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Potent toxins in Arctic environments – presence of saxitoxins and an unusual microcystin variant in Arctic freshwater ecosystems

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Abstract

Cyanobacteria are the predominant phototrophs in freshwater ecosystems of the polar regions where they commonly form extensive benthic mats. Despite their major biological role in these ecosystems, little attention has been paid to their physiology and biochemistry. An important feature of cyanobacteria from the temperate and tropical regions is the production of a large variety of toxic secondary metabolites. In Antarctica, and more recently in the Arctic, the cyanobacterial toxins microcystin and nodularin (Antarctic only) have been detected in freshwater microbial mats. To date other cyanobacterial toxins have not been reported from these locations. Five Arctic cyanobacterial communities were screened for saxitoxin, another common cyanobacterial toxin, and microcystins using immunological, spectroscopic and molecular methods. Saxitoxin was detected for the first time in cyanobacteria from the Arctic. In addition, an unusual microcystin variant was identified using liquid chromatography–mass spectrometry. Gene expression analyses confirmed the analytical findings, whereby parts of the *sxt* and *mcy* operon involved in saxitoxin and microcystin synthesis, were detected and sequenced in one and five of the Arctic cyanobacterial samples, respectively. The detection of these compounds in the cryosphere improves the understanding of the biogeography and distribution of toxic cyanobacteria globally. The sequences of *sxt* and *mcy* genes provided from this habitat for the first time may help to clarify the evolutionary origin of toxin production in cyanobacteria.

Keywords

Saxitoxin, microcystin, gene analysis, cyanobacterial mats, Arctic, climate change

1. Introduction

Few phototrophic organisms survive the harsh climate of the high Arctic regions. This includes some higher plants, mosses, lichens, various algal groups and cyanobacteria. Cyanobacteria are globally distributed, but in high Arctic freshwater ecosystems they represent the dominant primary producers [1,2]. Special features such as resistance to ultraviolet (UV) radiation, freeze-thaw cycle adaptation and nitrogen fixation allow their survival in these extreme environments [2]. During the polar summer, when both light and temperatures above the freezing point prevail, cyanobacterial communities thrive. They develop highly diverse benthic or floating mats in freshwater streams and ponds that can be several centimeters thick and extend over large areas [2,3]. These extensive mats form the basis of a small but diverse and dynamic ecosystem accommodating a variety of organisms such as nematodes, rotifers, tardigrades [4], mosses and moss-infecting oomycetes such as the recently-described *Pythium polare* [5].

Saxitoxins (STXs) (Figure 1A) are carbamate alkaloids, a group of fast-acting neurotoxins, inhibiting neuronal signal propagation of most higher organisms [6]. They are typically produced by marine dinoflagellates [7].However, planktonic and benthic cyanobacteria from temperate and tropical regions, e.g. *Aphanizomenon* spp., *Anabaena circinalis, Cylindrospermopsis raciborski, Planktothrix* spp., and *Lyngbya wollei* [8], are also known to produce STXs. Microcystins (MCs) (Figure 1B) on the other hand, represent a group of ≥80 structural heptapeptide variants with varying hepato-, renal-, and neurotoxicity [9,10]. They appear to act primarily via specific inhibition of serine/threonine phosphatases [8,10], thereby interfering with one of the most important regulatory mechanisms of the cell. MCs are produced by a large variety of planktonic and benthic cyanobacterial genera including *Microcystis, Nostoc*, *Planktothrix*, *Anabaena*, *Synechococcus* and *Snowella* [8,11].

The molecular basis for the production of both toxins are large and variable gene clusters, encoding enzymes involved in secondary metabolite production such as polyketide synthetases and/or non-ribosomal peptide synthetases [12–14]. The distribution of this gene cluster among different strains

of cyanobacteria does not necessarily correlate with the actual production of the toxins [15]. Therefore the presence of these genes in a given ecosystem is only an indication for the presence of the toxins and is therefore considered in this study as providing evidence for the 'potential to produce toxins'. These biosynthetic steps are energetically expensive for cyanobacteria [16], and this has prompted considerable speculation on their ecological function. To date the physiological function and ecological regulation of both STXs and MCs are poorly understood [17–19]. The current hypotheses aiming to explain this relatively enormous investment of energy in the synthesis of these toxins, include protection against grazing pressure, UV-radiation, and reactive oxygen species, as well as their function as signaling molecules in a quorum sensing-like manner [16,19–22]. The development of toxins as protection against grazers appears to be the least plausible hypothesis as the corresponding gene clusters appear to have been present in ancestral cyanobacteria species that have existed prior to the mesoproterozoic period [23–25], i.e. millions of years prior to the emergence of potential eukaryotic grazers of cyanobacteria. However it can not be excluded that new functions have developed in the course of evolution.

In view of the ancient origin and the high conservation of the toxin gene clusters it is not surprising that cyanobacteria inhabiting remote pristine areas, e.g. the rudimentary environments of the polar regions, could produce toxins. The presence of MCs in cyanobacterial mats has been reported for several locations in Antarctica [26–28], whereas this has only recently been demonstrated for Arctic cyanobacteria from northern Baffin Island [29]. Kleinteich *et al*. (2012) [29] demonstrated that culturing of cyanobacterial mats in the laboratory at increased temperatures caused a marked rise in the concentration of MCs in concert with shifts in the diversity of the cyanobacterial mat community composition. Saxitoxins, on the other hand, have never been reported in polar environments and cyanobacterial toxins in general remain understudied in this habitat.

In this study five cyanobacterial communities from the Arctic were screened for the presence of STX using enzyme-linked immunosorbent assay (ELISA) and further confirmation was undertaken using high performance liquid chromatography with fluorometric detection (HPLC-FLD). Furthermore an

unusual MC variant was identified using liquid chromatography-mass spectrometry (LC-MS). Samples were also screened for selected genes involved in the synthesis of MC and STX, thereby providing evidence of the toxin-producing potential of Arctic cyanobacterial communities.

2. Materials and Methods

2.1 Study sites and sampling

Five samples of cyanobacterial communities were collected during an expedition to northern Baffin Island in the vicinity of Cape Hatt (72°30' N and 79°47' W) in August to September 2009 from microbial communities on wet soil, small streams and ponds (see Supplemental Figure 1 for GPS coordinates). Samples for DNA extraction and toxin analysis were sealed in sterile tubes, those for RNA analysis stored in RNAlater (Qiagen, Hilden, DEU) and frozen (-20 °C) until further analysis.

2.2 Screening for saxitoxin and microcystin

2.2.1 Saxitoxin extraction

Frozen cyanobacterial material was lyophilized and their dry weight recorded. Sample for STX analysis were extracted as described by Smith *et al.* (2011) [30]. Briefly, 50 mg of lyophilized material was homogenized in 5 mL H_2O using a mortar and pestle and dried under nitrogen flow. The dried material was dissolved methanol (4 mL) acidified with acetic acid (0.1 %), vortexed (15 min), and placed in an ultrasonic water bath (15 min, ice cold). The suspension was centrifuged (30 min, 4,000 x *g*) and the supernatant transferred into a separate tube for HPLC-FLD analysis.

To ensure complete STX recovery from the residual pellet, 5 mL HCl (0.1 M) was added and the pellet boiled (5 min, 100 °C), directly followed by ultra-sonication (10 min, ice cold). Cell debris was removed by centrifugation (30 min, 3,000 x *g*), and the supernatant adjusted to pH 7.0 with NaOH. The supernatant was filtered through a 0.2 µm filter and pooled with the supernatant obtained in the first extraction. The pooled supernatant was dried under nitrogen gas flow and stored at -20 °C until HPLC-FLD analysis.

130

131 2.2.2 *Microcystin extraction*

132 Lyophilized material (173 mg) was homogenized to a powder using a mortar and pestle Methanol
133 (75 %, 5 mL) was added to the powder, the suspension placed in an ultrasonic water bath for 30 min
134 (ice-cold), centrifuged (30 min, 4,000 x *g*), and the supernatant removed for further processing. The
135 extraction was repeated three times. The supernatants were pooled, dried under nitrogen flow, re-
136 suspended in 15 mL H_2O, and loaded onto C18 cartridges (Sep-Pak, Waters, Dublin, IRL). MC was
137 eluted from the C18 cartridges with methanol (100 %), the methanolic eluate dried under nitrogen
138 gas flow, and resuspended in 3 mL methanol (20 %). The extract was centrifuged (20 min, 13,000 x *g*)
139 and the supernatant stored at -20 °C until further analyses.

140

141 *2.2.3 Saxitoxin analysis*

142 The STX (PSP) ELISA kit (ABRAXIS, Warminster, USA) was employed to analyze for STX according to
143 the manufacturer's protocol. This STX (PSP) ELISA has a limit of detection (LOD) of 0.0215 ng / mL.
144 Analytical data were generated based on three independent replicate analyses each with duplicate
145 technical replicates.
146 Saxitoxins were also analyzed using HPLC-FLD as described Smith *et al*. (2011) [30]. This HPLC
147 detection method had a LOD of 0.1 mg / kg total STXs.

148

149 2.2.4 Microcystin analysis

150 The extract of cyanobacterial sample A , which had previously tested positive for MC by A DDA-ELISA
151 [29], was analyzed by LC-MS in order to identify the MC congener/s present. LC-MS was undertaken
152 on a HPLC system (UltiMate 3000; Dionex) coupled to an AmaZon X (Bruker Daltonics) electrospray
153 ionization-ion trap mass spectrometer (ESI-IT-MS^n). Samples (20 µL) were separated on a C_{18} column
154 (Ascentis Express C_{18}, 100 × 2.1 mm, 2.7-µ; Supleco Analytical) using a gradient system of 98 % H_20 +
155 0.1 % formic acid (v/v; solvent A) and 98 % acetonitrile + 0.1 % formic acid (v/v; solvent B) with the
156 following gradient program; the sample was loaded in 10 % B; 10 % B was held for 1 min and

157 increased to 100 % B over 12 min; 100 % B was held for 2 min; the solvent concentration was
158 returned to 10 % B in 1 min and the column re-equilibrated for 4 min. The eluting compounds were
159 transferred into the IT-MS using a capillary voltage of 3.5 kV and a nebulizer pressure of 3.0 bar.
160 Desolvation was accomplished with a nitrogen flow of 8 L / min at 220 °C. Tandem MS (MS/MS)
161 spectra were gathered using the doubly- or singly-protonated ions of the target compounds and
162 collision-induced dissociation (CID) to induce fragmentation of the parent ion (collision amplitude of
163 1.0).
164
165 *2.3 Screening for genes involved in toxin synthesis*
166 *2.3.1 Nucleic acid extraction*
167 DNA was extracted from 5 - 10 mg of frozen material using the MO BIO PowerSoil® DNA Isolation Kit
168 following the manufacturer's recommendations. Due to the heterogeneity of the sample material,
169 three individual extractions were performed and the pooled extracts used for downstream
170 applications. RNA was extracted from 5 - 10 mg of material stored in RNAlater (Qiagen, Hilden, DEU).
171 RNAlater was removed by patting the material on a dry stack of paper, and RNA extracted using the
172 MO BIO PowerBiofilm® RNA extraction kit following the manufacture's protocol. RNA was eluted
173 with RNase-free water and stored at -80 °C.
174
175 *2.3.2 Detection of genes involved in toxin synthesis*
176 PCRs targeting the *mcy* and *sxt* operon for MC and STX synthesis respectively were performed with
177 primer pairs and at annealing temperatures as listed in Supplemental Table 2; primers were from
178 MWG eurofins (Ebersberg, DEU). For the reactions either the Master Mix™ (Fermentas, St. Leon-Rot,
179 DEU) or the Phusion™ polymerase mix (NEB, Ipswich, USA) was used supplemented with BSA, DMSO
180 and $MgCl_2$. Bands were excised from a 1.5 % agarose gel (TAE) using a sterile scalpel, purified with a
181 gel extraction kit (Fermentas, St. Leon-Rot, DEU) and sequenced bi-directionally using the primers
182 listed in Supplemental Table 1 at MWG eurofins (Ebersberg, DEU). Messenger-RNA of the *sxt* operon
183 was reverse transcribed into cDNA using gene-specific reverse primers (Supplemental Table 2) and a

184 standard protocol for reverse transcription (20 U RNAse Inhibitor, 0.8 mM dNTPs, 7 µL of extracted
185 RNA, 70 U M-MuLV). Enzyme and chemicals for RT-PCR were from NEB (Ipswich MA, USA). The cDNA
186 produced was used as template for PCR as described above. *Microcystis aeruginosa* CCAP 1450/16
187 served as a positive control for *mcy* genes, but no positive control of cyanobacterial origin was
188 available for the *sxt* genes. The obtained sequences were analyzed using Geneious™ software
189 (Geneious Pro 5.3.6) and the closest matches identified using NCBI's BLAST tools (mega-BLAST and
190 BLASTn). Phylogenetic trees using *sxtA* sequences were built using the Geneious™ tree builder
191 (Jukes-Cantor, Neighbour joining method). The obtained 657 bp product of the *sxtA* gene was
192 deposited in the GenBank database under the accession JX887897 (Supplemental Table 3). The
193 obtained 128 bp product of the *sxtA* gene is displayed in Supplemental Table 5 since GenBank does
194 not allow deposition of sequences shorter than 200 bp.
195
196 *2.4 Identification of toxin producer/s*
197 *2.4.1 Cloning of 16S rRNA and intergenic spacer region (ITS)*
198 Two samples that returned positive results from toxin analyses were selected for construction of 16S
199 rRNA gene and *intergenic spacer region* (ITS) region clone libraries. Amplification was achieved with
200 the cyanobacteria-specific primer pair 27F and 23S30R (Supplemental Table 2) in a 50 µL PCR
201 reaction (4 min, 95 °C; 35 x [92 °C, 60 s; 55 °C, 60 s; 72 °C, 120 s]; 72 °C, 10 min) containing the
202 Fermentas (St. Leon-Rot, DEU) Master Mix, 2.5 mM $MgCl_2$, 0.2 µg / µL BSA, 3 % DMSO and 0.5 µM of
203 each primer. The PCR products were separated on a TAE 1.5 % agarose gel and the bands excised
204 using a sterile scalpel. After purification with the GeneJET™ Gel Extraction Kit (Fermentas, St. Leon-
205 Rot, DEU) PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, USA)
206 following the standard protocol with a DNA to vector ratio of four to one. For each sample
207 approximately 40 clones were retrieved and conserved in sterile TE buffer. Each clone insert was
208 amplified in a PCR reaction (95 °C, 4 min; 35 x [92 °C, 30 s; 52 °C, 30 s; 72 °C ,70 s]; 72 °C, 5 min) using
209 the Fermentas PCR Master Mix (St. Leon-Rot, DEU), 0.25 µM of each TOPO-primer M13, 2 mM $MgCl_2$,
210 3 % DMSO and 0.3 µL of DNA template. Products were subjected to a Restriction Fragment Length

Polymorphism (RFLP) analysis using restriction enzymes AluI and ScrF1 (NEB, Ipswich, USA) (37 °C, 2 h) and subsequently visualized on an agarose gel (TAE, 2.5 % agarose) in order to identify groups of multiple clones of the same phylotype. Two to three representative clones of each individual RFLP pattern (phylotype) were selected and sequenced at GATC Biotech, Konstanz, Germany using the TOPO-primers T3 and T7 as well as the 16S-rRNA gene-specific primers 27F, 359F and 23S30R for verification. The resulting sequences were analyzed using Geneious™ software (Geneious Pro 5.3.6) and their closest matches identified via a Mega-BLAST search of the GenBank database. Sequences were deposited in GenBank under the accession numbers as indicated in Supplemental Table 3.

2.4.1 Morphological identification

Microscopic analysis was performed using a Nikon Eclipse TS 100 Microscope and images documented with a Nikon Digital Sight DS-5M camera. Image quality was improved using XnView for Windows Software (version 1.97.6; Libformat version 5.70) and scale bars included by Corel Photo Paint 11 for Windows (version 11.633). Species identification down to genera level was made using the taxonomic guides of Komarek and Anagnostidis [31,32].

2.5 Data evaluation

Data were evaluated using Graphpad Prism™ Software (Prism 5 for Windows, Version 5.04).

3. Results

3.1 Detection of toxins in Arctic cyanobacterial communities

3.1.1 Saxitoxin detection

One of the five samples analyzed with the STX ELISA tested positive (Sample E; 21 (SD=16) µg STX / kg dry weight; n=6) well above the detection limit of the STX ELISA and in the centre of the standard curve. However the concurrent HPLC-FLD analyses were not able to confirm this finding, possibly due to the detection limit of the individual STX variants (LOD between 0.5 – 13 µg / kg). Additionally different sample matrices may result in matrix suppression which may increase limits of detection (Pers. comm. Michael Boundy, Cawthron, October 2012). None of the other samples tested positive in the STX ELISA, either as original samples or when cultured in the laboratory at various temperatures (see Kleinteich *et al.* (2012) [29] for laboratory culture conditions). Subsequent PCR amplification of two segments (128 bp and 657 bp) of the *sxt*A gene provided for a positive signal in sample E but not for the other four samples (Table 1). Additionally, the *sxtA* gene mRNA was detected in sample E (Figure 2).

The *sxt*A gene encodes for a polyketide synthetase, which is part of the recently identified cyanobacterial *sxt* gene cluster [33]. The amplified 128 bp and 657 bp products of sample E were sequenced and compared to the GenBank data base. The 657 bp gene segment shared a high similarity with known *sxtA* genes from the freshwater cyanobacteria *Scytonema* cf. *crispum* (97 %, HM629429) and *Lyngbya wollei* (95 %, EU603711.1). In contrast, the 128 bp sequence was most similar to the *sxtA* gene of *Aphanizomenon* (99 %, HQ338481.1) and *Anabaena circinalis* (99 %, HQ338478.1) (Supplemental Table 3). A phylogenetic tree (Figure 3) was constructed using the 657 bp product of the *sxtA* gene in sample E, and the sequence grouped in the same clade as *Lyngbya wollei* (EU629174), and the *Scytonema* (HM629429) sequence. One *Cylindrospermopsis* (EU629178) and several *Anabaena* and *Aphanizomenon* sequences clustered in a different clade.

3.1.2 Microcystin detection

Liquid chromatography-MS analysis of sample A (Figure 4A) that contained 106 µg MC / kg dry weight in the ELISA assay, showed that multiple MC variants were present in the extract. One of these compounds had a similar retention time to that of MC-RR (Figure 4B), but yielded a doubly-protonated ion with *m/z* 526.7. Tandem MS of the *m/z* 526.7 ion revealed fragment ions resembling a di-arginated MC (minus CN_2H_2; [34]). Also observed in the fragment ions was a loss of 60 Da which suggested that the MC contained ADMADDA instead of the generally observed ADDA (minus HOAc; [35]). Assignment of the fragment ions indicated that this MC also contained alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu) and an 83 Da moiety (Mdha or Dhb) in the sequence Ala-Arg-Asp-Arg-ADMADDA-Glu-Mdha/Dhb (Table 2). This was most likely the known MC; [Asp^3, $ADMADDA^5$, Dhb^7] MC-RR (Figure 5), previously described in *Nostoc* [36].

There were several minor compounds present in Sample A that showed some structural similarity to MCs, but could not be identified using the current sample due to insufficient individual compound quantity. The four other samples which previously tested negative for MC by ELISA [29] tested negative for MC by LC-MS.

All five samples were tested for the presence of genes of the *mcy* gene cluster, responsible for MC synthesis. Three different genes involved in MC synthesis (*mcyA, mcyE* and polyketide synthase regions) were amplified for sample A, which had tested positive for MC in the ELISA and LC-MS analysis, providing a product of the correct size (Table 1). Subsequent sequencing of the products and GenBank comparison however resulted in only one product annotated to a gene involved in secondary metabolite production, i.e. an amino acid adenylation domain of *Clostridium* (Supplemental Table 3). The other two products did not result in a specific identification of an annotated gene.

283 For the other four samples (B-E), negative for MC in the ELISA and LC-MS analysis, at least one gene
284 involved in MC synthesis was amplified, sequenced and annotated in GenBank to a known gene
285 involved in MC synthesis (Supplemental Table 3) with similarities ranging between 60% and 99 %. In
286 total, seven sequences, annotated to genes involved in MC synthesis, were amplified. The genera
287 corresponding to these genes were *Microcystis*, *Nostoc* and *Microcoleus* (Supplemental Table 3).
288
289 *3.2 Potential toxin producers in Arctic cyanobacterial communities*
290 A clone library was constructed for the samples that contained either MC (sample A) or STX (sample
291 E) to identify the potential toxin producers. Genera were also identified using light microscopy.
292 Molecular characterization of the 16S-ITS region demonstrated that the species present in the STX
293 containing sample E were most similar to: *Nostoc puntiforme* (CP001037, 95 %), *Leptolyngbya*
294 *(frigida)* (AY493573, 97 %), *Calothrix* sp. (JN385289, 92 %), *Snowella littoralis* (AJ781040, 98 %), and
295 *Tolypothrix distorta* (GQ287651, 98 %). Fewer cyanobacterial signals were obtained for sample A,
296 and the sequences were most similar to; *Aphanizomenon gracile* (FJ424575, 94 %), *Leptolyngbya* sp.
297 (DQ431004, 94 %), and *Chroococcus* (FR798926, 97 %). Light microscopy showed that both samples
298 had a similar appearance, with dominating Nostocales embedded in a firm mucilaginous matrix.
299 Other orders, albeit in lower abundance, i.e. Oscillatoriales (e.g. *Leptolyngbya*) and Chroococcales
300 were also present. The Nostocales observed had cells of 3 – 6 µm in diameter and contained
301 heterocytes (Supplemental Figure 2). *Leptolyngbya* with a trichome width of approximately 1.7 µm
302 was present in both samples. In Sample A *Tolypothrix* was characterized by dark brown colored
303 sheaths and a trichome width of approximately 15 µm.
304
305 **4. Discussion**
306 Cyanobacterial toxin production is a worldwide phenomenon with concomitant widespread adverse
307 health effects in humans and wildlife of the temperate and tropical regions [37,38]. The adverse
308 effects are not only of acute nature but can also entail fundamental changes to whole ecosystems
309 upon chronic or intermittent acute exposure events [39,40]. Despite the high abundance of

cyanobacteria in the Arctic [2], at present there is only a single recent report of MCs in Arctic cyanobacteria [29] and in cyanobacteria associated lichen [41]. Although MC was below the limit of detection in most of the environmental samples analyzed by Kleinteich *et al*. (2012) [29], MC concentrations increased dramatically when cultured under laboratory conditions and at higher ambient temperatures. This was also associated with a profound change in the species diversity of the cyanobacterial mats investigated. Whether the higher toxin quantities produced and/or the higher temperatures induced the change in species diversity could not be determined. These data suggest that continued climatic change may led to increases in cyanotoxins in polar regions. In an extension this study, the same cyanobacterial communities from the Canadian Arctic were analyzed for the presence of STX and further characterization of the MC was undertaken.

4.1 Saxitoxins in Arctic cyanobacteria and their potential producers

The presence of STX was confirmed for the first time in a polar freshwater environment via immunological analyses as well as the detection of part of the *sxt* gene cluster and the corresponding mRNA product. The levels detected (21 µg STX / kg dry weight) were much lower than those reported for pure cultures of benthic cyanobacteria of temperate regions (*Scytonema* cf. *crispum*, 66 x 10^6 µg STX / kg dry weight [30]; *Lyngbya wollei*, maximum 58 x 10^6 µg STX / kg dry weight [42]). The latter difference is likely a result of the low abundance of the producer in a mixed environmental sample and low nutrient availability in contrast to a pure culture. Lack of STX detection using HPLC-FLD may be explained by the fact that approximately 26 different variants of STXs are known to date [43]. HPLC analysis relies on the detection of each individual STX variant. In contrast, multiple STX variants can be detected with the ELISA, albeit with different affinities according to the manufacturer (e.g. decarbamoyl STX with 29% and GTX 2 & 3 with 23%), thus giving a sum value of STXs present in the respective sample analyzed. Thus if multiple variants are present in a sample, reliable HPLC detection depends upon the concentration level of each variant present and not on the total STX concentration (as determined by STX-ELISA).

337 In contrast to MC which is widely distributed among cyanobacterial taxonomic groups [12], only a
338 few cyanobacterial species have been reported to produce the STXs, including *Aphanizomenon*,
339 *Planktothrix*, *Scytonema* and *Lyngbya* [30]. The limited number of STX producing cyanobacteria may
340 explain why STXs and the *sxt* genes were detected only in one out of five Arctic cyanobacterial
341 samples analyzed. Consequently, the distribution of STX producing species in the Arctic, at least in
342 the area studied, could be limited and solely dependent on the unique species composition of the
343 respective cyanobacterial community. Although the STX producing species could not be identified,
344 *Scytonema* or *Lyngbya* appeared likely candidates. The latter genus seems to have a widespread
345 distribution in the Arctic regions as it has been reported from the Canadian High Arctic [44,45], as
346 well as Spitsbergen [46]. The *sxtA* sequence obtained from this sample showed highest sequence
347 similarity to *Lyngbya* and *Scytonema* sequences. *Tolypotrix* or *Scytonema* were identified in the
348 sample by microscopy and *Tolypothrix* had the closest match in the 16S rRNA gene sequence library.
349 *Tolypothrix* and *Scytonema* both belong to the order of *Nostocales* and *Scytonema* has recently been
350 identified as a STX producer [30].
351
352 *4.2 Microcystins in Arctic cyanobacterial communities and their potential producers*
353 In a previous study MC was detected in one of the five cyanobacterial samples (Sample A) using an
354 ADDA specific ELISA assay [29]. In the present study the MC congener in Sample A was putatively
355 identified as [Asp^3, ADMADDA^5, Dhb^7] MC-RR. Although there are no studies available on the cross-
356 reactivity of ADMADDA with the ADDA specific antibody of the ADDA-ELISA kit, it is possible that the
357 concentration of the ADMADDA substituted MC variant is underestimated in this assay. The [Asp^3,
358 ADMADDA^5, Dhb^7] MC-RR variant has been reported before by Beattie *et al.* (1998) [36] in a *Nostoc*
359 strain (DUN901) isolated from brackish water in the United Kingdom and is reported here for the first
360 time in the Arctic region. A MC with the same mass, [Asp^3, ADMADDA^5] MC-RR, was recently
361 reported in lichen (*Peltigrea membranacea* / *Peltigrea hymenia*) associated cyanobacteria (most
362 likely *Nostoc* or *Nodularia*) from Scotland [41]. While Kaasalainen *et al.* (2012) reported the presence
363 of [Asp^3, ADMADDA^5] MC-RR, the characterization of the MC congeners identified was not reported.

As MS/MS alone cannot discriminate between Mdha and Dhb, it is possible that the MC identified in their study could have also contained a Dhb moiety.

MCs have been reported previously in a range of habitats in continental Antarctic (McMurdo Ice Shelf, Bratina Island and Dry Valleys; [26–28]). The MC analyses demonstrated the presence of new and unusual MC congeners, e.g. [Gly^{1}] MC-LR and -RR, some of which contained the acetyldesmethyl ADDA [ADMADDA^{5}] substitution [27] also observed in this study. Wood *et al.* (2008) identified the cyanobacterial genus *Nostoc* as an Antarctic MC producer [27] based on microscopy and molecular methods. The latter corroborated the reports by Beattie *et al.* (1998) and Kaasalainen *et al.* (2012) [36,41] suggesting that *Nostoc* could be the producer of the [Asp^{3}, ADMADDA^{5}, Dhb^{7}] MC-RR congener. Even though in this study all of the Arctic cyanobacterial communities were dominated by Nostocales, other potential MC producing genera of cyanobacteria were observed, e.g. *Leptolyngbya* [48]. Both *Nostoc* and *Leptolyngbya* were detected via genetic and microscopic analyses in samples A and E. Due to the unusual MC congener identified and the information provided by earlier publications [36,41,47], it is hypothesized that *Nostoc* could also be the MC producer in this Arctic sample. However, an exact identification of the MC producer is not possible based on the currently available data and attempts at sub-culturing the producer remained unsuccessful to date.

Genetic analyses of the Arctic cyanobacterial samples revealed that at least some species within the communities may have the potential to produce MCs. All of the samples contained at least one gene of the *mcy* gene cluster, involved in MC production (Table 1; Supplemental Table 3). The genes detected, included the *mcyA*, the *mcyB* and, in one case, the *mcyE* region (Table 1, Supplemental Figure 1), encoding for non-ribosomal peptide synthetases and a hybrid enzyme, respectively [49]. The signal intensity of *mcyA* was distinctly higher in sample A which tested positive for MC, suggesting a high abundance of the MC producer. When compared to the GenBank database however, some of the sequence similarities to known *mcy* genes were low (60 to 99 %; Supplemental Table 3). In general the cyanobacterial *mcy* operon is known to be highly variable [50,51] with

391 sequence similarities of the *mcy* genes between the two most studied MC producing species,
392 *Microcystis aeruginosa* and *Planktothrix aghardii,* only ranging between 53 and 86 % depending on
393 the gene region [50]. Low sequence similarities as detected in this study may therefore be a result of
394 the high geographic and phylogenetic separation between known MC producing cyanobacterial
395 species from temperate and tropical regions and the Arctic species of this study. More sequence data
396 and pure cultures are required to confirm genetic variations and phylogenetic relationships.
397
398 *4.4 Ecological implications of toxins in Arctic cyanobacterial communities*
399 The concentrations of STX and MC detected (21 µg STX / kg dry weight and 106 µg MC / kg dry
400 weight, [29]) are low when compared to those found in planktonic cyanobacterial blooms of
401 temperate and tropical regions (up to 65.6 x 10^6 µg STX / kg dry weight, [30]; and up to 20 x 10^6 µg
402 MC / kg dry weight, [52]) but are comparable to the levels detected in cyanobacterial mats of the
403 Antarctic (1 to 15,900 µg MC / kg dry weight, [27]). Nevertheless, the presence of these compounds,
404 albeit in low concentrations, could have a selective long-term effect on the organisms living in and
405 feeding on the cyanobacterial communities. Depending on the spatial distribution of the toxin
406 producer and the toxin itself within the mat, as well as on the feeding strategy, microorganisms and
407 metazoans may be differentially exposed to the toxins. Saxitoxin and MC containing cyanobacteria
408 have been reported to be toxic to nematodes, crustaceans and rotifers [53–57]; these groups are
409 also present in many cyanobacterial mats. Trophic interactions in these mat communities are
410 currently poorly understood and the effects of the toxins on the organisms present are largely
411 unknown. It has been suggested that rising temperatures could increase toxin concentrations in polar
412 cyanobacterial communities [29,58] and thus exacerbate potential effects of toxins on metazoan
413 organisms [54]. In the cryosphere many physiological processes happen just above a minimum
414 threshold level [59] and energy consuming production of secondary metabolites e.g. MC and STX
415 may be limited [29]. Kaebernick and Neilan (2001) [19] postulated that MC synthesis is increased
416 under optimal temperature and growth conditions, which are speculated to be around 20 °C for polar
417 cyanobacteria [60]. Thus a warmer climate as predicted for the Arctic in current climate change

418 models [61] could elevate the temperature above the minimum threshold level for toxin synthesis,
419 leading to an increased general metabolic activity and thus an increased level of toxin production
420 [29,62,63]. Monitoring toxins and changes in cyanobacterial diversity could help in understanding
421 climate change effects in the polar regions as well as assisting in evaluating the role of cyanobacterial
422 secondary metabolites in these environments.

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615

616 **Figure legends**

617

618 **Table 1: Genes for toxin production in five Arctic cyanobacterial samples.**

619 Detection of the *mcy* and *sxt* operon in five Arctic cyanobacterial communities suggesting the

620 potential for microcystin and saxitoxin production.

621

622 **Table 2: Identification of the microcystin (MC) variant by LC-MS.**

623 Fragment-ion pattern detected for [Asp^3, $ADMADDA^5$, Dhb^7] MC-RR when observed by electrospray

624 ionisation collision-induced dissociation.

625

626 **Fig 1: Chemical structures of saxitoxin (STX) and microcystin (MC)**

627 General structure of the STXs (A; adapted from Humpage *et al.* 2008 [8] and the general structure of

628 the MCs (B; adapted from [64].

629

630 **Fig 2: mRNA expression of the *stxA* gene.**

631 Detection of mRNA expression of the *sxtA* gene in Arctic cyanobacterial sample A. Two different
632 primer pairs sxta and sxtA were used resulting in a 650 bp and a 170 bp long product respectively.
633 Negative controls were performed excluding reverse transcriptase to reduce false positive signals of
634 DNA contamination.
635
636 **Fig 3: Phylogenetic analysis of *sxtA*.**
637 The 657 bp long sequence obtained is given in boldface; other sequences were obtained from
638 GenBank. Sequences were aligned and the tree constructed using Geneious™ software (version
639 5.5.6) as a Neighbor-Joining tree after the method of Jukes-Cantor. Substitutions per site are given
640 next to the branches.
641
642 **Fig 4: Identification of the microcystin (MC) variant by liquid chromatography – mass spectrometry**
643 **(LC-MS).**
644 High pressure LC-MS basepeak chromatograms (*m/z* 100-2000) of a methanolic extract of Artic
645 cyanobacterial Sample A (**A**) and of authentic standards of MC-RR, MC-YR and MC-LR (**B**) separated
646 on a C_{18} column.
647
648 **Fig 5: Chemical structure of detected and reference microcystins (MCs).**
649 Structures of MC-RR and [Asp^3, $ADMADDA^5$, Dhb^7] MC-RR.