most PST-producing cyanobacteria have primarily been deduced using these methods (Table 1). While HPLC-FLD can be automated and has overcome many of the limitations of the mouse bioassay, it has several

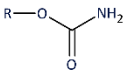
drawbacks. PSTs within samples must be derivatized into fluorescence products and the pre-column oxidation HPLC-FLD procedure requires that each sample is analyzed with two different oxidizing agents. In addition, some PSTs give the same oxidation product and produce two or three products, hindering data interpretation (Lawrence et al., 2005). Post-column oxidation HPLC-FLD enables separation of each toxin as an individual peak (Oshima, 1995). However, two chromatographic runs are required for each sample to separate all of the possible structural variants, and interfering compounds have been reported (Baker et al., 2003).

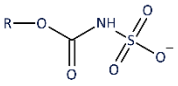
A hydrophilic interaction liquid chromatography (HILIC) method coupled with electrospray ionization tandem mass spectrometry (MS/MS) was developed by Dell'Aversano et al. (2005). The authors showed that this was suitable for chromatographic separation of PSTs without derivatization. This method avoids the need for ion pairing agents, which means ionization efficiency is not compromised. Most importantly, retention time specific ion transitions allow simultaneous determination of all common PSTs in a single 30 min analysis. The method has been utilized to identify the toxin profile of some cyanobacterial strains i.e., *R. brookii* D9 and *R. raciborskii* T3 (Soto-Liebe et al., 2010), marine algal PST-producers (Halme et al., 2012), contaminated shellfish (Boundy et al., 2015), and previously undetected PST analogues (Dell'Aversano et al., 2008). The most recent iteration of the HILIC-MS/MS method utilizes an optimized sample clean-up and preparation method which enables quantitation and determination of PSTs in matrices where polar contaminants, such as salts, would have previously hindered their detection (Boundy et al., 2015).

The aim of this study was to use the recent advances in HILIC-MS/MS methodology to re-evaluation variant profiles in five PST-producing cyanobacteria species. Additionally, we searched for structural variants that have not been commonly associated with or previously identified within cyanobacteria, but were likely to be present based on the proposed PST biosynthetic pathways. Accurate information on PST profiles for these strains will lead to increased accuracy for inferring biosynthetic function and assist in providing robust data for defining risk assessments.



OCOCH$_3$: 

OCONH$_2$: 

OCONHSO$_3^-$: 

Group (Charge state)	Analogue	R1	R2	R3	R4	R5	R6
C toxins (0)	C1	H	H	OSO_3^-	$OCONHSO_3^-$	OH	OH
	C2	H	OSO_3^-	H	$OCONHSO_3^-$	OH	OH
	C3	OH	H	OSO_3^-	$OCONHSO_3^-$	OH	OH
	C4	OH	OSO_3^-	H	$OCONHSO_3^-$	OH	OH
						OH	OH
GTXs (+1)	dcGTX2	H	H	OSO_3^-	OH	OH	OH
	dcGTX2	H	OSO_3^-	H	OH	OH	OH
	dcGTX1	OH	H	OSO_3^-	OH	OH	OH
	dcGTX4	OH	OSO_3^-	H	OH	OH	OH
	GTX2	H	H	OSO_3^-	$OCONH_2$	OH	OH
	GTX3	H	OSO_3^-	H	$OCONH_2$	OH	OH
	GTX1	OH	H	OSO_3^-	$OCONH_2$	OH	OH
	GTX4	OH	OSO_3^-	H	$OCONH_2$	OH	OH
	GTX5 (B1)	H	H	H	$OCONHSO_3^-$	OH	OH
	GTX6 (B2)	OH	H	H	$OCONHSO_3^-$	OH	OH
	M1α	H	H	OH	$OCONHSO_3^-$	OH	OH
	M1β	H	OH	H	$OCONHSO_3^-$	OH	OH
	M3	H	OH	OH	$OCONHSO_3^-$	OH	OH
	M5	Uncharacterised					
	LWTX1	H	H	OSO_3^-	$OCOCH_3$	H	OH
	LWTX2	H	H	OSO_3^-	$OCOCH_3$	OH	OH
	LWTX3	H	OSO_3^-	H	$OCOCH_3$	OH	OH
STXs (+2)	doSTX	H	H	H	H	OH	OH
	dcSTX	H	H	H	OH	OH	OH
	dcNEO	OH	H	H	OH	OH	OH
	STX	H	H	H	$OCONH_2$	OH	OH
	NEO	OH	H	H	$OCONH_2$	OH	OH
	11αOH-dcSTX (dcM2α)	H	H	OH	OH	OH	OH
	11βOH-dcSTX (dcM2β)	H	OH	H	OH	OH	OH
	M2α	H	H	OH	$OCONH_2$	OH	OH
	M2β	H	OH	H	$OCONH_2$	OH	OH
	M4	H	OH	OH	$OCONH_2$	OH	OH
	12,12-dido-dcSTX	H	H	H	OH	H	H
	12α-do-doSTX	H	H	H	H	OH	H
	12β-do-doSTX	H	H	H	H	H	OH
	12α-do-dcSTX	H	H	H	OH	OH	H
	12β-do-dcSTX (LWTX4)	H	H	H	OH	H	OH
	LWTX5	H	H	H	$OCOCH_3$	OH	OH
	LWTX6	H	H	H	$OCOCH_3$	H	OH

Figure 1: Structures of paralytic shellfish toxins, grouped by charge state.

Table 1. Examples of previously reported paralytic shellfish toxin profiles from known producers and initial characterization of their toxin profile.

Producer organism	Toxin profile	Original method of detection	Reference
Dolichospermum circinale AWQC131C	STX, GTX2, GTX3, GTX5, dcSTX, dcGTX2, dcGTX3, C1, C2	HPLC-FLD	(Llewellyn et al., 2001)
Aphanizomenon sp. NH-5	STX, NEO	HPLC-FLD	(Mahmood and Carmichael, 1986)
Raphidiopsis raciborskii T3	STX, GTX2, GTX3	HPLC-FLD RP-LCMS	(Lagos et al., 1999)
	STX, NEO, GTX5	RP-LCMS	(Kellmann et al., 2008)
	STX, NEO, dcNEO	HILIC-MS/MS	(Soto-Liebe et al., 2010)
Raphidiopsis brookii D9	STX, dcSTX, GTX2, GTX3, dcGTX2, dcGTX3	HILIC-MS/MS	(Soto-Liebe et al., 2012)
Microseira wollei (environmental material)	dcSTX, dcGTX2, dcGTX3, LWTX1-6	HPLC-FLD	(Onodera et al., 1997)
Scytonema cf. *crispum* CAWBG524=UCFS10	STX	HPLC-FLD	(Smith et al., 2011)
Scytonema cf. *crispum* CAWBG72=UCFS15	STX, GTX2, GTX3, dcSTX, dcGTX2, dcGTX3	HPLC-FLD	(Smith et al., 2012)

RP-LCMS: Reverse phase LCMS using C18 column; HPLC-FLD: high performance liquid chromatography coupled with fluorescence detection; HILIC-MS/MS: hydrophilic interaction liquid chromatography coupled with electrospray ionization tandem mass spectrometry.

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2. Materials and Methods

2.1. Strains and cultures

Aphanizomenon sp. (CAWBG01=NH-5; Mahmood and Carmichael (1986)), *S.* cf. *crispum* (CAWBG72=UCFS15; Smith et al. (2012)) and *S.* cf. *crispum* (CAWBG524=UCFS10; Smith et al. (2011)) were sourced from the Cawthron Institute Culture Collection of Micro-algae (CICCM; cultures.cawthron.org.nz). These cultures were grown in glass flasks (500 mL) in MLA medium (Bolch and Blackburn, 1996) under a light regime of 90 µmol s^{-1} m^{-2} with a 12:12 light:dark cycle, at a temperature of 17˚C ± 1. *D. circinale* AWQC131C was grown as described in D'Agostino et al. (2014b) and *R. raciborskii* T3 (Lagos et al., 1999) under a 24 hr light regime of 100 µmol s^{-1} m^{-2} at 25˚C ± 1 in BG-11. *M. wollei* biomass from a unicyanobacterial bloom in Guntersville Reservoir (Alabama, USA) was lyophilized, stored frozen (-20˚C) and used for subsequent toxin extraction.

2.2. Paralytic shellfish toxin extraction

All batch cultures were harvested in their stationary phase by centrifugation (2,000 × *g*, 5 min). Resulting pellets were lyophilized and weighed. Samples were extracted with 10 mL Milli-Q water (MQ; 18.2 Mohm), sonicated (40 kHz, 15 min), frozen (-20°C) overnight and then thawed. Samples were vortexed briefly mixer then centrifuged (3,200 × *g*, 10 min) to pellet cellular debris. An aliquot (4 mL) of each culture extract was loaded onto a MQ conditioned ENVI-Carb 250 mg/3 mL (Supelco) solid phase extraction cartridge. Cartridges were washed (MQ, 1 mL) and the combined load and wash fractions analyzed to determine if there was any breakthrough (i.e. toxins not retained on cartridge). Toxins were eluted from the cartridge with 2 mL of 20:80:1 acetonitrile/MQ/acetic acid. An aliquot (100 µL) of the eluent was diluted with 300 µL acetonitrile into a polypropylene autosampler vial. For HILIC-MS/MS analysis a 2 µL injection volume was used.

2.2. Paralytic shellfish toxin characterization and quantification

The toxin profiles were determined using the HILIC-MS/MS method described in Boundy et al. (2015). The method involves direct quantitation of 17 PST analogues against analytical standards available as certified reference standards (NRCC, CNC). Additional PST analogues were incorporated into the method, including; M-toxins (M1α, M1β, M2α, M2β, M3, M4, M5), LWTXs (LWTX-1, LWTX-2, LWX-3, LWTX-4, LWTX-5, LWTX-6) 12α-do-dcSTX, 12β-do-dcSTX, 12α-do-doSTX, 12β-do-doSTX and 12,12-dido-dcSTX. Quantitation was performed using the calibrations generated from their nearest structural analogue. Relative response factors were either experimentally determined, or where reference material was unavailable, assumed to be 1.0.

Chromatographic peak assignment for M-toxins and LWTXs were made based on reports by Dell'Aversano et al. (2008) and Foss et al. (2012). This included information on *m/z*, fragmentation spectra and relative retention times. Due to the low level and late retention time of the putative M4 peak, where interferences are known to elute, fragmentation spectra for M4 could not be obtained. To assist with assignment of the various 12α- and 12β-deoxy analogues, microscale chemical reactions were undertaken on purified toxin material (Cho et al., 2015). Briefly, 90 µL of 0.1% $NaBH_4$ in dimethylformamide was added to 10 µL of either

193 dcSTX, dcNEO, STX, NEO or doSTX. Samples were vortexed and after 5 min, 10 µL of the solution was
194 diluted with 490 µL 80% acetonitrile with 0.25% acetic acid and analyzed using full MS and product ion
195 scans at 5, 10, 15 and 20 eV collision energy. The chromatographic peaks formed from the micro-scale
196 reductions were assigned to the 12α- and 12β-deoxy analogues based on relative retention times (12α<12β)
197 (Cho et al., 2015; Koehn et al., 1981). Chromatography of non-commercial standards can be found in Figure
198 S1-S10.

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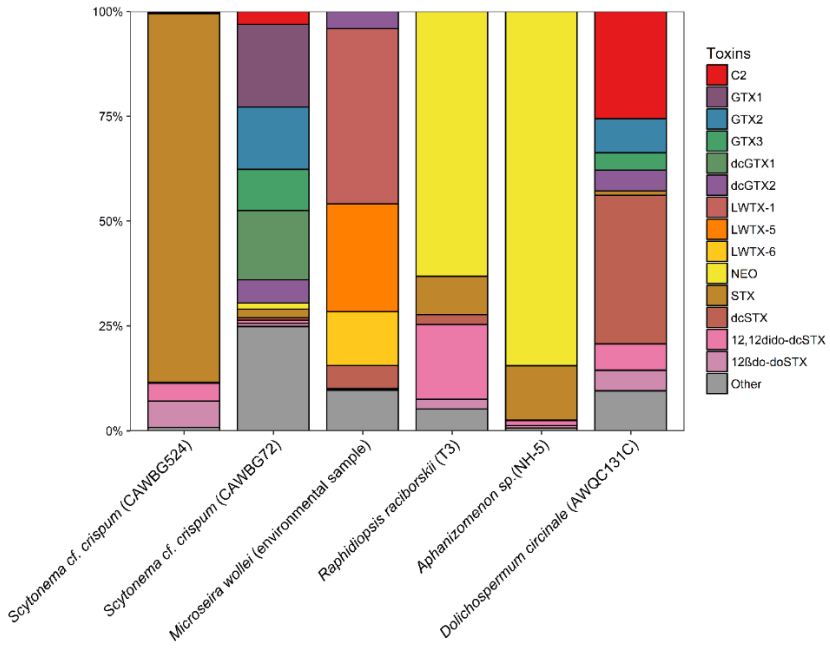
3. Results

A total of 35 different PST variants were detected in the five strains and one environmental sample (*D. circinale* AWQC131C, *Aphanizomenon* sp. NH-5, *R. raciborskii* T3, *M. wollei* (environmental material), *S.* cf. *crispum* CAWBG524 and CAWBG72). The highest number of variants detected was 23 in *D. circinale* AWQC131C, followed by *S.* cf. *crispum* CAWBG72 (22 variants), *M. wollei* (15 variants), *R. raciborskii* T3 (13 variants), *Aphanizomenon* sp. NH5 (11 variants) and *S.* cf. *crispum* CAWBG524 (10 variants). The highest total concentration of PST measured was in *D. circinale* (3020 ng mg^{-1} of lyophilized material) followed by *Aphanizomenon* sp. NH5 (852 ng mg^{-1}), whereas *S.* cf. *crispum* CAWBG72 contained the lowest (Table 2). *S.* cf. *crispum* CAWBG524, *R. raciborskii* T3 and *Aphanizomenon* sp. NH5 produced predominantly one PST variant each (STX or NEO; Figure 2). In contrast, 3-5 variants were present in high abundance in the other strains (Figure 2).

Fifteen of the variants detected in *D. circinale* AWQC131C, have not been reported from this strain previously (Tables 1 and 2). Of particular interest was the detection of M-toxins as these toxins have only previously been identified from shellfish where they were believed to be metabolites. Also, of note is the detection of variant GTX1/4 (albeit low levels) suggesting N-hydroxylation has occurred. Only trace levels were measured therefore detection would have been difficult or impossible using HPLC-FLD methods. We are confident of the assignment as retention times are correct and ratio of epimer pair matches that observed in the calibration standard (Figure S11-S12). The other cultures also produced variants that had not previously been detected in the strains: *S* cf. *crispum* CAWBG72 (14 variants), *S.* cf. *crispum* CAWBG524 (9), *Aphanizomenon* sp. NH5 (9), *R. raciborskii* T3 (7), and *M. wollei* (7).

The variants 12α/β-do-doSTX and 12,12-dido-dcSTX were detected in most taxa (Table 2). These were not completely resolved chromatographically, making identification of the lesser abundant 12α analogue difficult. Likewise, some other analogues are also difficult to identify with certainty as their mass transitions are identical (e.g. M1α with M1β; M2α with M2β). This is particularly problematic when one of the analogues is at high concentration, and the other analogues using the same transition are determined by only different chromatographic retention times.



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231 **Figure 2: Portion of Paralytic Shellfish Toxin variant detected in each strain or environmental sample**.

Table 2. **PST variants quantified (ng mg^{-1} of lyophilized material) by hydrophilic interaction liquid chromatography in cyanobacterial cultures.**

PST	*Scytonema* cf. *crispum* CAWBG524	*Scytonema* cf. *crispum* CAWBG72	*Microseira wollei*	*Raphiodiopsis raciborskii* T3	*Aphanizomenon* sp. NH-5	*Dolichospermum circinale* AWQC 131C
C1	ND	0.79	ND	ND	ND	**790**
C2	ND	0.37	ND	ND	ND	**570**
C3	ND	0.26	ND	ND	ND	ND
C4	ND	Trace	ND	ND	ND	ND
dcGTX2	ND	**0.68**	**8.8**	ND	ND	**110**
dcGTX3	ND	**0.25**	**4.2**	ND	ND	**50**
dcGTX1	ND	2	ND	ND	ND	ND
dcGTX4	ND	0.52	ND	ND	ND	ND
GTX2	0.051	**1.8**	ND	ND	ND	**180**
GTX3	0.031	**1.2**	ND	ND	ND	**93**
GTX1	ND	2.4	ND	ND	ND	0.53
GTX4	ND	0.97	ND	ND	ND	0.41
GTX5	ND	0.0089	ND	**0.29**	ND	**110**
GTX6	ND	ND	ND	2.3	ND	ND
doSTX	Trace	0.021	Trace	0.29	0.38	29
12αdo-dcSTX	0.021	0.01	0.013	0.31	0.19	10
12βdo-dcSTX (LWTX-4)	0.036	0.047	1	1.2	1	5.6
dcSTX	0.036	**0.073**	**12**	4	1.7	**790**
dcNEO	ND	0.14	ND	**4**	3.2	ND
STX	**14**	**0.25**	ND	**16**	**110**	**23**
NEO	ND	0.17	ND	**110**	**720**	ND
M1[d]	ND	ND	ND	Trace	ND	0.14
M2[d]	ND	ND	ND	ND	ND	0.29
M3	ND	ND	ND	ND	ND	0.93
M4[a]	ND	ND	ND	ND	0.27[b]	0.44
M5	ND	ND	ND	ND	ND	1.5
11-OH-dcSTX	ND	ND	0.0037	ND	ND	0.057
12,12dido-dcSTX	0.67	0.089	0.28	31	9.4	140
12αdo-doSTX	0.072	ND	0.019	0.74	0.53	3.7
12βdo-doSTX	1	0.099	0.63	4	5.3	110
LWTX-1	ND	ND	**91**	ND	ND	ND
LWTX-2	ND	ND	**8.6**	ND	ND	ND
LWTX-3	ND	ND	**7.2**	ND	ND	ND
LWTX-5	ND	ND	**56**	ND	ND	ND
LWTX-6	ND	ND	**28**	ND	ND	ND
Total	**15.9**	**12.1**	**222**	**174**	**852**	**3020**

[a] Putative peak, no reference standard available to confirm identity.

[b] NH-5 had 2 peaks present.

[c] Indicates variant detected previously, but not in this study.

[d] M1 and M2 exist as epimer pairs (M1α/M1β and M2α/M2β) and have not been chromatographically separated. Thus, they have been integrated as a single peak.

Bold indicates variant known to be produced based on previous studies on these strains.

ND: Not detected

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4. Discussion

The risk posed by PSP to human health has resulted in the need for monitoring of areas where potential PST-producers might occur. Identification of which PST variants are produced is essential as each varies in its toxicity, and this information can be used by monitoring authorities to refine risk assessments. PST-producing cyanobacteria with a characterized *sxt* cluster (Kellmann et al., 2008; Mihali et al., 2011; Mihali et al., 2009; Stucken et al., 2010) have become model organisms for studies investigating the physiology of PST production e.g., proteomics studies (D'Agostino et al., 2014b, 2016; Ongley et al., 2015; Plominsky et al., 2009; Soto-Liebe et al., 2012). Many PST profiles from these model cyanobacteria strains were originally described using HPLC-FLD. These methods have several disadvantages such as the need to derivatize the PSTs into fluorescent products, some toxins having multiple fluorescent products and naturally fluorescent products. Further, several of the variants do not form fluorescent products. This ambiguity in defining the profiles of PST-producing cyanobacteria limits the confidence of researchers to propose functions within the *sxt* gene cluster. In this study a HILIC-MS/MS chromatographic separation method developed by Boundy et al. (2015) was used to revisit PST variant production by the six most common PST-producing cyanobacteria. This method has been utilized in multiple studies for the detection of PSTs in dinoflagellates and contaminated shellfish (Cho et al., 2015; Thomas et al., 2017; Turner et al., 2015), however its application to PST variant characterization in cyanobacteria is limited.

In general, the toxin profile of each cyanobacterial strain investigated in this study was similar to that described previously (Table 1) with respect to the most abundant toxins (Figure 2). However, other PSTs were also found in much lower abundance for the first time. For example, *Aphanizomenon* sp. NH-5 was thought to produce only STX and NEO, while these were the most abundant PSTs in this organism, it was also found to produce dcNEO, dcSTX, doSTX, 12αdo-dcSTX and 12βdo-dcSTX (LWTX-4). Further, *M. wollei* was thought to produce only dcGTX2/3, dcNEO, dcSTX and LWTX1-6 but analysis in the present study detected the production of deoxy- and C11-hydroxy derivatives of these toxins. Most notable was the detection of GTX1/4 in *D. circinale* AWQC131C, a strain that was previously believed to be unable to produce N1-hydroxlated PSTs. The very low abundance of these congeners has likely contributed to their previous lack of detection using other methods. In the future, we envisage that the HILIC-MS/MS method can be used characterize cyanobacterial PST profiles at the inter-species level while utilizing the profiles of these model organisms a reference. Work in this area has recently begun, such as the morphological, genetic and toxin interspecies study of PST-producing *D. circinale* by Pereyra et al. (2017) and within cylindrospermopsin-producing *R. raciborskii* (Willis et al., 2016). As the use of genomics for toxigenic analysis increases, large intergenic studies comparing toxin profiles within each species will be required to further elucidate the connection between toxicity and ecotype.

Of particular interest in this study was the PST profile of *R. raciborskii* T3. The initial toxin profile of *R. raciborskii* T3 was reported as a 1:9 ratio mixture of STX and GTX2/3 as detected by HPLC-FLD and reverse phase LCMS (RP-LCMS; Lagos et al. (1999)), and later to STX, NEO and GTX5 by Kellmann et al. (2008). However, the *R. raciborskii* T3 profile was later reinvestigated by Soto-Liebe et al. (2010) *via* a HILIC-MS/MS approach, which was found to differ from Lagos et al. (1999) and Kellmann et al. (2008) with the sole identification of STX, NEO and dcNEO. The analysis in the present study identified STX, NEO and

dcNEO in the same relative amounts described by Soto-Liebe et al. (2010) but also detected dcSTX, GTX5, GTX6 and doSTX as minor constituents. Since these toxins are in minor concentrations compared to the most abundant toxins, it is likely they were below the limit of detection of the previous respective studies. Soto-Liebe et al. (2010) also predicted the non-functional state of SxtN, putatively responsible for sulfotransfer to the N-carbomoyl moiety, based on their failure to detect N-sulfonated PSTs. Thus, our results contradict those of Soto-Liebe et al. (2010) based on our detection of GTX5 and GTX6 in *R. raciborskii* T3, with the possibility of culture conditions, extraction methods and analytical instruments having a profound effect on the detection of toxins in low abundance. Recently, there has been a push towards identification of the STX biosynthetic intermediates (Chun et al., 2018; Tsuchiya et al., 2014, 2015, 2016; Tsuchiya et al., 2017) and it is evident that accurate biochemical characterization of PST enzymes responsible for biosynthesis of PST analogues will be targeted in the near future. Accurate analysis and descriptions of PST profiles is essential to support these biochemical studies.

PST molecules known as the M-toxins were identified within extracts of *D. circinale* AWQC131C, *R. raciborskii* T3 and *Aphanizomenon* sp. NH-5. Structurally, the M-toxins (M1-M4; M5 has not been structurally characterized), are mono- or di-hydroxylated derivatives of STX or GTX5 (Figure 1). The M-toxins were initially believed to be bivalve metabolites since they have repeatedly been identified within shellfish but not within dinoflagellate extracts (Boundy et al., 2015; Dell'Aversano et al., 2008; Vale, 2010a, b). In the present study, we report the detection of M-toxins within cyanobacteria for the first time. Recently, biochemical proof of M2 (C11-hydroxySTX) as a true cyanobacterial PST has been reported by Lukowski et al. (2018) via biosynthesis by the Rieske oxygenase SxtDIOX (also referred to as GtxA). While biosynthesis of M1 has not been confirmed *in vitro*, this molecule is also likely to be a true PST intermediate based on the recent characterization of the N-21 sulfotransferase SxtN (Cullen et al., 2018). However, it must be noted that the origin of M3-M5 as either biosynthetic intermediates or metabolic byproducts remains unsolved. Interestingly, N1-hydroxylated M-toxin type PSTs have yet to be identified. The previous lack of detection of the M-toxins within cyanobacteria and dinoflagellates is unsurprising, since these molecules are undetectable using HPLC-FLD and extremely sensitive MS approaches are needed (Dell'Aversano et al., 2008). While all 5 M-toxins that we monitored were detected in *D. circinale* AWQC131C, these were in very low abundance and it is likely the amount of toxin within other species may be below the limit of detection. Further, the detection of 12,12-dido-dcSTX (also detected by Kellmann et al. (2008) as intermediate E'), 12α/βdo-dcSTX, doSTX and 12α/βdo-doSTX in most cyanobacterial taxa also hint towards their being biosynthetic intermediates of STX or dcSTX production.

The presence of PSTs within two kingdoms of life, cyanobacteria and dinoflagellates, has long fascinated scientists interested in the evolution of these molecules. A genetic basis for the production of PSTs in dinoflagellates was first described by Stüken et al. (2011) upon the discovery of nuclear-encoded *sxtA*. Later discoveries have since identified a range of other *sxt* genes in dinoflagellates, although they are sometimes also detected in non-toxic strains (Hackett et al., 2012; Orr et al., 2013; Wang et al., 2016). In regard to detection of toxins, only the most common PSTs such as decarbomoyl-PSTs, STX, NEO, GTXs and C-toxins have been reported in both groups of organisms. However, PST varieties including doSTX, 12,12-dido-dcSTX PST derivatives originally thought to be only produced by dinoflagellates (Cho et al., 2015;

Oshima et al., 1993; Turner et al., 2015) were also detected in all cyanobacterial strains analyzed in this study. While 12,12-dido-dcSTX has previously been detected in *R. raciborskii* T3 by Kellmann et al. (2008), this is the first time it has been detected in other cyanobacterial strains. The ever-increasing number of identical PST molecules within cyanobacteria and dinoflagellates further supports an evolutionary link between these organisms.

5. Conclusions

Re-analysis of PST variants in five cyanobacterial cultures and an environmental sample using HILIC-MS/MS identified 35 PST variants, including some not previously described from cyanobacteria. Future research on the *sxt* cluster will lead to the eventual biochemical characterization of catalytic steps resulting in formation of the PSTs. Well-characterized PST variant profiles are essential for this research, and will assist in refining risk assessments

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