Diversity and Ecophysiology of Cyanobacterial Mat Communities in Arctic and Antarctic Ecosystems

DISSERTATION

Zur Erlangung des
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der Naturwissenschaften

an der

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Vorgelegt von

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“Curiosity is the one thing invincible in nature.”

Freya Stark

I. Publications and Honours

A. Publications

Kleinteich J, Wood SA, Küpper FC, Camacho A, Quesada A, Frickey T, Dietrich DR: Temperature related changes in polar cyanobacterial mat diversity and toxin production. Nature Climate Change. 2012 May; 2: 356-360.

Kleinteich J, Wood SA, Puddick J, Schleheck D, Küpper FC, Dietrich DR: Potent toxins in Arctic environments – presence of saxitoxins and an unusual microcystin variant in Arctic freshwater ecosystems. Chemico-Biological Interactions. Manuscript accepted.

Kleinteich J, Wood SA, Cirés S, Quesada A, Pearce D, Convey P, Küpper, FC, Dietrich, DR: Widespread distribution of cyanobacterial toxins in Antarctica and implications of climate change. Manuscript ready for submission.

B. Conference talks

Common patterns in Antarctic microbial biodiversity and vulnerability to environmental change, 10th Antarctic Funding Initiative Workshop, 2011, Cambridge, UK.

Climate change induced shift in cyanobacterial species in Arctic and Antarctic mats. Byers Meeting 2009, Miraflores, Spain

C. Posters

Buhmann M, **Kleinteich J**, Dietrich DR, Kroth P, Schleheck D: Cultivation of Defined Photoautotrophic / Heterotrophic Biofilm Communities. Biofilms4 International Conference Southampton 2010.

Kleinteich J, Quesada A, Camacho A, Küpper FC, Wood S, Dietrich DR: Cyanobacterial Diversity and Toxin Production in Arctic and Antarctic Freshwater Ecosystems. IPY Oslo Science Conference 2010.

D. Honours, Awards and Fellowships

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II. Deutsche Zusammenfassung

Diversität und Ökophysiologie cyanobakterieller Mattengemeinschaften in den Ökosystemen der Arktis und Antarktis

Im Rahmen dieses Dissertationsprojekts wurde das Ökosystem der bisher wenig beschriebenen ‚cyanobakteriellen Matten' aus den Süßwassersystemen der Arktis und Antarktis untersucht. Der Fokus dabei lag auf ihrer Diversität, Toxizität und den Auswirkungen des Klimawandels auf diese beiden Parameter.

Cyanobakterien (auch bekannt als ‚Blaualgen') sind weltweit verbreitete, photosynthetische Bakterien, die hauptsächlich im Süßwasser aber auch in marinen Küstengewässern verbreitet sind. Als Pionier-Organismen sind sie auch an extremen Standorten zu finden und stellen so in den terrestrischen Polargebieten den Hauptteil der Primärproduzenten, womit ihnen eine entscheidende Rolle im Ökosystem zukommt. In den verschiedenen Süßwassersystemen der Arktis und Antarktis wachsen sie im polaren Sommer zu mehreren Quadratmeter umfassenden Matten heran, die einer Vielzahl von anderen Organismen (vor allem Proto- und Metazoen) als Lebensraum und Nahrungsgrundlage dienen.

In dieser Arbeit wurde die Diversität (Artzusammensetzung) mehrerer Matten-Gemeinschaften aus der Arktis und Antarktis, die auf verschiedenen Expeditionen im Vorfeld und während der Dissertation gesammelt wurden, phylogenetisch beschrieben. Die Charakterisierung geschah durch morphologische Identifizierung sowie der Sequenzierung des ribosomalen RNA Gens sowie der variableren ITS-Region (genetischer Fingerabdruck). Dabei wurden konventionelle molekularbiologische Methoden (Klon-Bibliotheken, Automated-Ribosomal-Intergenic-Spacer-Analysis) angewandt, aber auch die Methode des sehr fortschrittlichen „Next-Generation-Sequencing" basierend auf der 454® Technologie, das bei sehr hohem Durchsatz eine großen Probentiefe ermöglicht. So konnte ein umfassendes Bild der phylogenetischen Diversität gezeichnet und Vergleiche innerhalb einer Region sowie zwischen Arktis und Antarktis gezogen werden (Manuskript 1).

Cyanobakterien synthetisieren eine Reihe von Sekundärmetaboliten, von denen einige toxisch für die meisten höheren Organismen sind, einschließlich des Menschen. Durch das massenhafte Auftreten von Cyanobakterien, so genannten Blüten, kommt es in gemäßigten und tropischen Regionen immer wieder zu gesundheitsgefährdenden oder sogar tödlichen Zwischenfällen. Es konnte in dieser Arbeit nachgewiesen werden, dass auch in den cyanobakteriellen Matten der Arktis und Antarktis die cyanobakteriellen Toxine zu finden sind. So konnte eine ungewöhnliche Variante des cyanobakteriellen Lebertoxins Microcystin (Manuskript 1 & 2), das als Inhibitor zellulärer

Proteinphosphatasen (PP 1, 2A, 4 und 5) wirkt, sowie das neurotoxische Saxitoxin, das die neuronale Reizweiterleitung durch eine Blockade der Na^{2+}-Kanäle inhibiert, erstmalig in der Arktis nachgewiesen werden (Manuskript 2). Weiterhin konnte Cylindrospermopsin, ein Inhibitor der Proteinsynthese, erstmalig in der Antarktis detektiert werden (Manuskript 3). Diese Befunde lassen darauf schließen, dass das Potenzial zur Toxinbildung in den cyanobakteriellen Mattengemeinschaften der Arktis und Antarktis größer ist als bisher angenommen.

Die Polarregionen sind einem starken Wandel unterworfen: Der Klimawandel lässt hier die Temperaturen schneller steigen als in jedem anderen Teil der Erde. Es ist zu erwarten, dass sich mit dem Klimawandel auch das Ökosystem der Mattengemeinschaften, bestehend aus Cyanobakterien und darin lebenden Proto- und Metazoen, weitgehend verändert, die durch ihren einfachen trophischen Aufbau als ideales Modellsystem für die Untersuchung klimabedingter Veränderungen fungieren. In einem Laborexperiment wurde daher die Auswirkung des Klimawandels auf die Artenzusammensetzung sowie die Toxizität der Cyanobakterien untersucht. Es konnte nachgewiesen werden, dass erhöhte Temperaturen (8 - 16 °C) eine verstärkte Produktion von giftigen Sekundärmetaboliten in kultivierten Cyanobakterien der Arktis und Antarktis auslösen. Ferner findet eine Umstrukturierung ihrer Diversität stattfindet (Manuskript 1).

Basierend auf diesen Ergebnissen sowie denen anderer vorangegangener Studien ist zu erwarten, dass in den Polarregionen wie auch in unseren Breiten der Klimawandel Auswirkungen auf die Toxizität von Cyanobakterien zeigen wird, wobei mit einer steigenden Verbreitung giftiger Cyanobakterienarten, sowie mit einem Anstieg der Gift-Produktion selbst zu rechnen ist. Im Hinblick auf die zunehmende Wasserknappheit und die intensivierte Nutzung von Wasserspeichern zur Trinkwassergewinnung, Bewässerung, Aquakultur und Freizeit ist daher mit einer Verschärfung des Problems und somit mit einer erhöhten Toxinbelastung beim Menschen zu rechnen.

III. Summary

Cyanobacteria (more commonly known as blue-green algae) are photosynthetic bacteria with a worldwide distribution. They are most common in the freshwater environment but are also present in many marine waters. As they are pioneer-organisms and they can often be found in extreme environments. They also constitute the dominant primary producers in the terrestrial Polar Regions and therefore have a pivotal role in polar ecosystems. In freshwater streams, ponds, and lakes that are formed during the polar summer, they occur as benthic or floating mats several millimetres to centimetres thick, sometimes covering several square meters. These mats are the nutritional basis and the micro-habitat for several other types of organisms (primarily proto- and metazoa).

This study describes the species diversity of cyanobacterial mat communities from the Arctic and Antarctic that were collected prior to and during the thesis using molecular phylogenetic techniques. The characterization was accomplished by morphological identification as well as the sequencing of the ribosomal RNA genes and the more variable ITS-region (intergenic spacer region). Conventional molecular biological methods (clone-library, automated ribosomal intergenic spacer analysis (ARISA)) were applied as well as „Next-Generation-Sequencing“ based on the 454® technology. The latter allows thousands of sequences to be obtained from a single sample. Using these methods a comprehensive picture of the phylogenetic diversity could be obtained and mats from the Arctic and the Antarctic region compared with and within each other.

Cyanobacteria synthesize multiple secondary metabolites, some of which are toxic to most higher organisms including humans. Health hazards or even life threatening incidents regularly occur in the temperate and tropical regions during bloom events (mass-occurrences of cyanobacteria).

This study demonstrated that cyanobacterial toxins are present in cyanobacterial mats of the Arctic and the Antarctic. In the Arctic two cyanobacterial toxins were recorded for the first time: An unusual variant of the cyanobacterial hepatotoxin microcystin. This toxin acts as an inhibitor of cellular protein phosphatases (PP 1, 2A, 4 und 5). The second toxin identified was the neurotoxic saxitoxin which inhibits neuronal signal propagation by blocking Na^{2+} channels. Cylindrospermopsin, a protein synthesis inhibitor was detected for the first time in the Antarctic. These results indicate that the potential for toxin production in the cyanobacterial communities of the Arctic and Antarctic is higher than previously thought.

The Polar Regions are currently subject to profound change: Temperatures are increasing at higher rates than anywhere else on the planet. In is expected that a consequence of climate change will be that the composition of the mat communities

comprising cyanobacteria and associated proto- and metazoans, are affected. Because of their simple trophic structure and their sensitivity to change, they are an ideal model system for the exploration of climate induced changes. Therefore the effect of climate change on the diversity and toxicity of cyanobacterial mats was studied here in a laboratory based approach. Increased temperatures (8 - 16 °C) resulted in a higher concentration of microcystin as well as in structural changes of the community composition. Based on these results as well as on previous studies, it can be expected that climate change will affect the toxicity of cyanobacteria in the Polar Regions and in lower latitudes. A general increase and a wider distribution of toxic cyanobacteria, as well as an increase of the rate of toxin production may be expected.

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V. Table of Content

VI. Table of Figures

Supplementary Figures

VII. Table of Tables

Supplementary Tables

VIII. Abbreviations

AA, Amino acid; Adda, 3-amino-9-methoxy-2,6,8 trimethyl-10-phenyldeca-4,6 dienoic acid; ADMAdda, 9-acetoxy-3-amino-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; Ala, alanine; ANOVA, analysis of variance; *aoa*, cylindrospermopsin gene cluster; ARISA, Automated Ribosomal Intergenic Spacer Analysis; Arg, arginine; Asp, aspartic acid; BAS, British Antarctic Survey; bp, base pairs; BLAST, basic local alignment search tool; BMAA, β-methylamino-L-alanine; BSA, bovine serum albumin; C18, octadecyl carbon chain; CCAP, Culture Collection for Algae and Protozoa; cDNA, complementary deoxyribonucleic acid; CGS, Collaborative Gearing Scheme, CYN, cylindrospermopsin; *cyr*, cylindrospermopsin gene cluster; Cys, cysteine; Da, Dalton(s); DEU, Deutschland; DEPC, diethylpyrocarbonate; Dhb, dehydrobutyrine; DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; dNTPs, deoxyribonucleotides; dw, dry weight; EtOH, ethanol; EDTA, ethylenediaminetetraacetic acid; ELISA, Enzyme-linked immunosorbant assay; F, phenylalanine; g, gram(s); Glu, glutamic acid or glutamate; h, hour(s); HPLC, High-performance liquid chromatography; H_2O, water; ITS, intergenic spacer; k, kilo (10^3); L, Litre; L, leucine; LC, liquid chromatography; LD_{50}, lethal dosage ; LOD, limit of detection; log, logarithm; LPS, lipopolysaccharides; Lys, lysine; m, milli (10^{-3}) or metre(s); M, molar or moles per litre; MC, microcystin; *mcy*, microcystin gene cluster; MeOH, methanol; Mdha, *N*-methyldehydroalanine; MDS, multidimensional scaling; mg, milligram; $MgCl_2$, magnesium chloride; min, minute(s); mRNA, messenger ribonucleic acid; mrpA, microcystin related protein A; MS, mass spectrometry; MuLV, Moloney Murine Leukemia Virus Reverse Transcriptase; *m/z*, mass-to-charge ratio; N, nitrogen; *n*, sample size; n, nano (10^{-9}); NCBI, National Center for Biotechnology Information; NERC, National Environmental Research Council; NRPS, non-ribosomal peptide synthetase; nd, not detected; NMR, nuclear magnetic resonance; P, inorganic phosphate; PCR, polymerase chain reaction; pH, hydrogen ion concentration PKS, polyketide synthase; PP, protein phosphatase; ppm, parts per million; RFLP, restriction fragment lengths polymorphism; RNA, ribonucleic acid; rRNA, ribosomal ribonucleic acid; ROS, reactive oxygen species; RT-PCR, Reverse transcription PCR; R, arginine; RT, room temperature; s, second(s); SAMS, Scottish Association for Marine Science; SD, standard deviation; SDS, Sodium dodecylsulfate; ser, serine; STX, saxitoxin; *sxt*, saxitoxin gene cluster; TAE, Tris base, acetic acid and EDTA; thr, threonine; Tm, annealing temperature; U, units; UV, ultra violet; v/v, volume per volume ratio; W, tryptophan; WHO, World Health Organisation; w/v, weight per volume ratio; °C, degrees Celsius; μ, micro (10^{-6}); λ, wavelength; Δ, difference; 2D / 3D, 2-dimensional / 3-dimensional.

1 INTRODUCTION

1.1 Cyanobacteria

Cyanobacteria are a phylum of phototrophic gram negative prokaryotes that inhabit almost every illuminated biotope and form an integral part of the marine and freshwater phytoplankton (Whitton and Potts, 2012). By their abundance and their diversity they are amongst the most important organisms on earth with a conservative estimate of 3×10^{14} g C or a thousand million tons (10^{15} g) of wet weight biomass (Garcia-Pichel et al., 2003). Cyanobacteria have a long evolutionary history with an estimated age of at least 3.5×10^{9} years (Schopf, 2012). Their ancestors were the first organisms to perform oxygenic photosynthesis, using light to reduce water for carbon fixation under the emission of oxygen, and thereby forming the early oxygenated atmosphere (Schopf, 2012). For photosynthesis they posses accessory pigments besides chlorophyll (e.g. phycoerythrin, phycocyanin) due to the often blue colour of which their more common name “blue-green algae” is derived. Due to their ancient origin, preliminary forms of the cyanobacteria are considered as the early ancestors of plant chloroplasts (Adams et al., 2012).

Modern taxonomy recognizes the orders of Oscillatoriales, Nostocales, Chroococcales, Pleurocapsales, but also Gloeobacterales, Prochlorales, and Stigonematales (NCBI, 2012). The phylogeny of cyanobacteria is however not yet fully resolved and is subject to constant amendment. This is in particular due to new sequencing methods and the availability of an increasing set of, as well as more detailed, genetic data (Whitton and Potts, 2012).

Cyanobacterial species occur ubiquitously in almost every type of environment, albeit they are most abundant in the freshwater and marine environment. Here most cyanobacterial species occur dispersed throughout the water column, forming an integral part of the phytoplankton, but they may be also present as benthic or floating mats (Callieri et al., 2012; Paerl, 2012; Scott and Marcarelli, 2012). Certain environmental factors favour the sudden and exponential growth of cyanobacteria, leading to surface or subsurface blooms (Oliver et al., 2012). In the temperate regions, this phenomenon mainly occurs in

eutrophic stratified freshwater lakes in the late summer that are exposed to high solar radiation (Oliver et al., 2012).

Figure 1-1: Cyanobacterial bloom in a German lake (Templiner See), in August 2010 (Photograph courtesy of Julia Kleinteich).

Due to their broad habitat adaptability cyanobacteria are pioneer organisms and often thrive in extreme or rapidly changing environments, as for example endolithically in deserts (Bhatnagar and Bhatnagar, 2005), as benthic mats in hot springs (Miller et al., 2007) or in the Polar Regions (Jungblut et al., 2009). Besides free-living forms, some species of cyanobacteria live in symbiotic association with fungi, bryophytes, gymno- or angiosperms (Chorus and Bartram, 1999). The high diversity of cyanobacterial habitats and their ability to thrive in extreme environments is the result of special adaptations (Chorus and Bartram, 1999). As such cyanobacteria can fix inorganic nitrogen by special nitrogenase enzymes, and are therefore largely independent from organic nitrogen supply (Oliver et al., 2012). In order to survive long periods of darkness and other unfavourable conditions, some cyanobacterial species can evolve resting stages (e.g. akinetes) (Whitton and Potts, 2012). Finally, cyanobacteria produce a range of toxic second metabolites, and these are discussed in the following paragraphs.

1.1.1 Cyanobacterial Toxicity

A wide range of second metabolites are produced by cyanobacteria, some of which are toxic to higher organisms (Chorus and Bartram, 1999). The ability to produce the toxic metabolites is a widespread phenomenon throughout most phylogenetic groups of cyanobacteria, and a wide range of habitats including marine, freshwater and terrestrial habitats worldwide are affected (Chorus and Bartram, 1999). The high structural and functional diversity of cyanobacterial toxins include: microcystins (MCs), saxitoxins (STXs), cylindrospermopsins (CYNs), and anatoxins. These cyclic polypeptides or alkaloids act as hepatotoxins, neurotoxins, dermatotoxins, or as general cytotoxins (Table 1-1) (for review

see Pearson et al. 2010 or Humpage 2008). The quality and quantity of the produced toxins in a given environment however depends largely on the species composition and environmental factors. The biological benefit of the toxins for the cyanobacterial cell has not yet been resolved (see paragraph 1.1.8).

Table 1-1: Selected cyanobacterial toxins and their mode of action.

Structure	Toxin	Target organ	Mode of action	Toxic structure	Species (examples)
Cyclopeptides	Microcystin	Liver, Kidney, Neuronal system (?)	Inhibition of ser/thr specific PPs	ADDA	*Microcystis Nostoc Planktothrix*
	Nodularin	Liver, Kidney	Inhibition of ser/thr specific PPs	ADDA	*Nodularia*
Alkaloids	Saxitoxin	Neuronal system	Blockage of Na^+ channels		*Anabaena Aphanizomenon*
	Anatoxin-a	Neuro-muscular blocking	Postsynaptic cholinergic nicotine agonist	Agonist acetyl-choline	*Anabaena Aphanizomenon Planktothrix*
	Anatoxin-a(S)	Neuronal system	Inhibition of acetyl-choline-esterase activity	Agonist acetyl-choline	*Anabaena*
	Cylindro-spermopsin	All tissues	Inhibition of protein- and glutathione- synthesis, DNA damage	Uracil bridge	*Cylindrospermopsis Aphanizomenon*
	BMAA (β-Methylamino-L-Alanin)	Most tissues	Binding to Glu-receptors, protein integration	Glu-agonist	Most species of cyanobacteria
Lipopoly-saccharides	LPS	Skin, Mucosa	Inflammatory	Fatty acid	All gram(-) bacteria

1.1.2 Implications for human and animal health

Adverse health consequences of cyanobacterial toxins for humans and livestock have been reported in many temperate and tropical countries (de Figueiredo et al., 2004; Dietrich and Hoeger, 2005; Stewart et al., 2008) affecting all living organisms including humans, cattle, fish, zoo- and phytoplankton, as well as higher plants (Chorus and Bartram, 1999; Briand et al., 2003; de Figueiredo et al., 2004). Several deaths and severe human poisonings have been connected to acute intoxication with cyanobacterial toxins (reviewed

in Dietrich et al. 2008b; de Figueiredo et al. 2004) while chronic exposure has been reported in connection with increased cancer rates and is hypothesized to induce neurological diseases (Ueno et al., 1996; Feurstein et al., 2011; Qiu et al., 2012). The most common, acute and chronic, exposure route is probably contaminated drinking water (Dietrich, Fischer, Michel, and Hoeger, 2008). When considering the increase of the human population and the need for clean drinking water resources, this becomes especially problematic. Especially in developing countries exploration of drinking water affected with cyanobacteria often co-occurs with poor or insufficient water treatment. Global warming exacerbates this problem as it limits existing water resources and simultaneously is predicted to result in an increased number and abundance of toxic cyanobacterial blooms as described in chapter 1.1.9 (Paul, 2008; O'Neil et al., 2012).

Figure 1-2: Lake closed for recreational activities due to toxic cyanobacteria, Germany in August 2009 (Photograph courtesy of Julia Kleinteich).

Other routes of exposure which may lead to ingestion of cyanobacterial toxins include contaminated fish or shellfish, but also vegetables that have been irrigated with contaminated water (de Figueiredo et al., 2004). Recreational activities in contaminated water bodies, inhalation of aerosols in sauna applications, and more recently the consumption of food supplements based on algae (BGAS) have also been discussed as possible ways of exposure (Dietrich, Fischer, Michel, and Hoeger, 2008). While acute exposure to cyanobacterial toxins may be circumvented with relatively simple methods (control of water bodies, appropriate drinking water treatment, or avoidance of contaminated recreational water bodies), a chronic low dose exposure, for example by the consumption of drinking water or BGAS, is a much more likely scenario (Dietrich, Fischer, Michel, and Hoeger, 2008) that has been given much less attention. Long term studies on the chronic exposure to cyanobacterial toxins have not been undertaken.

Consequently, next to regular monitoring of water bodies for toxic cyanobacteria, research is needed to understand the current distribution of toxic cyanobacteria and the environmental factors triggering toxin production, to better predict future developments of toxic cyanobacterial blooms.

1.1.3 Microcystins: Structure, Toxicity, Occurrence, Case studies

The cyclic heptapeptide microcystin (MC) comprises the largest group of cyanobacterial toxins with more than 110 structural variants known to date (Neilan et al., 2012; Puddick, 2012). In freshwater, MCs are also the most common cyanobacterial toxins (Chorus and Bartram, 1999). Their structural diversity is based on two variable amino acids on position 2 and 4, as well as side chain modifications such as methylation (Figure 3-1). Single congeners can thereby exhibit different toxicities (Watanabe et al., 1988; Blom et al., 2001; Dittmann and Wiegand, 2006).

The toxicity of MCs is mainly based on the unusual amino acid 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4(E),6(E)-dienoic acid (ADDA) that is exclusively found in the cyanobacterial metabolites MC and nodularin. Toxic variants of MCs act by an irreversible inhibition of the active centre of serin/threonin specific protein phosphatases, wherby a covalent binding is formed between the Mdha of the MC and a Cystein-residue of the PPs (MacKintosh et al., 1995). They are therefore toxic to most eukaryotic organisms (Dittmann and Wiegand, 2006; Pegram et al., 2008). Most MC variants can not penetrate the bi-lipid layer of cellular membranes, but require the active uptake via specific transporters (Fischer et al., 2005). Organ toxicity in higher animals including humans is therefore primarily based on the presence / absence of these transporters. MC leads to severe liver damage in humans under acute exposure and is hypothesized to be carcinogen (Yu, 1995; Ueno et al., 1996; Fleming et al., 2002) and neurotoxic (Feurstein et al., 2009, 2011) under chronic exposure.

Microcystin production is mainly associated with bloom forming planktonic cyanobacteria (e.g. *Microcystis* and *Planktothrix*) but has also been reported from benthic species (e.g. *Oscillatoria limnosa,* Humpage 2008) or in cyanobacteria that live in symbiosis as lichen (i.e. *Nostoc*) (Kaasalainen et al., 2012). The toxins have been found in water bodies worldwide (Chorus and Bartram, 1999). The most severe incident of MC intoxication known has occurred in 1996 in a haemodialysis unit in Caruaru, Brazil, where more than 120 dialysis patients were exposed with poorly treated water intravenously from a contaminated resource. More than 60 patients died as a consequence of intoxication with cyanobacterial toxins, now known to be mainly MCs (Pouria et al., 1998).

1.1.4 Saxitoxins: Structure, Toxicity, Occurrence, Case studies

Saxitoxin (STX) and its variants are carbamate alkaloids (Figure 3-1) that are primarily neurotoxic to all higher organisms (Dittmann and Wiegand, 2006). Saxitoxins are more commonly known as paralytic shellfish poisons when produced by marine dinoflagellates (Pegram et al., 2008). More than 30 naturally occurring isoforms of STX are documented, some of which are exclusively produced by freshwater cyanobacteria (Humpage, 2008; Wiese et al., 2010). Saxitoxins are blocking agents of voltage-gated Na^+ and Ca^{2+} channels and modify K^+ channels. They thereby act as potent inhibitors of neuronal signal propagation (Murray et al., 2011) causing numbness and paralysis or even death by respiratory arrest (Humpage, 2008).

Few planktonic and benthic cyanobacterial species have been verified to produce STXs (Murray et al., 2011; Smith et al., 2011), including planktonic *Anabaena circinalis*, *Cylindrospermopsis raciborskii* (Humpage et al., 1994; Lagos et al., 1999), and benthic *Lyngbya wollei* (Onodera et al., 1998) species.

Acute STX intoxications majorly occur through contaminated seafood with an estimated number of 2,000 intoxications per year and mortality rates of app. 15 % (Hallegraeff, 2003). The economic losses are therefore considerable. These incidences however, are caused by marine dinoflagellates, whereas chronic intoxication via drinking water of saxitoxin producing freshwater cyanobacteria is less well understood (Humpage, 2008).

1.1.5 Cylindrospermopsin: Structure, Toxicity, Occurrence, Case studies

Cylindrospermopsin (CYN) is a cytotoxic alkaloid with an uracil moiety (Figure 4-1) of which only three structural variants are known: CYN, 7-epi-CYN and 7-deoxy-CYN. The primary toxic function is an irreversible inhibition of cellular protein synthesis leading to cell death. It seems to be metabolically activated by Cyt P450, as inhibition of this enzyme leads to decreased toxicity, albeit not to regenerated protein synthesis, suggesting at least two toxic mechanisms (Humpage et al., 2005). Cylindrospermopsin has also been reported to inhibit glutathione synthesis (Runnegar et al., 1995) and to be genotoxic (Humpage et al., 2005; Pegram et al., 2008). Acute intoxications are described to induce liver necrosis, as well as adverse effects on the kidney and other tissues (Humpage, 2008). Carcinogenic effects by chronic exposure can however, not be excluded. CYN has a circumpolar distribution but has not yet been detected in the Polar Regions (Sinha et al., 2012). Few producers of CYN have been identified, including the planktonic *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Anabaena bergii*, *Umezakia natans*, *Raphidiopsis curvata* (Humpage, 2008) and the benthic *Oscillatoria* sp. (Mazmouz et al., 2010). However, the number of known CYN producing species increases constantly and more non-identified

species are suspected to produce the toxic compound (Humpage, 2008; Mazmouz et al., 2010).

The toxin was first identified in 1979 in an intoxication event on Palm Island, Queensland, Australia. More than 100 people were treated for hepatoenteritis, connected to a bloom of *C. raciborskii* in a freshwater reservoir (Griffiths and Saker, 2003). Numerous drinking water sites have been found to be contaminated with cylindrospermopsin (Falconer and Humpage, 2005) so that chronic long-term exposure and resulting effects cannot be excluded.

1.1.6 Other cyanobacterial toxins

Other toxic second metabolites produced by cyanobacteria are the hepatotoxic nodularin, the neurotoxic anatoxin-a, and anatoxin-a(S), the tumor promotors lyngbyatoxin and aplysiatoxin, as well as β-methylamino alanine that is suspected to induce neurodegenerative disorders (Okle et al., 2012). Many other cyanobacterial secondary metabolites have not yet been described in detail but are suspected to have bioactive or even toxic potential (Humpage, 2008). With increasing research and public interest, more and more toxic compound are being identified in a wider range of habitats. As in the natural environment cyanobacterial toxins often do not occur as single compounds but as a mixture of different metabolites, the potential synergistic effects are not well understood, but could increase the toxic potential beyond the simple addition of single effects (Dietrich, Fischer, Michel, Hoeger, et al., 2008).

1.1.7 Genetics of cyanobacterial toxins

Many cyanobacterial secondary metabolites including MCs, STXs, and CYNs are produced non-ribosomally by complex and energy intensive pathways involving multi-enzyme-complexes. These often include non-ribosomal peptide synthetases (NRPS), and polyketide synthases (PKS) (Neilan et al., 2008). The enzymes are encoded on large gene clusters containing multiple genes. The *mcyS* (55 kb) as well as the *sxt* (25.7 - 36 kb) gene cluster for MC and SXT synthesis respectively, have been fully sequenced in several cyanobacterial species including *Microcystis* sp., *Anabaena* sp. and *Planktothrix* sp. for MC and *Aphanizomenon* sp., *Anabaena circinales* and *C. raciborskii* for SXT (Kellmann, Mihali, Jeon, et al., 2008; Neilan et al., 2008, 2012; Pearson et al., 2010; Murray et al., 2011). The *mcyS* cluster comprises 10 genes (*mcyA-J*) on two individual operons, coding genes for MC synthesis as well as an ABC transporter-like protein (*mcyH*) putatively responsible for the transport of the toxin (Neilan et al., 2008). The *sxt* gene cluster carries 33 genes encoding biosynthetic enzymes, transporters and regulatory proteins (Neilan et al., 2012). Phylogenetic analysis has shown that both gene clusters (*mcyS* and *sxt*) are very ancient and older than eukaryotic life (Rantala et al., 2004; Murray et al., 2011).

The gene cluster for CYN production *cyr/aoa* (43 kb) has only recently been sequenced in, to date four different genera (*C. raciborskii*, *Aphanizomenon* sp., *Oscillatoria* sp., and *R. curvata;* Jiang et al. 2012; Mazmouz et al. 2010; Mihali et al. 2008; Méjean et al. 2010; Stüken and Jakobsen 2010). In contrary to the *mcyS* and *sxt* gene clusters, which are variable between species (Christiansen et al., 2003; Rouhiainen et al., 2004; Kellmann, Mihali, Michali, et al., 2008; Wiese et al., 2010), the *cyr* gene cluster seems to be more conserved, even though gene rearrangements must have occurred (Stüken and Jakobsen, 2010). Whereby the sequences of *Cylindrospermopsis raciborskii* and *Aphanizomenon* sp. showed high similarities, the sequence of *Oscillatoria* sp. is most distant from the other known genera (Jiang et al., 2012). The different genetic diversities are also reflected as the number of variants known from each of the toxin groups: Whereas around 110 congeners of MC (Puddick, 2012) and 57 of STX (Wiese et al., 2010) are known, to date only three have been identified for CYN (Jiang et al., 2012).

1.1.8 Biological role of cyanobacterial toxins

Many theories have been postulated about the physiological function and ecological regulation of cyanobacterial toxins (Kaplan et al., 2012). The toxic second metabolites have been hypothesized for a long time to act as anti-grazing agents. In the light of the recently discovery ancient origin of MC and STX genes, with an estimated age older than eukaryotic life (Rantala et al., 2004; Murray et al., 2011), a primarily role as grazing protection seems however questionable. Rantala et al. (2004) suspect that the *mcy* operon was present in all ancient cyanobacteria and that its present erratic distribution can be explained by prevalent gene-loss events. Despite the unknown initial function of cyanobacterial toxins, it is possible that during the later evolutionary process, under the selection pressure of grazing and competition, the anti-grazing component has gained importance. The high conservation of the gene clusters responsible for toxin production over millions of years, as well as the high energy investment into the expensive synthesis of the compounds may imply that the initial function is still present in cyanobacteria.

As many MCs have a cysteine-binding residue on their methyl-dehydroalanine (MdhA) amino acid they covalently bind to cysteine containing proteins such as protein phosphatases (MacKintosh et al., 1995). The covalent binding to thiol-groups of other proteins than protein phosphatases was shown in another study (Zilliges et al., 2011) and was increased under high light conditions and oxidative stress. In that study it was therefore hypothesized that the primarily function of MCs, is the protection of intracellular proteins of the cyanobacterial cell against oxidative stress under high light conditions (Zilliges et al., 2011). Moreover, the promoter region between *mcyA* and *mcyD* seems to have a binding site for regulators of ferric uptake as well as nitrogen, implicating a sensitivity of *mcy* transcription towards the iron and nitrogen availability and therefore its redox status

(Kaplan et al., 2012). These theories are strengthened by the fact that MCs are to a major part located intracellular. The presence of a hypothetical ABC-transporter on the *mcy* operon (*mcyH*) and the release of small amounts of the toxin into the medium could however suggest other possible functions (Kaplan et al., 2012): Small molecules produced by microbes in low concentrations have been discussed recently to act as messenger molecules between cells rather than for antibiosis (Davies, 2007) and to regulate gene expression, thereby influencing community interactions (Yim et al., 2007). In this context MCs have been discussed as signalling molecules in a quorum sensing-like manner (Kaebernick and Neilan, 2001; Schatz et al., 2007; Kaplan et al., 2012). Studies with a *mcyB* deficient mutant of *M. aeruginosa* revealed the protein MrpA (microcystin-related protein A) that seems to be related to cell-signalling proteins of other microorganisms (Kaplan et al., 2012). Moreover, a strong upregulation of MC synthesis was observed in *Microcystis* after the addition of media supernatant of lysed cells (Schatz et al., 2007).

In summary it can be speculated that MCs have differential intracellular and extracellular functions. Intracellular they might act as protecting molecules of cellular proteins (Zilliges et al., 2011), whereas, when released into the media, they may be messenger molecules to other cyanobacterial cells (Schatz et al., 2007; Kaplan et al., 2012). The role of different congeners of MCs is thereby yet unclear.

The ecological role of STX is even less well understood than that of MC. It has recently been discovered that the *sxt* gene cluster has a similarly ancient origin as the *mcy* gene cluster, and that K^+ rather than Na^+ channels were initially the target of the molecule (Murray et al., 2011). A primarily function as a neurotoxic agent may thus be excluded. Other potential functions that have been discussed are nitrogen storage, DNA metabolism, or chemical signalling (Murray et al., 2011). The synthesis of STX seems to be influenced by environmental factors such as temperature, conductivity, light, and nitrogen (Neilan et al., 2012), a specific association between environmental factors and STX production could however not yet been elucidated. The recent description of the *sxt* gene cluster along with transcriptomic studies should help in clarifying the biological function of this toxin.

For CYN at least one biological function seems to be resolved. When produced by *Aphanizomenon* sp. cells under P limiting conditions the toxin is described to induce alkaline phosphatase synthesis and excretion in other algae. This increases extracellular P levels and gives a growth advantage to the CYN producer under low P conditions (Bar-Yosef et al., 2010). The authors state that the synthesis of CYN for the cyanobacterial cell is energetically less expensive than the production of alkaline phosphatases (Bar-Yosef et al., 2010; Kaplan et al., 2012).

In general cyanobacterial secondary metabolites seem to be produced under conditions favourable for cyanobacterial growth and proliferation (Neilan et al., 2008, 2012). Unfavourable conditions, that hardly sustain cyanobacterial growth, may not allow

the cyanobacterial cell to invest energy in the expensive production of secondary metabolites. Indeed, toxin production is often linked to rapid cyanobacterial proliferation and bloom formation. In summary, the biological reason for cyanobacterial toxin production remains to be elucidated but the clarification of the involved gene clusters and new and more efficient molecular methods may help to answer this question.

1.1.9 Climate Change and Cyanobacteria

On a worldwide scale the frequency, intensity, and distribution of toxic cyanobacterial blooms is expected to increase as an effect of eutrophication of water bodies as well as a warming global climate (Paerl and Huisman, 2008; El-Shehawy et al., 2012; Neilan et al., 2012; Paerl and Paul, 2012; Sinha et al., 2012). Toxic cyanobacteria are expected to produce higher concentrations of toxins as a direct effect of temperature, supporting higher growth rates, as well as an indirect effect by increased stratification and eutrophication of many water systems (O'Neil et al., 2012; Paerl and Paul, 2012; Posch et al., 2012). Higher concentrations of P and N are reported to sustain higher biovolumes of cyanobacteria (Dolman et al., 2012), however, also re-oligotrophication may sustain certain toxin producing species (Ernst et al., 2009; Posch et al., 2012). A warming climate facilitates the invasion of toxic cyanobacterial species from tropical to temperate regions, due to more favourable growth conditions. This problem is intensified by species transitions through increased human mobility and activity (Frenot et al., 2007). The establishment of non-native species in a new environment has often severe consequences on the local ecosystem. In the case of toxic cyanobacteria also health hazards as well as economic losses must be considered (Sukenik et al., 2012). For cyanobacteria the invasion of the CYN producing *C. raciborskii* to sub-tropical and temperate regions is probably the best documented case and has been associated with eutrophication as well as climate change (Wiedner et al., 2007; Sinha et al., 2012; Sukenik et al., 2012). The species formerly confined to tropical and sub-tropical areas has now been increasingly reported from Northern Europe, North America and New Zealand (Sinha et al., 2012). Although better methods of detection and intensified research may bias these findings, future water bodies will most likely be subjected to an increased number of toxic cyanobacterial blooms with health consequences for humans.

1.2 The Arctic and Antarctic

The Polar Regions are, next to the deep sea, the most hostile as well as understudied places on Earth. More than 99 % of the Antarctic and most of the high Arctic are covered in ice and snow (SCAR, 2012). Extreme cold and the absence of light for half of the year allow only few organisms to survive. Whereas the Arctic is an Ocean that is surrounded by continental land masses (Siberia, Alaska, Northern Canada, and Northern Europe), the continent of Antarctica is surrounded by the southern ocean and is the highest, driest, and coldest continent on earth (SCAR, 2012). Their extreme climate as well as their geographic separation makes the polar ecosystems one of the last pristine environments on earth that have been relatively untouched by human activity. The Polar Regions are not deserted of life, but on the contrary host some of most extraordinary life forms that bear astonishing adaptations to a life at the edge. The majority of this life is microbial. Microorganisms survive and reproduce here in all kinds of habitats: bare rock and soil, melt water lakes, on the snow, or even within the ice itself (Bell, 2012). More recently subglacial lakes that have potentially been separated from the rest of the world for several million years are discussed as a resource of previously unseen forms of life (Alekhina et al., 2007).

1.2.1 Polar freshwater systems

The Polar Regions are diverse in their freshwater systems, ranging from minute inclusions in the ice, to medium-sized melt water ponds to the large streams of the Arctic e.g. the Mackenzie-River (for review see Vincent et al. 2008). In this work only the smaller melt water ponds and streams will be discussed which appear seasonally both in the Arctic and the Antarctic. They are being formed by snow melt during the polar summer, creating a highly dynamic landscape of moist and dry habitats of short temporal duration and high inter-annual variation (Hawes et al., 1999; Rochera and Camacho, 2012). These habitats can form on substrates composed of rocks or soil as for example in the Dry Valleys (Wood, Rueckert, et al., 2008; Jungblut, Wood, et al., 2012) and the Antarctic Peninsula (Fernández-Valiente et al., 2007; Yergeau, Newsham, et al., 2007), but also on large glaciers and ice shelves such as the McMurdo Ice Shelf (de los Rios et al., 2004; Jungblut et al., 2008).

Temperatures in larger polar freshwater bodies, e.g. seasonal or perennial ice covered lakes, are usually stable throughout the year and lie below 5 °C with higher temperatures reached only in the surface layers (Vincent et al., 2008). Smaller water bodies on the other hand, can be subjected to intense daily and seasonal temperature fluctuations, with temperatures ranging from below freezing to up to 20 °C depending on the input of solar radiation and heat flux (Vincent et al., 2008; Quesada and Vincent, 2012). On yearly

average, freshwater systems at the poles receive less solar radiation than in the temperate regions; maximal values for irradiance in the summer however, can be extreme, especially in small ponds and streams that do not possess a protective ice or snow cover (Hawes et al., 1999).

Nutrient levels, salinity and pH largely depend on the catchment area of a freshwater system and are characterized by very high, spatial and temporal, variability in the Polar Regions. In general, nutrient levels are considered low, as terrestrial primary production is insignificant and biogeochemical processes are slowed down by low temperatures and low moisture (Vincent et al., 2008). Local variables such as bird or seal colonies in the catchment area can result in extreme spatial variation. The nutrient status has therefore been discussed as the most important factor, next to temperature and irradiance, controlling primary production in polar freshwater systems (Vincent et al., 2008).

Due to the constraints of temperature, irradiance and nutrient status, trophic levels in polar freshwater systems are usually extremely simple compared to those in the temperate regions, with short life cycles and high turn-over rates (Christoffersen et al., 2008). Whereas in Arctic lakes and rivers large predators such as fish occur, they are completely absent (except for a single crustacean species in coastal lakes) in the Antarctic (Christoffersen et al., 2008). In both, the high Arctic and the Antarctic, the trophic basis of most freshwaters are benthic phototrophic microorganisms. These communities often dominate total productivity and biomass of a given ecosystem and can reach immense standing stocks, as the result of gradual accumulation over many seasons in the stable bottom waters of seasonal or perennially ice-covered lakes (Vincent, 2000a). In shallow streams and ponds they develop highly diverse benthic or floating mats that can be several centimetres thick and extend over numerous square meters (Figure 4-3; Supplementary Figure 2-I; Supplementary Figure 2-II; Vincent 2000a; Zakhia et al. 2008). The temporary and variable character of this environment thereby requests high growth rates and the presence of opportunistic species that thrive in a habitat present only for few weeks of the year (Hawes et al., 1999). The mats provide a microclimate, with often higher ambient concentrations of dissolved nutrients and temperatures. They thus serve as a habitat and nutritional basis for several other types of organisms including phototrophic and heterotrophic protists (e.g. Chlorophyta, Dinophyta, Bacillariophyta, Haptophyta, Chrysophyta, and Cryptophyta) and metazoans (e.g. nematodes, rotifers, and tardigrades) and thus fuel the food chain of many polar freshwaters (Zakhia et al., 2008; Cary et al., 2010; Jungblut et al., 2010; Jungblut, Vincent, et al., 2012).

1.2.2 Cyanobacteria in Polar Ecosystems

The benthic communities of Arctic and Antarctic freshwater systems are often dominated by cyanobacteria (Vincent, 2000a). By their cellular biomass and the

extracellular mucilaginous matrix they form so called 'cyanobacterial mats' with a complex three-dimensional structure and functional differentiation (Paerl et al., 2000; de los Rios et al., 2004). Biochemical processes within the mats are adapted to microscale chemical gradients i.e. oxygen, pH, and light (Paerl et al., 2000; Stal, 2001; Vincent and Quesada, 2012). Nutrient concentrations within the interstitial fluids of the mats are often much higher than in the surrounding water column (Jungblut and Neilan, 2012). Whereas oxygenic processes e.g. photosynthesis happen in the upper layers of the mats and may reach a peak in the deep chlorophyll maximum, the lower layers are usually anoxic (Jungblut and Neilan, 2012). Different organisms and cyanobacterial species may be found in the different layers, according to their biochemical prerequisites (Paerl et al., 2000). Non-heterocystous, filamentous cyanobacterial genera (e.g. *Phormidium*, *Oscillatoria*) as well as diatoms, with strong pigmentation and therefore increased UV resistance, usually occur in the upper layers, whereas diazotrophs may be present in lower layers (Paerl et al., 2000). The basal layers are usually heterotrophic and dominated by anaerobic nitrate or sulphate reduction processes (Vincent and Quesada, 2012) by various phyla of bacteria e.g. *Apha-, Beta-, Gamma-Proteobacteria, Actinobacteria,* or *Bacteriodetes* (Varin et al., 2012).

Figure 1-3: Sampling of cyanobacteria in an Antarctic meltwater stream on Anchorage Island, in January 2011 (Photograph courtesy of Prof. FC Küpper).

1.2.3 Cyanobacterial Diversity and Endemism

In Antarctica cyanobacteria were first described more than 100 years ago by the first Antarctic explorers (Vincent, 2000a; Jungblut and Neilan, 2012). Since then many studies have been undertaken to investigate their diversity (e.g. Jungblut et al. 2012b; Jungblut et al. 2008; Taton et al. 2003; Vézina and Vincent 1997; Webster-Brown et al. 2010; Wood et al. 2008b; Zakhia et al. 2008). Cyanobacterial mats from both Polar Regions are in general

described to be rich in diversity. In the Antarctic they are dominated by Oscillatoriales, i.e. *Phormidium* sp. (*P. autumnale*, *P. deflexum*), *Oscillatoria* sp. (*O. limosa, O*. cf. *fragile, O. priestleyi*), *Lyngbya* cf. *limnetica* (Jungblut and Neilan, 2012), and *Leptolyngbya sp.* (Vincent and Quesada, 2012). Also Nostocales, i.e. *Nostoc* spp. and *Nodularia* sp. (Quesada et al., 2008; Jungblut and Neilan, 2012; Jungblut, Wood, et al., 2012) are present. Chroococalles and Stigonematales are less frequently found. A significant number of studies though have been based on morphological characterization, which uses the plasticity of the organism relies on the competence of the identifier (Komarek and Komarek, 2012). Small and single celled organisms may thereby not be as prominent and easy to identify as large filamentous species. Moreover, many taxonomic criteria refer to literature from the temperate and tropical regions that are often not applicable (Komarek and Komarek, 2012). More recently, the application of molecular tools such as the analysis of the 16S rRNA gene, as a genetic fingerprint, has simplified the identification of species. This trend is now being renewed by the introduction of high-throughput next-generation sequencing technologies. Despite new technologies, we still need to keep in mind that molecular data solely do not give any morphological and ecological information, whereas morphological characteristics are often insufficient in describing the genetic variability (Komarek and Komarek, 2012). Only a combined approach of both techniques may therefore reveal the full diversity of Arctic and Antarctic cyanobacteria.

Also the question whether Antarctic cyanobacteria are truly endemic has not yet been resolved. Comparative molecular analyses have been made on Arctic and Antarctic cyanobacteria that suggested different levels of geographic distribution (Jungblut and Neilan, 2012). Whereas some cyanobacterial species are present in both polar as well as in the temperate regions (e.g. *Phormidium autumnale*, *Leptolyngbya frigida* or *Nostoc commune*), many phylotypes detected, have been reported exclusively from the Antarctic continent, indicating at least a certain degree of endemism (Jungblut and Neilan, 2012; Kleinteich et al., 2012; Vincent and Quesada, 2012).

1.2.4 Ecophysiology of cyanobacteria

Many habitats in the Polar Regions are occupied by cyanobacteria: They occur in lake phytoplankton and benthic mats, endolithically or on rocks, on the ice of glaciers and ice shelves, as well as in symbiosis with fungi as lichen (Vincent, 2000a). In contrast they are almost absent the polar oceans. As pioneer organisms cyanobacteria are often the primary colonizer of a new environment and play a pivotal role in many established communities for nitrogen and carbon cycling. Their success in the harsh polar environment is a result of their resistance towards several environmental stressors such as extreme cold and freezing, desiccation, variable salinity and light conditions, as well as high UV radiation (Vincent et al., 2008).

Even though adaptation to cold may be expected one of the most important features, polar cyanobacteria are considered cryotolerant rather than cryophile with average growth optima of around 20 °C (range from 5 °C to 30 °C; Tang et al. 1997) and optimal values for photosynthesis and nitrogen fixation around 15 °C - 25 °C (Davey, 1989; Velázquez et al., 2011). Therefore conditions for the growth of cyanobacteria in most polar freshwater habitats are suboptimal throughout the year, with low average water temperatures and regular freezing. The seasonal and daily variations in this environment can be extreme (Quesada and Vincent, 2012). Water temperatures in some polar cyanobacterial habitats, such as shallow lakes and streams, or the top layers of deeper lakes, can reach maximum temperature values exceeding 15 °C (Vincent 2000; Supplementary Figure 4-I); ideal conditions for the growth of many polar cyanobacteria. Periods of cold-inhibition thus alternate with conditions close to the physiological maximum of the organisms present. This requires a fast adaptation of the organisms to this rapidly changing environment (Jungblut and Neilan, 2012).

Freezing often correlates with desiccation of the cells and simultaneously results in gradually increasing salinity in the remaining water body (Hawes et al., 1999; Vincent, 2000a). Both factors (salinity and desiccation) as well as the formation of ice crystals are stressors that require specific cold-adaptation mechanisms (Jungblut and Neilan, 2012). Recently Varin et al. (2012) have provided a metagenomic study detecting several genes for the functional response to environmental stress (e.g. cold shock proteins and membrane modifications) in microbial mat communities of the High Arctic and Antarctica (Varin et al., 2012). High UV radiation is met by DNA repair mechanisms and the formation of accessory pigments to protect the cyanobacterial cells (Jungblut and Neilan, 2012). Also the ability to fix inorganic nitrogen is advantageous for some cyanobacterial species e.g. *Nostoc* over competing organisms. Biological inorganic nitrogen fixation by cyanobacteria is estimated to contribute approximately 30 % to the total nitrogen requirement of ice-based cyanobacterial mats (Howard-Williams et al., 1989; Jungblut and Neilan, 2012). Cyanobacteria may survive the long and dark winter months by forming resting stages, such as akinetes. They may also survive in a freeze-dried status from they have been reported to recover rapidly (Davey, 1989; Hawes et al., 1992). In water bodies that remain partly liquid throughout the year, cyanobacteria may also remain active and even photosynthetic (Hawes et al., 1999). The survival of viable cells provides for an inoculum for the population of the next growth season. The described features also provide cyanobacteria with the ability to travel over long distances in the atmosphere, not affected by high solar radiation, desiccation, or low temperatures, thereby allowing long-distance dispersal.

It has been argued that a broad habitat tolerance is a pre-requisite for success in the highly dynamic habitats of many polar freshwater systems (Sutherland, 2009; Jungblut, Wood, et al., 2012). Competitive sorting along environmental gradients may therefore not

play a major role in community selection but a high overlap in the spatial range certain species occupy is expected. Attempts have been made to determine how environmental factors affect the composition of benthic mats in Antarctic moist terrestrial habitats, and conductivity, nutrients and pH have emerged as significant variables (Jungblut, Wood, et al., 2012). Biotic interactions on the other hand, have been assigned a subordinate role in terrestrial polar habitats (Hogg et al., 2006). Improved understanding of these communities requires a better appreciation of how environmental conditions and other organisms compete in determining community characteristics.

1.2.5 Toxicity

Only a small number of studies have investigated the toxicity of cyanobacteria in the Polar Regions. The first record was made by Hitzfeld et al. in the year 2000, for benthic mats of melt water ponds of the McMurdo Ice Shelf. The toxin MC was recorded in this study in a range of mats, however, no characterization of the producers or the exact MC variant was made. Jungblut et al. (2006) described the congeners [Asp^3] MC-LR and MC-LR in the same habitat. No producer of MC could be identified, however, *Phormidium, Oscillatoria, Anabaena* and *Nostoc* were described to occur in that habitat. These species are known to produce MCs in non-polar benthic microbial communities (Jungblut and Neilan, 2012). Genes for non ribosomal peptide synthetases (NRPS) and polyketidesynthase (PKS), involved in secondary metabolite production, were detected in that study, with highest genetic similarities (68 % and 75 %) to *Nostoc punctiforme* (Jungblut et al., 2006). Several new and unusual MC variants, containing [Gly^1] rather than alanine as well as [$ADMAdda^5$] substitutions were reported by Wood et al. (2008) in the Dry Valleys and Bratina Island in the continental Antarctic. *Nostoc* was identified as the most likely candidate to produce MC in that study. This was supported by the detection of sequences of the cyanobacterial *mcyE* gene (involved in MC synthesis) which were most closely related to the species of *Nostoc* (Wood, Mountfort, et al., 2008). Prior to the current study, no cyanobacterial toxins were known from the Arctic and no cyanobacterial toxin other than MC has ever been described from any polar environment. Only recently MC was reported in lichen associated cyanobacteria (*Nostoc*) in Svalbard (Kaasalainen et al., 2012), which is next to this study the only other record of cyanobacterial toxins in the cryosphere.

1.2.6 Climate Change in the Polar Regions

Global average surface temperatures have increase by about 0.74 °C over the past hundred years (between 1906 and 2005) with strong seasonal and regional variations; whereby eleven out of the twelve warmest years have occurred in the last twelve years (1995 to 2006) (Trenberth et al., 2007). Models state that next to natural variation, human-made action (e.g. the emission of greenhouse gases to the atmosphere) have contributed to

this trend (Trenberth et al., 2007). As a consequence the global climate is changing with already visible effects on the ecosystems worldwide.

Some regions on Earth are in particular subjected to this warming trend, including the Arctic as well as parts of the Antarctic. Surface temperatures on the Antarctic Peninsula have warmed at a rate of 0.5 °C per decade over the past 50 years (Turner et al., 2005). This region has therefore been designated as an area of rapid regional climate change (Vaughan, Marshall, et al., 2003). The warming has resulted in a general loss of ice cover (Rignot et al., 2008; Velicogna, 2009), increased the flow rate of many glaciers in the region (Pritchard et al., 2009), and has lead to the breakup of large ice shelves such as the Larson A and B Ice Shelf in 1995 and 2002, which is predicted to increase the flow rates of connected glacial systems even further (Rott et al., 2002; Pritchard et al., 2012). In the Arctic, the warming is mainly represented as the extreme loss of sea ice. Within the past 40 years the summer sea ice extent has shrunk to almost half of its initial size (satellite monitoring started in 1979). September 2012 even provided for a new minimum record after the record year of 2007 (NSIDC, 2012). Summer (and therefore multiyear) sea ice is predicted to disappear from the Arctic within a few decades (NSIDC, 2012). Glacial retreat, ice shelf breakup, as well (Rignot et al., 2008; Post et al., 2009; Velicogna, 2009).

1.2.7 Consequences of climate change for polar ecosystems

The effects of these changes on the marine and terrestrial ecosystems are already observable and have been summarized in studies for the Antarctic (Kennedy, 1996; Walther et al., 2002; Convey et al., 2003; Convey, 2006), and for the Arctic (Post et al., 2009). In general, an increased rate of local colonization processes as well as the invasion of species from warmer climates is expected, with the consequences of increased terrestrial diversity, biomass, and trophic complexity (Convey, 2006).

As the Arctic is better connected to the surrounding continents and has a more complex trophic web, terrestrial ecosystem changes are more conspicuous than in the Antarctic. The thaw of permafrost, the rise of the tree line to more northerly latitudes, the northern spread-out of species, or the loss of hunting grounds for polar bears, are just some examples of Arctic terrestrial ecosystem changes (Convey et al., 2003; Post et al., 2009; Steltzer et al., 2011).

In the Antarctic, changes attract less attention. In this remote continent, the spread-out of the only two native Antarctic flowering plants species, *Deschampsia antarctica* and *Colobanthus quitensis,* to more southerly habitats is one of the few observational studies that describe a direct ecological consequence of climate change (Smith, 1994). Other studies are more predictive: In a field manipulation study using enclosures Wynn-Williams et al. (1996) showed that pioneer soil microalgal colonization and community development were changed when exposed to higher temperatures *in situ* over a six years period (Wynn-

Williams, 1996a). This is one of the few long term studies of that kind. Similar results are reported by other field based studies (Kennedy, 1994; Smith, 1994; Convey and Wynn-Williams, 2002; Convey et al., 2002) indicating a rapid adaptation of terrestrial communities to changing environmental conditions. Also laboratory based experiments can help to understand the effects of single parameters on selected organisms (Velázquez et al., 2011; Kleinteich et al., 2012).

1.2.8 Climate change and polar freshwater ecosystems

Polar freshwater systems have been described as especially sensitive towards the effects of climate warming (Quayle et al., 2002). This in concert with their simple trophic setup, means they are considered early indicators of environmental change in the Arctic and Antarctic (Camacho et al., 2012; Rochera and Camacho, 2012). Vincent et al. (2010) postulate that the changing environmental conditions in the Arctic may significantly affect freshwater microbial systems (Vincent, 2010). The exploration of this has been one of the core topics of the International Polar Year (IPY) 2007-2008 under the multinational program 'Microbiological and ecological responses to global environmental changes in the Polar Regions' (MERGE) in which parts of this study were integrated under the Spanish LIMNOPOLAR project.

As an effect of climate change an earlier breakup of the ice-cover and a longer growth season are expected. A study by Pearce (2005) describes the transition of the phytoplankton communities in three Antarctic lakes over the season and suggests that the earlier breakup of the ice-cover may have substantial impact on the bacterio-plankton community (Pearce, 2005). This observation is confirmed by Rochera et al. (2012) who report an intense phytoplankton bloom after the ice-breakup of a lake on the Antarctic Peninsula, the time point of which is dependent on annual climate variations (Rochera and Camacho, 2012).

Changes in diversity and metabolism are predicted for cyanobacterial mat ecosystems. In detail, endemic psychrophilic species are forecast to be replaced by a more cosmopolitan diversity and the predominance of psychrotolerant species (Pringault et al., 2001; Vincent, 2010; Velázquez et al., 2011), as it was demonstrated with physiological studies on benthic phototrophic communities from the maritime Antarctic (Velázquez et al., 2011). In that study, also higher growth rates and changes in metabolism, i.e. nitrogen and carbon fixation, at higher temperatures were reported (Velázquez et al., 2011). Similarly, benthic mats in Fryxell Stream, Antarctica, have responded with positive metabolic activity to an artificial temperature increase (Vincent and Howard-Williams, 1989).

An indirect effect of rising temperatures is a generally higher freshwater availability, which does not only provide new habitats for freshwater communities but simultaneously changes the water balance of pre-existing lakes (Convey et al., 2003; Convey, 2006; Hawes,

Safi, Sorrell, et al., 2011). Specifically, a recent trend for warm summers has increased water influx, and, as most of the lakes occupy closed basins (no outflow), an increased water loading has resulted in a gradual increase in lake level. This results in a change of the light regime within the water column and thus a transition in the vertical zonation pattern of microbial communities as for example in Lake Joyce, Antarctica (Hawes, Sumner, et al., 2011).

In summary, the expected long term effects of climate change on polar freshwater ecosystems are, a higher biodiversity, increased growth rates, and therefore trophic complexity (Convey, 2006; Yergeau, Newsham, et al., 2007). Consequently, perturbations such as climate shift could affect the composition of the simple mat ecosystems as well as their role in polar ecological processes.

1.3 Aim of the Study / Need for Research

On a worldwide scale toxic cyanobacteria are expected to produce higher concentrations of toxins and to invade new habitats as a consequence of climate change (O'Neil et al., 2012), with to date unknown consequences on freshwater reservoirs and therefore human health. Temperatures on the Antarctic Peninsula as well as in the Arctic are increasing with higher rates than anywhere else on the planet (Trenberth et al., 2007). As freshwater systems are especially sensitive towards the effects of climate change (Williamson et al., 2009) they are considered as early sentinels of climate change induced ecosystem changes in the Polar Regions (Camacho et al., 2012). For cyanobacteria are the dominant primary producers of these freshwater systems (Vincent, 2000a) they are the ideal model organisms to study climate change in the Polar Regions.

This study hypothesises that toxic cyanobacteria are more widespread in terrestrial and freshwater ecosystems of the cryosphere than current data indicate. Moreover this toxicity could increase as a long term effect of climate change induced warming in the Polar Regions. For a better understanding of the baseline-distribution of various cyanobacterial toxins in the Polar Regions, cyanobacterial mats from several Arctic and Antarctic ecosystems were screened for the presence of various cyanobacterial toxins. Concurrently, the genetic basis for toxin production was evaluated. To simulate the effects of climate change on cyanobacterial communities and their toxicity a laboratory based experiment on cyanobacteria from the Arctic and the Antarctic was performed. Based on the results of this study it may be possible to predict future ecosystem changes in cyanobacterial dominated habitats induced by climate change in the cryosphere and worldwide.

2 MANUSCRPT 1

2.1 Temperature-related changes in polar cyanobacterial mat diversity and toxin production

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2.2 Main Text

One of the fastest rates of recent climate warming has been reported for the Arctic and the maritime Antarctic (Trenberth et al., 2007); for example, mean annual temperatures increased by 0.5 °C per decade over the Antarctic Peninsula during the past 50 years (Turner et al., 2005). Owing to their comparatively simple and highly sensitive food webs (Camacho, 2006a), polar freshwater systems, with cyanobacterial mats representing the dominant benthic primary producers (Vincent, 2000a), seem well suited for monitoring environmental perturbation, including climate change (Quayle et al., 2002). Prolonged climate change may challenge the resilience, plasticity and adaptability and thus affect the community composition of cyanobacterial mats. We demonstrate that exposing polar mat samples to raised temperatures for six months results in a change in species predominance. Mats exposed to a constant temperature of 8 °C or 16 °C showed high cyanobacterial diversity, commensurate with an increased presence of cyanobacterial toxins. In contrast, mats held at 4 °C and 23 °C seemed low in diversity. Our data thus indicate that a temperature shift to 8–16 °C, potentially reached during summer months in Polar Regions at the present warming rate, could affect cyanobacterial diversity, and in some instances result in a shift to toxin-producing species or to elevated toxin concentrations by pre-existing species that could profoundly alter freshwater polar ecosystems.

Cyanobacteria are considered the most important and dominant phototrophs in polar freshwater and terrestrial ecosystems and primarily occur in benthic or floating microbial mat communities up to several centimetres thick (Vincent, 2000a; Quesada et al., 2008). These mats are highly structured (de los Rios et al., 2004), typically dominated by two filamentous orders of cyanobacteria, Nostocales and Oscillatoriales (Vincent, 2000a), yet represent a simple ecosystem with few trophic levels (Cary et al., 2010). Antarctic cyanobacteria have been reported to produce microcystins (Hitzfeld et al., 2000). Microcystins are inhibitors of protein phosphatases and thus toxic to most eukaryotic organisms (Chorus and Bartram, 1999). However, the microcystin levels found in Antarctic cyanobacterial mats are much lower (Hitzfeld et al., 2000) than those reported for cyanobacteria of temperate regions (Chorus and Bartram, 1999). Contrary to Antarctic cyanobacteria (Hitzfeld et al., 2000), this is the first report on microcystins in Arctic cyanobacterial mats. Cyanobacterial mat communities also provide habitat and/or nutrition for other organisms including prokaryotes (archaea, heterotrophic bacteria and viruses), eukaryotic phototrophs (diatoms and green algae) and eukaryotic heterotrophs (nematodes, rotifers and tardigrades) (Quesada et al., 2008; Cary et al., 2010). Consequently, perturbations such as climate shift could affect the composition of these simple mat ecosystems as well as their role in polar ecological processes. Freshwater

habitats in the Polar Regions are increasingly subjected to the effect of climate change, for example a relative increase in water temperature (ΔT) 2-3-fold higher than the corresponding ΔT of the local air (Quayle et al., 2002). Concomitant with a higher nutrient input, an extended growth season and increased freshwater availability (Convey, 2006), changes in biological communities of freshwater ecosystems have previously been suggested (Pearce, 2005), and may lead to increased biodiversity, growth rates and thus trophic complexity (Convey, 2006; Yergeau, Newsham, et al., 2007). Moreover a shift from endemic psychrophilic species towards a more cosmopolitan diversity and predominance of psychrotolerant species could occur (Pringault et al., 2001; Vincent, 2010; Velázquez et al., 2011), as demonstrated with physiological studies on benthic phototrophic communities from the maritime Antarctic (Velázquez et al., 2011). In conjunction with changing growth conditions, the predominance of microcystin-producing cyanobacteria species may be favoured or new and toxin-producing species may establish themselves. The advantage of producing toxins at high energy expense, amongst many possibilities (Ginn et al., 2010), might be that microcystins provide protection from grazing (Lürling, 2003) especially when environmental conditions become less harsh and colonization by new species or facilitation for present grazers could alter the grazing pressure (Camacho, 2006b). Alternatively, microcystins are discussed to serve as quorum-sensing or –signalling molecules, thus employed by cyanobacteria to indicate changing growth conditions (Dittmann et al., 2001). Irrespective of their function, increased microcystin concentrations in mats, whether by an increase in number or relative abundance of pre-existing or newly established microcystin-producing cyanobacteria, could influence the species composition of the immediate mat community as well as the polar freshwater ecosystems in general.

Consequently, we reason that a continuously increased ambient temperature will shift the diversity and/or species predominance of cyanobacteria in microbial Antarctic and Arctic mats concomitant with an increase of microcystin concentrations.

Cyanobacterial mat samples were collected from five locations in the Arctic (North Baffin Island) and the Antarctic (Byers Peninsula, Livingston Island, South Shetland Islands). Antarctic cyanobacterial mats, with Oscillatoriales as the predominant cyanobacterial order, grew on wet soil substrate and in shallow ponds, forming stratified mats of up to several millimetres in thickness and appeared to have high species diversity (Supplementary Figure 2-I). Arctic cyanobacterial mats, predominated by Nostocales, were gathered from small streams and appeared to be more homogeneous in their community composition (Supplementary Figure 2-II, Supplementary Figure 2-I). Microscopic observation of all mat samples revealed a high variety of organisms, including cyanobacteria, ciliates, nematodes, tardigrades, rotifers, diatoms and green algae, thus corroborating earlier studies of eukaryotic diversity in cyanobacterial mats (Cary et al., 2010) and emphasizing the importance of these habitats within the polar ecosystems.

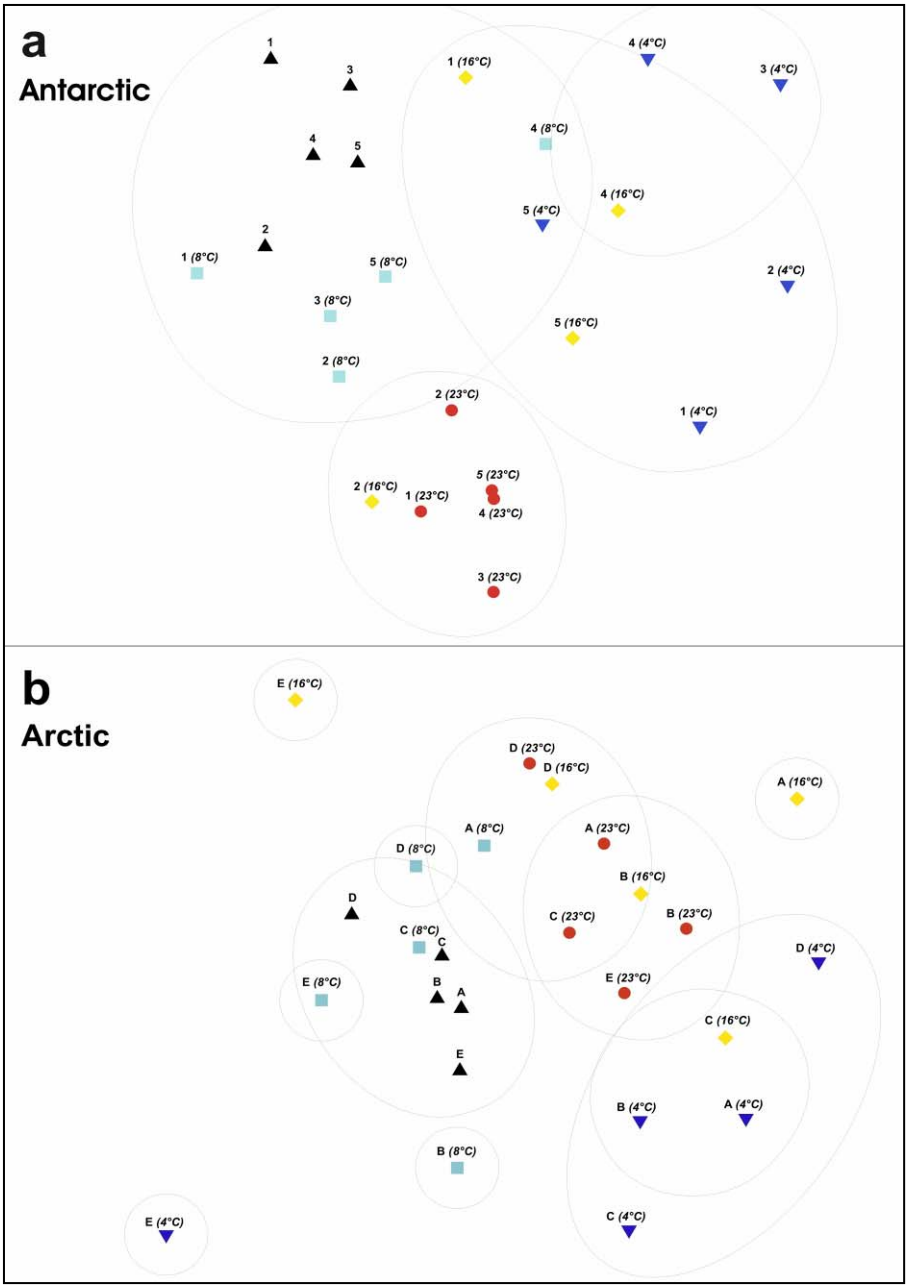


Figure 2-1: Community composition respective to temperature and origin of cyanobacterial mats and cultures.

a, b, Community composition of cyanobacterial mats (black triangles) and cultures (blue down triangles, 4 °C; blue squares, 8 °C; yellow diamonds, 16 °C; red circles, 23 °C) from the Antarctic (a) and Arctic (b), shown as a two-dimensional non-metric multidimensional scaling ordination (stress value of 0.12) based on Bray–Curtis similarities of ARISA fingerprints. Points within a circle cluster at 20 % similarity.

To elucidate the effects of increased temperature on cyanobacterial mat communities, we cultured small representative samples of the different cyanobacterial mats for six months at identical laboratory conditions but at different constant temperatures, thereby simulating the single influence of temperature on the individual communities.

Within the six-month time frame, cultured cyanobacterial mats grew at all temperatures; however, mats cultured at 8 °C attained a slightly lower biomass than those cultured at lower or higher temperatures (data not shown). As mats were cultured in BG-11 media (provided at the start but not replenished over the duration of the experiment) using cotton/aluminium stoppers, no additional species could have entered the flasks during culturing. Consequently, the changes in species observed for the same mat sample cultured at the different temperatures are a reflection of the influence of the temperature. The BG-11 media has a N/P ratio of 67.6:1, which is in excess of the 16:1 originally reported by Redfield for marine plankton (Barrett et al., 2007). However this N/P ratio of 67.6:1 is consistent with the range of N/P ratios (1.6:1-296:1) in the terrestrial surface waters of the Dry Valleys in McMurdo, Antarctica (Barrett et al., 2007), and more importantly lies within the range of N/P ratios reported for interstitial water of cyanobacterial mats in situ (range 53:1-121:1; Quesada et al. 2008).

Visual inspection of cultures indicated a temperature-dependent shift in relative pigment content or community composition, as cultures at low temperatures were pigmented red and orange whereas cultures at warmer temperatures had an intense dark green colouration (Supplementary Figure 2-III).The data collected from automated ribosomal RNA intergenic spacer analysis (ARISA) and 16S-rRNA gene sequence clone library analysis demonstrated a temperature-dependent difference in cyanobacterial community composition (Figure 2-1). The ARISA method not only compares shifts in community composition but, under the presumption that two to three ARISA fragment lengths (AFLs) are present in one cyanobacterial species (Boyer et al., 2001), also allows the number of AFLs to be used as a proxy for the total number of species present. However, as ARISA, that is AFLs, cannot determine whether or not a specific species was not detected because it was lost from culture or not detectable owing to very low abundance, the results as presented are a testimony of detectable species at the respective temperatures. It is noteworthy that, for both the Antarctic (Figure 2-1) and the Arctic (Figure 2-1) samples, AFLs of the original mats cluster in close proximity to the AFLs of the respective mats cultured at 8 °C (Figure 2-1), thus indicating that the original mats and the respective samples incubated at 8 °C had a similar community composition and species number (Figure 2-2) and within each sample group (Arctic or Antarctic) they converge to a similar community structure.

Moreover, AFLs of Arctic and Antarctic samples (irrespective of the culture temperature) do not cluster together, thereby indicating that the cyanobacterial clusters

from the two poles are distinctly different (Supplementary Figure 2-IV). The original mats and those cultured at 8 °C had a significantly higher detectable species number than observed in mats cultured at 4 °C, 16 °C and 23 °C (Figure 2-2). High temperatures (23 °C) resulted in a low number of AFLs, whereas the AFLs of cultures at 4 °C and 16 °C provided for a less clustered pattern. 16S-rRNA sequences derived from the clone libraries established from the original and cultured mats demonstrated that some of the species and orders, for example *Phormidium autumnale*, Nostocales and *Leptolyngbya* sp. , identified in the original Antarctic and Arctic mats were also almost exclusively found in the 8 °C mat cultures (Figure 2-3). This finding corroborated the earlier interpretation that the 8 °C conditions resemble the mean temperatures at the sampling sites during the growth season. An average temperature in the air and within cyanobacterial mats ranging between 0-3 °C and 0-7 °C, respectively, was observed in Antarctic cyanobacterial habitats, albeit the daily temperatures oscillated between 0 °C and 18 °C (data not shown). Thus, an average temperature of 8 °C most likely favours growth of a diverse cyanobacterial community (Vincent and Howard-Williams, 1989; Pringault et al., 2001; Velázquez et al., 2011), whereas higher temperatures, 16 °C and 23 °C, may be selective, as indicated by the presence of only four detectable species at 23 °C (Figure 2-3 and Supplementary Table 2-I).

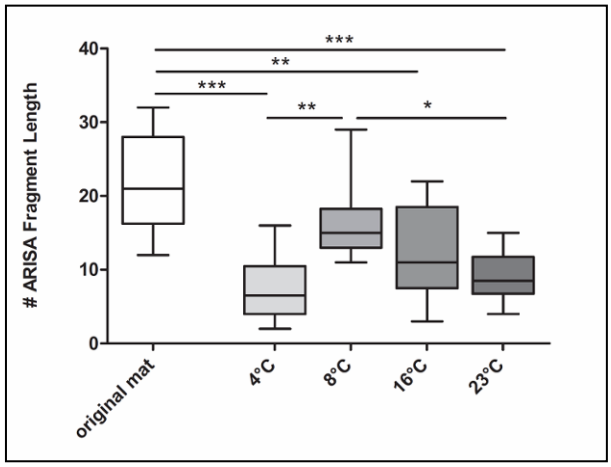


Figure 2-2: Temperature effects on detectable species numbers (number of ARISA fragments) in cyanobacterial mat cultures.

The diversity of 8 °C cultures is significantly higher than of mats cultured at 4 and 23 °C. Conversely, original Antarctic and Arctic mats show a significantly higher diversity than that observed in 4 °C, 16 °C and 23 °C cultures, but no difference to the 8 °C cultures. One-way analysis of variance, Bonferroni post-test, *n*=9–10; mean, 75 % percentile, min–max (*P<0:05; **P<0:01; ***P<0:001).

Regular exposure to oscillating temperature conditions would seem to inhibit the growth of highly competitive psychrotolerant species, sustain slow growing psychrophile species and thus result in a high biodiversity in polar cyanobacterial mats (Vincent and Howard-Williams, 1989; Upton et al., 1990). Some of the species found in the original mats,

for example *Leptolyngbya frigida*, were found in nearly all culture temperatures, demonstrating their adaptability to changes of environmental conditions. In fact, the high initial N/P ratio of 67.6:1 in BG-11 medium could possibly have influenced the community of diazotrophs, for example *Nostoc*, to shift towards non-nitrogen-fixing species such as *Leptolyngbya*. Conversely, specific species clusters were observed primarily in mats cultured at 4 °C and 23 °C, for example a psychrophile strain of *Leptolyngbya antarctica* at 4 °C and a high-temperature-adapted *Leptolyngbya antarctica* and multiple representatives of *Planktothrix* sp. as well as Oscillatoriales at 23 °C. Whether the `psychrophile' and the `high temperature adapted' *Leptolyngbya antarctica* represent subspecies or possibly truly different species can not be determined at present.

Although culturing cyanobacterial mats in the laboratory certainly does not directly compare to the situation present at the respective locations in the Antarctic and Arctic, it does allow an assessment of how temperature may influence diversity and growth of specific species, especially as all conditions (for example, transport, storage, light and medium) except temperature were similar. A similar manipulation under field experimental conditions, especially in the Polar Regions, would unfortunately be impossible. Our laboratory data demonstrate that the ambient temperature has a clear (direct or indirect) effect on the species composition of polar cyanobacterial mats. Obviously the mat cultures used were not axenic monocultures; consequently, other factors, for example bacterial degradation and viral activity, may have been influenced by the temperature as well and in turn have influenced the growth of individual cyanobacteria (Pearce, 2005). However, this is also assumed to occur under field conditions and would contribute to the overall temperature-mediated change in species diversity as indicated with our laboratory experiments.

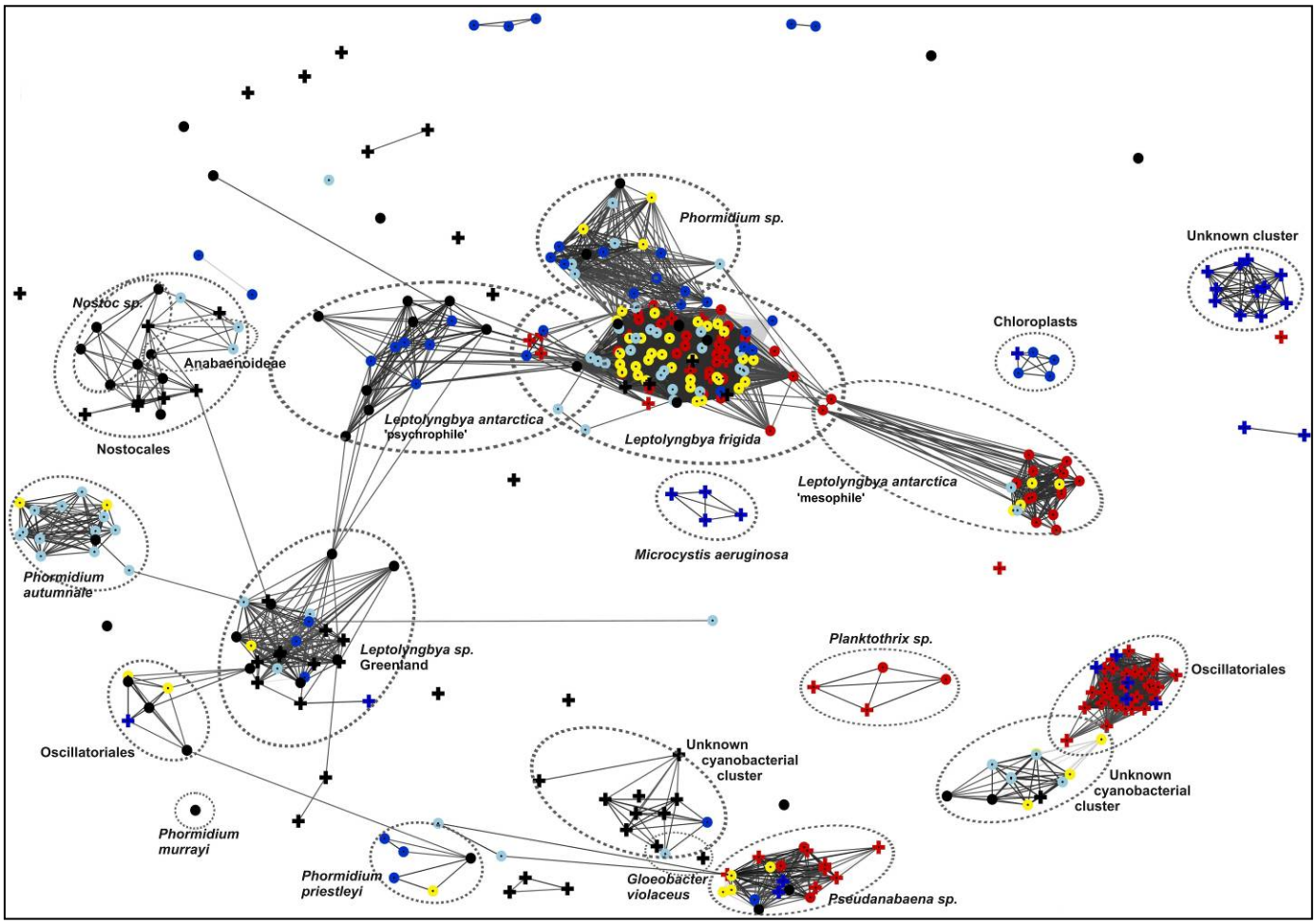


Figure 2-3: Cyanobacterial species distribution in original and cultured mat samples.

Two-dimensional illustration (from a three-dimensional map) of 16S-rRNA pairwise sequence similarities of cyanobacterial clones derived from original Arctic (plus symbols) and Antarctic (circles) cyanobacterial mats (black symbols) and their respective cultures (dark-blue symbols, 4 °C; light-blue circles, 8 °C; yellow circles, 16 °C; red symbols, 23 °C). Clones with a high sequence similarity cluster in close proximity to one another in the three-dimensional map. Lines connecting the sequences and the clusters represent the pairwise sequence similarity and indicate strong similarity of the sequence to at least one sequence in the cluster (the third dimension complicates visualization).

Concomitant with above observations, cultured mat samples all had higher microcystin concentrations than their corresponding original mats. Although the increased formation of N-rich metabolites, for example microcystins, could have derived from the culture conditions, the extremely high nutrient concentrations as well as the high N/P ratios found in the interstitial waters of cyanobacterial mats under natural conditions, commonly ranging between 53:1 and 345:1 in this area (Quesada et al. 2008; Quesada et al., unpublished data), indicate that under natural conditions macronutrients may be non-limiting in cyanobacterial mats. Indeed, despite similar nutrient availability and comparable conditions in the flasks (constant light and temperature as well as absence of perturbation), microcystin concentrations were significantly higher (Supplementary Table 2-II and Supplementary Figure 2-V; P<0:05) in the 8 °C and 16 °C cultures when compared with those incubated at 4 °C and 23 °C (Figure 2-4). Moreover, the 8 °C cultures attained the lowest mean biomass at the six-month time point (data not shown), thus possibly indicating that the 8 °C conditions resulted in a shift towards a predominance of microcystin-producing cyanobacteria (for example, *Phormidium* sp. and Nostocales) or a change in the microcystin production of pre-existing species, whereby a proportion of available nutrients and energy was invested in microcystin synthesis. Microcystin production as protection against grazing pressure (Lürling, 2003) would seem questionable, as few to no predators were observed under culture conditions. However, the observed differences in species predominance (Figure 2-2 and Supplementary Table 2-I) at the different culture temperatures would support microcystins serving as quorumsensing or -signalling molecules for changing growth conditions, as suggested earlier (Dittmann et al., 2001; Wood et al., 2011).

The production of a complex cyclic heptapeptide toxin that has high environmental stability and thus capability of prolonged signalling capacity could be advantageous. Consequently a moderate increase of mean temperatures in Polar Regions could favour predominance of microcystin-producing cyanobacteria (Figure 2-2, Supplementary Table 2-I, Supplementary Figure 2-V), a potential reduction of cyanobacterial mat diversity and thus instability of the mat ecosystem. This could facilitate emergence and establishment of highly competitive species and thereby have a great impact on freshwater ecosystems of the Polar Regions.

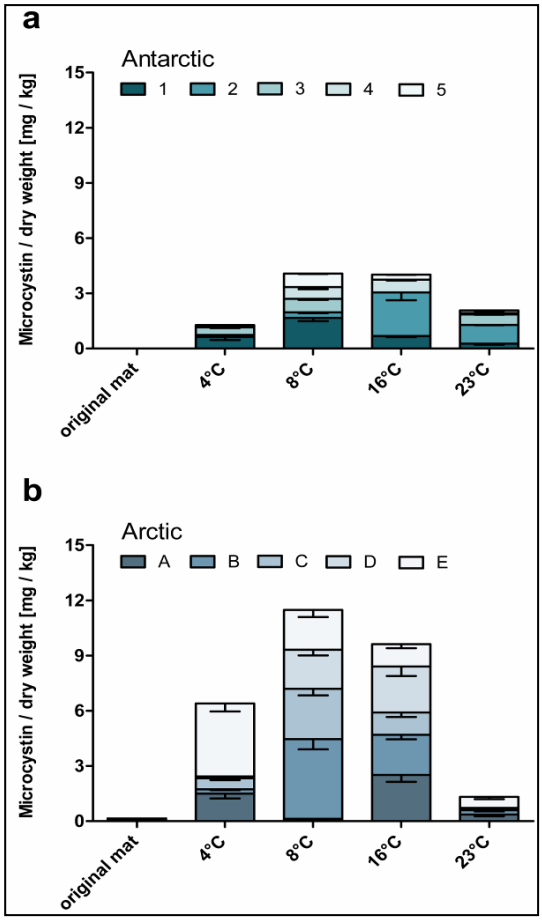


Figure 2-4: Microcystin concentrations detected in original and cultured cyanobacterial mat samples.

a,b, Microcystin concentration found in five Antarctic (**a**) and five Arctic (**b**) original cyanobacterial mats and from the respective mats cultured at 4 °C, 8 °C, 16 °C and 23 °C. Whereas original mats contained only low to non-detectable concentrations of microcystins (as confirmed by liquid chromatography with tandem mass spectrometry, data not shown), the highest concentrations were found in mats cultured at 8 °C and 16 °C. Mean with s.e.m., *n*= 4–8.

2.3 Methods

2.3.1 Site description and sampling.

Cyanobacterial samples were collected from five different sampling sites (samples 1-5) in the Antarctic Specially Protected Area (ASPA) No. 126 of Byers Peninsula, Livingston Island (62°34’35’’ to 62°40’ 35’’ S and 60°54’14’’ to 61°13’07’’ W), South Shetland Islands, Antarctic Peninsula, during an expedition in February 2009. Arctic samples (samples A-E) were collected during an expedition to northern Baffin Island in the vicinity of Cape Hatt (72°30’ N and 79°47’ W) in August-September 2009. Microbial mats were probed using a sterile spatula, sealed in sterile plastic bags or tubes and stored frozen (-20 °C) for DNA extraction and toxin analysis, or were kept in the dark at 4 °C for 9 (Antarctic) or 3 (Arctic) months for cultivation.

2.3.2 Cultivation.

Of each sample, an aliquot of approximately 5 mg wet-weight mat material was transferred into sterile 150 ml standard BG-11 medium (Stanier et al., 1971) and incubated at 23 °C, 16 °C, 8 °C and 4 °C under sterile conditions in cotton-sealed flasks and constant light (23 mol photons m^{-2} s^{-1}, 16:8 h light-dark cycle). During the incubation period the material grew to approximately 200 mg biomass; no additional nutrients were supplied over the whole culturing period. Cultures were collected after six months and stored frozen.

2.3.3 Microscopy.

Microscopic analysis was carried out using a Nikon Eclipse TS 100 microscope and images were documented with a Nikon Digital Sight DS-5M camera.

2.3.4 DNA extraction.

DNA was extracted from 5 to 10 mg of frozen original mat material or dried (24 h, 42 °C) and homogenized cultured material using the MoBio PowerSoil DNA Isolation Kit following the manufacturer's recommendations. Owing to the heterogeneity of the mat material, original samples were extracted in three replicates and subsequently combined.

2.3.5 ARISA.

Total community DNA was amplified in a PCR reaction (Supplementary Information) with a set of cyanobacteria-specific oligonucleotides (Wood, Rueckert, et al., 2008). Intergenic spacer lengths were detected and statistically analysed as described in detail previously (Wood, Rueckert, et al., 2008). A signal intensity of below 200 fluorescence units was considered to be background noise and those peaks were discarded. Only fragments of >250 base pairs were regarded to be true intragenic transcribed spacer signals and considered for the statistical analysis using the Primer-E 6 Software (PRIMER-E).

2.3.6 Cloning of 16S rRNA.

The 16S-rRNA gene was amplified in a PCR reaction (Supplementary Information) using cyanobacteria-specific primers (Saker et al., 2005). PCR products were cloned the same day using the TOPO TA Cloning Kit following the standard protocol with a DNA to vector ratio of four to one. Several hundred clones were obtained. For each sample a minimum of 40 clones were retrieved and conserved in sterile TE buffer. The correct size of the insert was controlled in a PCR reaction (Supplementary Information). PCR products were subjected to a restriction fragment length polymorphism analysis using the enzymes AluI and ScrF1 (NEB) at 37 °C for 2 h. Two to three clones of each individual restriction fragment length polymorphism pattern were sequenced at GATC Biotech. All sequences were deposited with the GenBank database (Supplementary Table 2-III).

2.3.7 16S-rRNA gene sequence analysis.

The 16S-rRNA sequences were analysed in a two-step process. First the sequences were compared to a database of known bacterial and cyanobacterial 16S-rRNA sequences (SILVA database v104; Pruesse et al. 2007) using CLANS (Frickey and Lupas, 2004). BLASTN (Altschul, 1997) pairwise alignment average score-per-column values (SC-values) were used as a metric to visualize pairwise similarities. Sequence clusters in which at least one classified sequence from the SILVA database and one sequence from our unknown species of interest were present were used to assign putative species to our unclassified 16S-rRNA sequences. In a second step the 16S-rRNA sequences, now with assigned putative taxonomic information, were compared to one another using CLANS to identify species or genus bias in the cultures grown under the various temperatures. An SC-value cutoff of 1.5 was chosen for the final figure as it provided the best resolution between the various sequence groups present in the data set. This two-step process enabled species to be assigned to unknown sequences and for these sequences to then be compared without contaminating signal from the sampling bias present in the SILVA database.

2.3.8 Toxin analysis.

Toxins were extracted from ground 50 mg dry-weight material from frozen cyanobacterial mats or respective cultures (Supplementary Information). Toxins were analysed using the microcystin ADDA ELISA Microtiter Plate from ABRAXIS following the standard protocol. The low concentration of microcystins found by the ADDA ELISA in one of the Arctic mats was confirmed by liquid chromatography with tandem mass spectrometry analyses using a previously published method (Altschul, 1997) (data not shown).

2.3.9 Statistical analysis.

Subsequent to an analysis for outliers (Grubbs test for outlier analysis) of the number of AFLs as well as the concentrations of microcystin a one-way analysis of variance and Bonferroni post-test with an *n*=9-10 was carried out (**P* <0:05; ***P* <0:01; ****P* <0:001).

2.3.10 Acknowledgements

For financial support of the Antarctic expedition we would like to acknowledge the Deutsche Forschungsgemeinschaft (DFG)-funded project DI698/18-1 Dietrich, as well as the Spanish Ministry of Science and Technology through project LIMNOPOLAR (POL2006-06635 and CGL2005-06549-C02-01/ANT to A.Q., as well as CGL2005-06549-C02-02/ANT to A.C., the last of these co-financed by European FEDER funds). We thank the TOTAL Foundation for funding the expedition to Baffin Island and within this context O. Dargent, Nice, France, and P. van West, University of Aberdeen, UK, for collecting and photographing Arctic specimens on Baffin Island. We would also like to thank the Carl Zeiss Stiftung and the Excellence Initiative of the University of Konstanz, Germany, for funding the PhD project of J.K. and APECS for their support to conference contributions. Furthermore, we acknowledge the support of the European Community research infrastructure action under the FP7 `capacities' specific programme ASSEMBLE No.227788. For technical support and new ideas we are very grateful to D. Schleheck, H. Bastek and K. Leinweber from the University of Konstanz, Germany, and A. Jungblut from the Natural History Museum, London, UK.

2.3.11 Author contributions

J.K. planned and carried out all presented experiments, and prepared the data, figures and the manuscript. S.A.W. assisted in experimental procedures and analysed the ARISA samples. F.C.K. organized and collected the samples of the Arctic expedition. A.Q. and A.C. were in charge of the organization and collected the samples during the Antarctic expedition. T.F. carried out the bioinformatic analysis of sequence data. D.R.D. was the project coordinator, supervisor of experiments, planned and carried out some experiments and prepared the manuscript. All authors contributed equally to critically reviewing the manuscript and discussing experiments and results.

2.3.12 Additional information

The authors declare no competing financial interests. Supplementary information accompanies this paper on www.nature.com/natureclimatechange. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to D.R.D.

2.4 Supplemental Information

2.4.1 Materials and Methods

Toxin Analysis

Dried and ground material (24 h, 42 °C) was extracted with 3 x 5 ml of 75 % methanol. During each extraction step the suspension was kept in an ultrasound bath for 30 min (ice-cold) and subsequently centrifuged (4.000 x *g*, 30 min). The supernatant was combined, air dried and re-suspended in H_2O and purified on C18 cartridges (Sep-Pak, Waters, Dublin). The methanolic eluate was air dried to complete dryness and resuspended in 600 µl methanol and 2.4 ml H_2O. The suspension was centrifuged (13.000 x *g*, 20 min) and the supernatant used for further analysis.

Toxins were analyzed using the microcystin-ADDA ELISA Microtiter Plate from ABRAXIS (Warminster, US) following the standard protocol. The ELISA reaction was inhibited by unknown compounds in the extracts. This was verified by spiking the samples in variable concentrations of microcystin standards supplied with the ELISA. To minimize inhibitor activity, several dilutions for each sample were tested and the final ELISA was performed using the selected optimal dilution.

PCR reactions

All PCR reactions were performed on a Primus 96 plus Thermocycler. All primers were purchased from Eurofins MWG operon, Ebersberg, Germany, other reagents are from Fermentas, St. Leon-Rot, Germany, unless stated differently.

PCR for ARISA:

Reaction mix (2 x 50 µl): 1x Fermentas Master Mix; 3 mM $MgCl_2$, 0.4 mg / ml BSA, 0.4 µM primer Cy-ARISA / 23S30R, 2.5 µl extracted DNA.

PCR conditions: 94 °C 4 min, [94 °C 60 s, 50 °C 60 s, 72 °C 2 min] x 35, 72 °C 7 min.

PCR products were visualized on a TAE 1.8 % agarose gel and purified using the Fermentas PCR purification Kit.

PCR for clone library:

Reaction mix (25 µl): 1x Fermentas Master Mix, 2.5 mM $MgCl_2$, 0.2 µg / µl BSA, 0.5 µM primer 27F / 809R, 1 µl extracted DNA.

PCR conditions: 95 °C 4 min, [92 °C 30 s, 50 °C 45 s, 72 °C 90 s] x 35, 72 °C 10 min.

PCR products were visualized on a TAE 1.5 % agarose gel. Bands of the right size were excised using a sterile scalpel and purified with the Fermentas Gel extraction Kit.

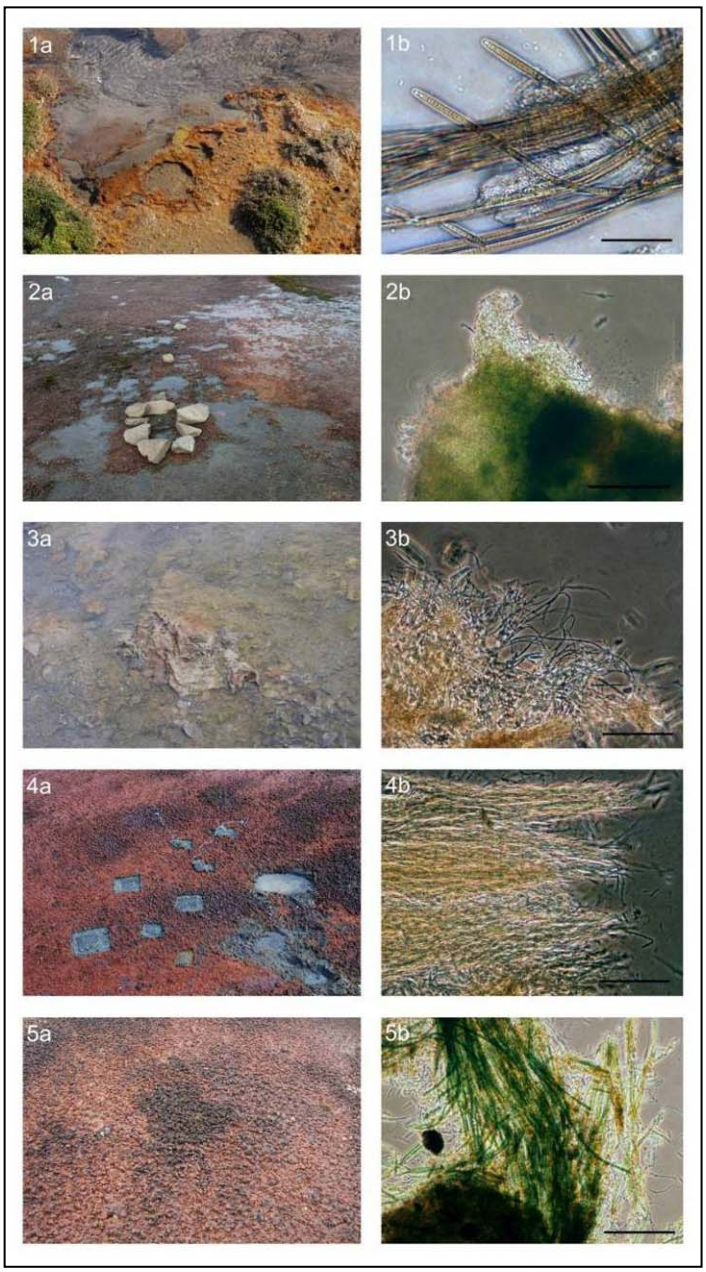
PCR of clone colonies:

Reaction mix (20 µl): 0.02 U / µl Phusion high-fidelity DNA polymerase (Finnzymes), 1x HF buffer (Finnzymes), 2 mM $MgCl_2$, 2 % DMSO (Finnzymes), 200 µM dNTPs, 0.4 µM vector specific primers M13, 0.5 µl DNA template.

PCR conditions: 98 °C 2 min, [98 °C 20 s, 58 °C 30 s, 72 °C 40 s] x 35, 72 °C 10 min.

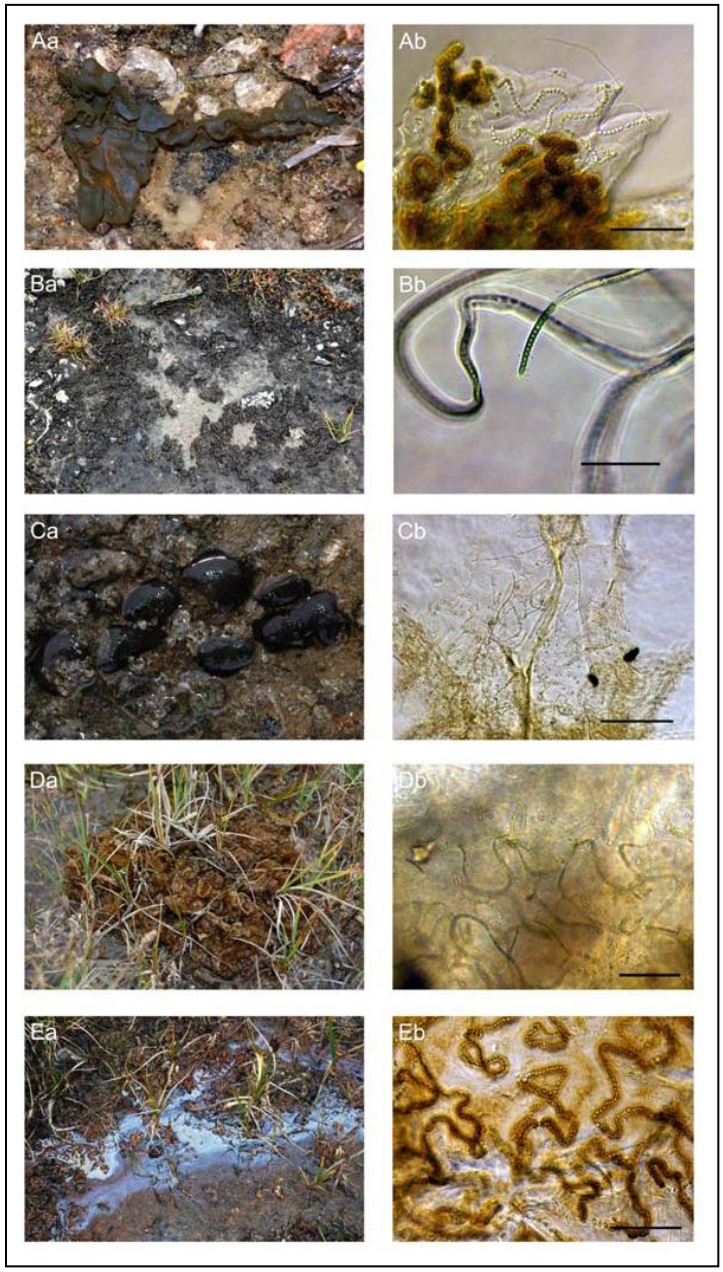
PCR Products were visualized on a TAE 1.5 % agarose gel.

2.4.2 Supplementary Figures and Tables:



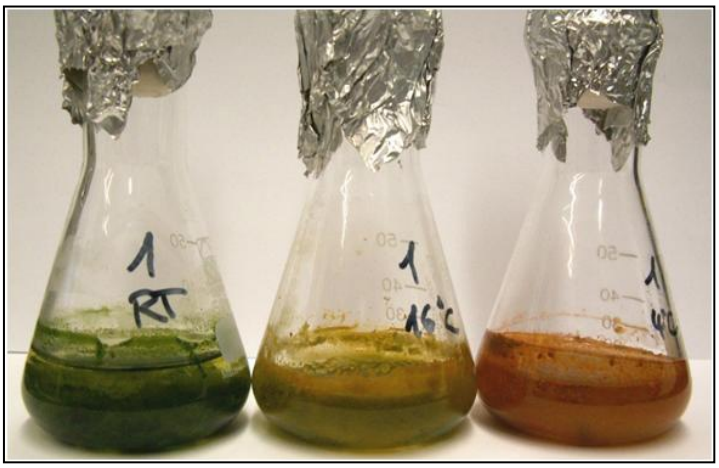
Supplementary Figure 2-I: Photographs of cyanobacterial mats 1-5 on Byers Peninsula, Antarctica (left) and respective microphotographs of selected organisms (right) of each mat.

Scale bars represent 100 µm in 1b and 50 µm in 2b – 5b. Photographs of Antarctic mats were kindly provided by Dr. Daniel Dietrich.



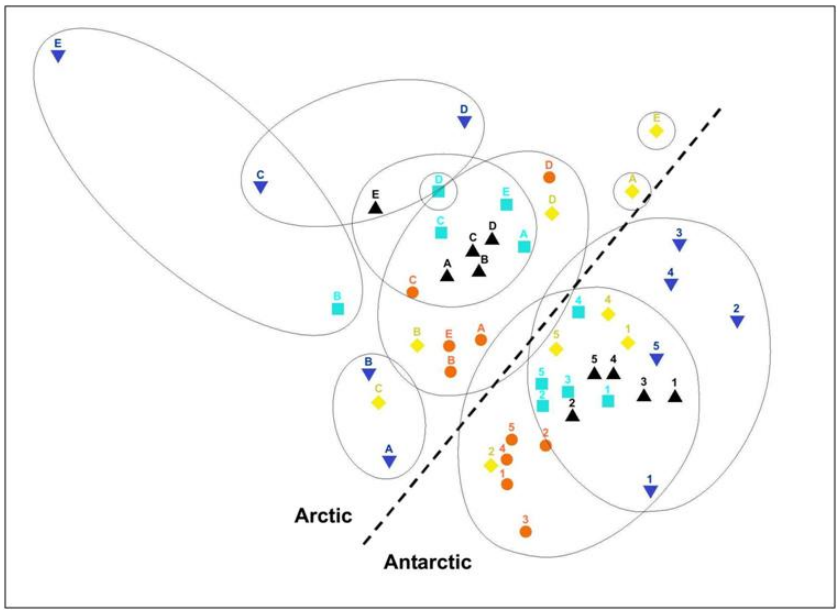
Supplementary Figure 2-II: Photographs of cyanobacterial mats A-E on Baffin Island, Canadian Arctic (left) and respective microphotographs of selected organisms (right) of each mat.

Scale bars represent 50 µm in Ab, Bb, Cb and Eb and 400 µm Db. Photographs of Arctic mats were kindly provided by Dr. Pieter van West (University of Aberdeen) and Olivier Dargent (Nice, France).



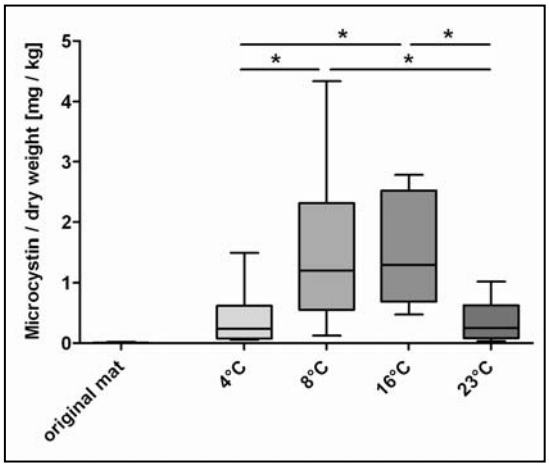
Supplementary Figure 2-III: Cultures of a cyanobacterial mat from Baffin Island incubated at 4 °C, 8 °C, 16 °C and 23 °C.

Cultures were held for six months in BG-11 standard cyanobacterial medium and stable light conditions (23 µmol Photons m^{-2} s^{-1}, 16 - 8 h light-dark cycle). Cultures at 23 °C showed a dark and intense green pigmentation. At lower temperatures cultures were increasingly dominated by orange and red pigmented species.



Supplementary Figure 2-IV: Community composition of cyanobacterial mats (▲) and cultures (▼ 4 °C; ■ 8 °C; ◆16 °C; ● 23 °C) from the Antarctic and Arctic,

displayed as a two-dimensional non-metric multidimensional scaling ordination (stress value of 0.12) based on Bray-Curtis similarities of Automated Ribosomal Intergenic Spacer Analysis (ARISA) fingerprints. Points within a circle cluster at 15 % similarity.



Supplementary Figure 2-V: Microcystin concentrations in extracts from the original cyanobacterial mats (Arctic and Antarctic) and from mats cultured at 4 °C, 8 °C, 16 °C, and 23 °C.

The toxin concentration of cultures at 8 °C and 16 °C is significantly elevated when compared to 4 °C and 23 °C cultures (*, P< 0.05) and to the original mats (P< 0.001, not indicated in figure). Grubbs outlier test; One Way ANOVA, Bonferroni post test, *n*= 9 - 10; Mean, 75 % percentile, Min-Max.

SupplementaryTable 2-I: Species identified in cyanobacterial mats from the Antarctic (1-5) and the Arctic (A-E) and their respective cultures at 4 °C, 8 °C, 16 °C, and 23 °C.

The 16S rRNA gene of extracts from cyanobacterial material was amplified and cloned. Sequences were clustered according to their pair wise similarity (Figure 3). A library of published sequences was used to determine species or group identities (SILVA database v104). Numbers (1-5) and letters (A-E) indicate the samples in which the respective species were present. *extracted from culture directly and analysed by BLAST search.

Species / culture	original mat	4 °C	8 °C	16 °C	23 °C
Phormidium sp.	2,5	1,2,5	5	2,5	-
Phormidium autumnale / Microcoleus	3	4*	1,2	1	-
Phormidium priestleyi	5	2,4	-	1	-
Phormidium murrayi	4	-	-	-	-
Planktothrix sp.	5,B	-	-	-	-
Pseudanabaenaceae	1,4,E	4,C,E	4,5	1,4	1,3,E
Oscillatoriales	-	A,B,C	-	-	3,A,C,D
Nostoc sp.	2,3	-	-	-	-
Nostocaceae	2,3,A,B,C,D,E	-	3	-	-
Anabaenoidae	3	-	3	-	-
Leptolyngbya frigida	2,3,4,5,A,C,D,E	2,4	2,3,4,5	1,2,4,5	2,3,4,5,B,D
Leptolyngbya antarctica I	-	-	3	2	E,1,2,3,4,5
Leptolyngbya antarctica II	1,2,3,4,5	2,4,5	3	-	-
Leptolyngbya greenland	1,5,A,C,D,E	1,2,4	2,3	1	-
Gleobacter violaceus	A,B	-	-	-	-
Microcystis aeruginosa	-	C	-	-	-
Unknown cyanobacterium	A,B,C,D,E	2	2	-	-
Total	**14**	**9**	**10 (A-E n.d.)**	**7 (A-E n.d.)**	**4**

SupplementaryTable 2-II: Microcystin concentration in Arctic and Antarctic original mats and respective cultures.

Microcystin concentrations are given as mg Microcystin per kg dry weight mat or culture material. Cultures at 8 °C and 16 °C contained a significantly higher toxin concentration when compared to the original mat (***, P< 0.001) and to the cultures incubated at 4 °C and 23 °C (*, P< 0.05). One way ANOVA Bonferroni post test, *n*= 9 - 10. # replicates excluded as outliers following analysis with the Grubbs Outlier test.

Sample	1	2	3	4	5	A	B	C	D	E	Mean ± SD
original mat	0,00	0,00	0,00	0,00	0,00	0,11#	0,02	0,00	0,00	0,00	0,01±0,01
4 °C	0,63	0,10	0,42	0,06	0,05	1,50	0,24	0,61	0,09	3,96#	0,41±0,47
8 °C	1,67	0,30	1,07	0,64	0,67	0,13	4,33	2,74	2,12	2,97	1,66±1,37***,*
16 °C	0,95	2,37	n.d.	0,69	0,47	2,51	2,18	1,02	2,50	1,21	1,55±0,83***,*
23 °C	0,26	1,02	0,66	0,08	0,15	0,36	0,24	0,03	0,09	0,60	0,35±0,32

Supplementary Table 2-III: GenBank Accession numbers of Clone sequences derived in this study.

Sample	Location	Culture Temperature [°C]	Clone #	GenBank Accession number
1	Antarctic	4	1.4.2	JQ310492
1	Antarctic	4	1.4.7	JQ310506
1	Antarctic	8	1.1.2	JQ310485
1	Antarctic	8	1.1.3	JQ310507
1	Antarctic	8	1.1.4	JQ310499
1	Antarctic	8	1.1.6	JQ310511
1	Antarctic	8	1.2.4	JQ310484
1	Antarctic	8	1.2.5	JQ310521
1	Antarctic	8	1.5.2	JQ310509
1	Antarctic	16	1.1.1	JQ310514
1	Antarctic	16	1.1.2	JQ310495
1	Antarctic	16	1.1.3	JQ310518
1	Antarctic	16	1.1.4	JQ310487
1	Antarctic	16	1.1.5	JQ310503
1	Antarctic	16	1.2.7	JQ310502
1	Antarctic	16	1.3.1	JQ310516
1	Antarctic	16	1.3.4	JQ310486
1	Antarctic	16	1.3.5	JQ310488
1	Antarctic	16	1.4.2	JQ310500
1	Antarctic	16	1.4.7	JQ310493
1	Antarctic	16	1.4.8	JQ310519
1	Antarctic	16	1.5.1	JQ310525
1	Antarctic	16	1.5.3	JQ310504
1	Antarctic	16	1.5.5	JQ310517
1	Antarctic	23	1.1.4	JQ310513
1	Antarctic	23	1.1.6	JQ310489
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1	Antarctic	23	1.5.3	JQ310505
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2	Antarctic	4	2.1.6	JQ310476
2	Antarctic	4	2.1.7	JQ310478
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2	Antarctic	4	2.2.3	JQ310480
2	Antarctic	4	2.2.6	JQ310473
2	Antarctic	4	2.2.7	JQ310441
2	Antarctic	4	2.3.2	JQ310454
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2	Antarctic	8	2.4.7	JQ310477
2	Antarctic	8	2.4.8	JQ310445
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2	Antarctic	23	2.1.1	JQ310431
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A	Arctic	23	A.1.6	JQ310299
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A	Arctic	23	A.2.5	JQ310285
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A	Arctic	23	A.2.7	JQ310294
A	Arctic	23	A.3.1	JQ310295
A	Arctic	23	A.3.5	JQ310289

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A	Arctic	23	A.5.5	JQ310279
A	Arctic	23	A.5.6	JQ310298
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A	Arctic	original mat	A.3.8	JQ310284
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A	Arctic	original mat	A.5.4	JQ310290
A	Arctic	original mat	A.5.5	JQ310296
A	Arctic	original mat	A.5.6	JQ310291
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B	Arctic	23	B.4.2	JQ310258
B	Arctic	23	B.4.3	JQ310273
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B	Arctic	original mat	B.2.6	JQ310262
B	Arctic	original mat	B.3.1	JQ310275
B	Arctic	original mat	B.3.6	JQ310261
B	Arctic	original mat	B.4.3	JQ310277
B	Arctic	original mat	B.4.5	JQ310265
B	Arctic	original mat	B.4.6	JQ310260
B	Arctic	original mat	B.5.3	JQ310269
C	Arctic	4	C.1.6	JQ310236
C	Arctic	4	C.2.5	JQ310251
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C	Arctic	4	C.3.9	JQ310249
C	Arctic	4	C.5.2	JQ310238
C	Arctic	4	C.5.6	JQ310252
C	Arctic	4	D.1.7	JQ310256
C	Arctic	4	D.2.3	JQ310254
C	Arctic	4	D.2.6	JQ310255
C	Arctic	23	C.1.2	JQ310242
C	Arctic	23	C.1.3	JQ310237
C	Arctic	23	C.1.4	JQ310240
C	Arctic	23	C.1.8	JQ310239
C	Arctic	23	C.3.1	JQ310253
C	Arctic	23	C.3.3	JQ310241
C	Arctic	23	C.3.6	JQ310244
C	Arctic	23	C.4.3	JQ310233
C	Arctic	23	C.5.2	JQ310247
C	Arctic	original mat	C.1.1	JQ310245
C	Arctic	original mat	C.1.5	JQ310248
C	Arctic	original mat	C.1.8	JQ310246
C	Arctic	original mat	C.2.6	JQ310243
C	Arctic	original mat	C.3.6	JQ310250
C	Arctic	original mat	C.4.7	JQ310235
D	Arctic	4	D.1.1	JQ310229
D	Arctic	4	D.1.2	JQ310227
D	Arctic	4	D.1.5	JQ310232
D	Arctic	4	D.2.3	JQ310225
D	Arctic	4	D.3.1	JQ310226
D	Arctic	4	D.3.2	JQ310228

D	Arctic	4	D.3.3	JQ310230
D	Arctic	4	D.3.4	JQ310231
D	Arctic	23	D.1.1	JQ310212
D	Arctic	23	D.1.2	JQ310208
D	Arctic	23	D.1.4	JQ310216
D	Arctic	23	D.2.2	JQ310211
D	Arctic	23	D.2.3	JQ310220
D	Arctic	23	D.3.1	JQ310210
D	Arctic	23	D.3.2	JQ310219
D	Arctic	23	D.3.3	JQ310223
D	Arctic	23	D.3.4	JQ310214
D	Arctic	23	D.4.2	JQ310224
D	Arctic	23	D.4.5	JQ310207
D	Arctic	23	D.4.6	JQ310215
D	Arctic	original mat	D.1.5	JQ310213
D	Arctic	original mat	D.2.2	JQ310221
D	Arctic	original mat	D.2.6	JQ310217
D	Arctic	original mat	D.3.2	JQ310218
D	Arctic	original mat	D.4.2	JQ310222
D	Arctic	original mat	D.5.2	JQ310209
D	Arctic	original mat	D.5.6	JQ310206
E	Arctic	4	E.1.2	JQ310197
E	Arctic	4	E.1.3	JQ310205
E	Arctic	4	E.2.8	JQ310204
E	Arctic	4	E.4.4	JQ310192
E	Arctic	23	E.1.2	JQ310203
E	Arctic	23	E.1.5	JQ310194
E	Arctic	23	E.2.1	JQ310190
E	Arctic	23	E.3.2	JQ310189
E	Arctic	23	E.3.3	JQ310191
E	Arctic	23	E.3.6	JQ310201
E	Arctic	23	E.5.2	JQ310199
E	Arctic	original mat	E.1.1	JQ310185
E	Arctic	original mat	E.1.3	JQ310187
E	Arctic	original mat	E.1.5	JQ310186
E	Arctic	original mat	E.1.7	JQ310198
E	Arctic	original mat	E.1.8	JQ310196
E	Arctic	original mat	E.2.2	JQ310193
E	Arctic	original mat	E.3.2	JQ310202
E	Arctic	original mat	E.5.1	JQ310200
E	Arctic	original mat	E.5.2	JQ310188
E	Arctic	original mat	E.5.7	JQ310195

3 MANUSCRIPT 2

3.1 Potent toxins in Arctic cyanobacterial mats

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3.1.2 Abstract

Cyanobacteria are the predominant phototrophs in polar freshwater communities where they commonly form extensive benthic mats. The species comprising these mats have shown to produce the cyanobacterial toxin microcystin (MC). In the Arctic cyanobacterial mats are likely to be affected by climate change, as indicated by recent research that showed increased temperature can give resulted in elevated toxin production and changes in species diversity within mats.

Five Arctic cyanobacterial mats samples were screened for saxitoxin and microcystin using immunological, analytical as well as molecular methods. Saxitoxin (STX) was detected for the first time in cyanobacterial mats from the Arctic. In addition, an unusual MC variant was identified via LC-MS/MS. Gene expression analyses confirmed the analytical findings, whereby parts of the sxt and mcy operon involved in STX and MC synthesis, were detected in one and five of the Arctic cyanobacterial mat samples tested respectively. In conjunction with a change of environmental conditions conducive to toxin production or the spread of

toxin-producing cyanobacteria, the latter provides the biological prerequisites for dramatic alterations of freshwater ecosystems of the Polar Regions in the near future, especially when the assumed future trends of climate change become reality.

3.1.3 Keywords

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

3.2 Introduction

Few phototrophic organisms are adapted to 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 (Wynn-Williams, 1996b; Vincent, 2000a). Special features such as resistance to ultraviolet (UV) radiation, freeze-thaw cycle adaptation and nitrogen fixation allow their survival in these extreme environments (Vincent, 2000a). During the polar summer, when both light and temperatures above freezing prevail, cyanobacterial communities thrive. They develop highly diverse benthic or floating mats in freshwater streams and ponds that can be several centimetres thick and extend over large areas (Vincent, 2000b; Zakhia et al., 2008). These extensive mats form the basis of a small but diverse and dynamic ecosystem accommodating a variety of organisms such as nematodes, rotifers, tardigrades (Cary et al., 2010), mosses and moss-infecting oomycetes such as the recently-described *Pythium polare* (Tojo et al., 2012).

Saxitoxins (STXs) (Figure 3-1) are fast-acting neurotoxins which are typically produced by marine dinoflagellates (Alexander et al., 2009). However, planktonic and benthic cyanobacteria from temperate and tropical regions, e.g. *Aphanizomenon* spp., *Anabaena circinalis*, *Cylindrospermopsis raciborskii*, *Planktothrix* spp., and *Lyngbya wollei* (Humpage, 2008), are also known to produce STXs. Microcystins (MCs) (Figure 3-1), represent a group of ≥80 structural heptapeptide variants with varying hepato-, renal-, and neurotoxicity (Vichi et al., 2012) that appear to act primarily via a specific inhibition of serine/threonine phosphatases (Dietrich and Hoeger, 2005; Humpage, 2008). MCs are produced by a large variety of planktonic and benthic cyanobacterial genera including *Microcystis, Nostoc, Planktothrix, Anabaena, Synechococcus* and *Snowella* (Humpage, 2008; O'Neil et al., 2012).

The basis for the production of both toxins are large and variable gene clusters, encoding enzymes involved in secondary metabolite production such as polyketide synthases or non-ribosomal peptide synthetases (Neilan et al., 2008; Moustafa et al., 2009; Al-Tebrineh et al., 2010). More importantly, these biosynthetic steps are energetically expensive for cyanobacteria (Kaplan et al., 2012), and thus prompted considerable speculation on their ecological function. To date the physiological function and ecological regulation of both STXs and MCs is poorly understood (Dittmann et al., 2001; Kaebernick and Neilan, 2001; Babica et al., 2006). The more common theories for investing energy in the synthesis of these compounds, include protection against grazing pressure, UV-

radiation, and reactive oxygen species, as well as signalling functions in a quorum sensing-like manner (Kaebernick and Neilan, 2001; Schatz et al., 2007; Kleinteich et al., 2012).

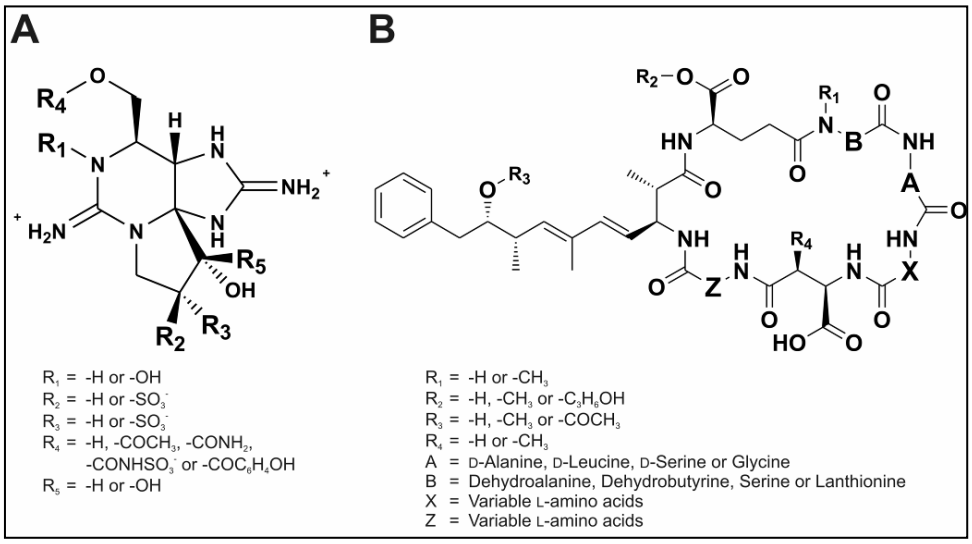


Figure 3-1: General structure of (A) saxitoxins and (B) microcystins.

(A); General structure of SXT adapted from Humpage, 2008 and the general structure of the MCs (B); adapted from Puddick, 2012.

However, among the many theories, the specific development of toxins as protection against grazers appears a priori to be the least plausible as, since for both toxins the corresponding gene cluster appears to have been present in ancestral cyanobacteria species that have existed prior to the mesoproterozoic period (Rantala et al., 2004; Kurmayer and Christiansen, 2009; Murray et al., 2011). Correspondingly, SXTs and MCs were produced by cyanobacteria millions of years before the emergence of eukaryotic organisms that could potentially have grazed on them.

In view of the ancient origin and the high conservation of the toxin gene clusters is not surprising that cyanobacteria of remote pristine areas e.g. the rudimentary environments of the Polar Regions, could possibly produce toxins. The presence of MCs in cyanobacterial mats has been reported for several locations in Antarctica (Hitzfeld et al., 2000; Jungblut et al., 2006; Wood, Mountfort, et al., 2008), whereas this has only recently been demonstrated for Arctic cyanobacterial mats from northern Baffin Island (Kleinteich et al., 2012). In Kleinteich et al. (2012) it was demonstrated that culturing of cyanobacterial mats in the laboratory at increased temperatures provided for a dramatic rise in the presence of detectable MCs. Saxitoxins on the other hand have never been detected in the polar environment, however due to the ancestral origin and high conservation of the sxt gene cluster, the presence of STX in polar environments seems likely.

In this study five cyanobacterial mat samples from the Arctic were screened for the presence of STX using enzyme-linked immunosorbent assay (ELISA). Samples from this study

that tested positive for STX and from a previous study that tested positive for MC (Kleinteich et al., 2012) were further investigated using liquid chromatography-mass spectrometry (LC-MS) or high performance liquid chromatography with fluorometric detection (HPLC-FLD). 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 mats.

3.3 Materials and Methods

3.3.1 Study sites and sampling

Five cyanobacterial mat samples 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 mats 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.

3.3.2 Screening for saxitoxin and microcystin

Saxitoxin extraction

Frozen mat material was lyophilized and their dry weight recorded. Sample for STX analysis were extracted as described by Smith and colleagues, 2011. 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 for 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.

Microcystin extraction

Lyophilized material (173 mg) was homogenized to a powder using a mortar and pestle Methanol (75 %, 5 mL) was added to the powder, the suspension placed in an ultrasonic water bath for 30 min (ice-cold), centrifuged (30 min, 4,000 *x g*), and the supernatant removed for further processing. The extraction was repeated three times. The supernatants were pooled, dried under nitrogen flow, re-suspended in 15 mL H2O, and loaded onto C18 cartridges (Sep-Pak, Waters, Dublin, IRL). MC was eluted from the C18 cartridges with methanol (100 %), the methanolic eluate dried under nitrogen gas flow, and resuspended in 3 mL methanol (20 %). The extract was centrifuged (20 min, 13,000 *x g*) and the supernatant stored at -20 °C until further analyses.

Saxitoxin analysis

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

Microcystin analysis

The extract of cyanobacterial mat sample A (sample A), which had previously tested positive for MC by Adda-ELISA (Kleinteich et al., 2012), was analyzed by LC MS in order to confirm the presence of MC congener/s. LC-MS was done on a high performance liquid chromatography (HPLC) system (UltiMate 3000; Dionex) coupled to an AmaZon X (Bruker Daltonics) electrospray ionisation ion trap mass spectrometer (ESI-IT-MSn). Samples (20 µL) were separated on a C18 column (Ascentis Express C18, 100 × 2.1 mm, 2.7 µ; Supleco Analytical) using a gradient system of 98 % H_20 + 0.1 % formic acid (v/v; solvent A) and 98 % acetonitrile + 0.1 % formic acid (v/v; solvent B) with the following gradient program: The sample was loaded in 10 % B; 10 % B was held for 1 min and increased to 100 % B over 12 min; 100 % B was held for 2 min; the solvent concentration was returned to 10 % B in 1 min and the column re equilibrated for 4 min. The eluting compounds were transferred into the IT-MS using a capillary voltage of 3.5 kV and a nebulizer pressure of 3.0 bar. Dissolution was accomplished with a nitrogen flow of 8 L / min at 220 °C. Tandem MS (MS/MS) spectra were gathered using the doubly- or singly-protonated ions of the target compounds and collision-induced dissociation (CID) to induce fragmentation of the parent ion (collision amplitude of 1.0).

3.3.3 Screening for genes involved in toxin synthesis

Nucleic acid extraction

DNA was extracted from 5 - 10 mg of frozen material using the MO BIO PowerSoil® DNA Isolation Kit following the manufacturer's recommendations. Due to the heterogeneity of the mat material, three individual extractions were performed and the pooled extracts used for downstream applications. RNA was extracted from 5 - 10 mg of material stored in RNAlater (Qiagen, Hilden, DEU). RNAlater was removed by patting the material on a dry stack of paper, and RNA extracted using the MO BIO PowerBiofilm® RNA extraction kit following the manufacture's protocol. RNA was eluted with RNase-free water and stored at -80 °C.

Detection of genes involved in toxin synthesis

PCRs targeting the mcy and sxt operon for MC and STX synthesis, respectively were performed with primer pairs and at annealing temperatures as listed in Supplemental Table 2; primers were from MWG eurofins (Ebersberg, DEU). For the reactions either the Master Mix™ (Fermentas, St. Leon-Rot, DEU) or the Phusion™ polymerase mix (NEB, Ipswich, USA) was used supplemented with BSA, DMSO and $MgCl_2$. Bands were excised from a 1.5 % agarose gel (TAE) using a sterile scalpel, purified with a gel extraction kit (Fermentas, St. Leon-Rot, DEU) and sequenced bi-directionally using the primers listed in Supplemental Table 1 at MWG eurofins (Ebersberg, DEU). Messenger-RNA of the sxt operon was reverse transcribed into cDNA using gene-specific reverse primers (Supplemental Table 2) and a standard protocol for reverse transcription (20 U RNAse Inhibitor, 0.8 mM dNTPs, 7 µL of extracted RNA, 70 U M-MuLV); enzyme and chemicals for RT-PCR were from NEB (Ipswich MA, USA). The cDNA produced was used as template for PCR as described above. *Microcystis aeruginosa* CCAP 1450/16 served as a positive control for mcy genes, but no positive control of cyanobacterial origin was available for the *sxt* genes. The obtained sequences were analyzed using Geneious™ software (Geneious Pro 5.3.6) and the closest matches identified using NCBI's BLAST tools (mega-BLAST and BLASTn). Phylogenetic trees using *sxtA* sequences were built using the GeneiousTM tree builder (Jukes-Cantor, Neighbour joining method). The obtained 657 bp product of the *sxtA* gene was deposited in the GenBank database under the accession JX887897 (Supplemental Table 3). The obtained 128 bp product of the *sxtA* gene is displayed in Supplemental Table 4 since GenBank does not deposit sequences shorter than 200 bp.

3.3.4 Identification of toxin producer/s

Cloning of 16S rRNA and intergenic spacer region (ITS)

Two samples that returned positive results from toxin analyses were selected for construction of 16S rRNA gene and intergenic spacer region (ITS) region clone libraries. Amplification was achieved with the cyanobacteria-specific primer pair 27F and 23S30R (Supplemental Table 2) in a 50 µL PCR 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 Fermentas (St. Leon-Rot, DEU) Master Mix, 2.5 mM $MgCl_2$, 0.2 µg / µL BSA, 3 % DMSO and 0.5 µM of each primer. The PCR products were separated on a TAE 1.5 % agarose gel and the bands excised using a sterile scalpel. After purification with the GeneJET™ Gel Extraction Kit (Fermentas, St. Leon-Rot, DEU) PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, USA) following the standard protocol with a DNA to vector ratio of four to one. For each sample approximately 40 clones were retrieved and conserved in sterile TE buffer. Each clone insert was 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 the Fermentas PCR Master Mix (St. Leon-Rot, DEU), 0.25 µM of each TOPO-primer M13, 2 mM $MgCl_2$, 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.

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 (2005) and Komarek and Anagnostidis (1982).

3.3.5 Data evaluation

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

3.4 Results

3.4.1 Detection of toxins in Arctic cyanobacterial mats

Saxitoxin detection

One of the five samples analyzed with the STX ELISA tested positive (Sample E; 21 ng STX/ g dry weight; *n*=6), 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, October 2012). None of the other samples tested positive in the STX ELISA, either as original mats or when cultured in the laboratory at various temperatures (see Kleinteich et al., 2012 for laboratory culture conditions). Subsequent PCR amplification of two segments (128 bp and 657 bp) of the *sxtA* gene provided for a positive signal in sample E but not for the other four samples (Table 3-1). Moreover, *sxtA* gene mRNA was detected in sample E (Figure 3-2), thereby confirming the positive detection of the STX ELISA.

Table 3-1: Table 1 Detection of the mcy and sxt operon in five Arctic cyanobacterial mat samples suggesting the potential for MC and STX production.

Sample	16S	*mcyA*	*mcyA*	*mcyB*	*mcyE*	PKS	*sxtA*	*sxtA*
A	+	+	+	-	+	+	-	-
B	+	+	-	+	-	-	-	-
C	+	+	-	-	+	-	-	-
D	+	+	+	+	-	+	-	-
E	+	+	+	+	+	+	+	+

The *sxtA* gene encodes for a polyketide synthetase, which is part of the recently identified cyanobacterial *sxt* gene cluster (Kellmann, Mihali, Michali, et al., 2008). 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 3-3) 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.

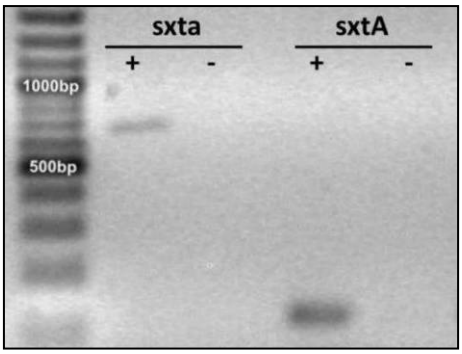


Figure 3-2: Detection of mRNA expression of the sxtA gene in Arctic mat sample A.

Two different primer pairs sxta and sxtA were used resulting in a 650 bp and a 170 bp long product respectively. Negative controls were performed excluding reverse transcriptase to reduce false positive signals of DNA contamination.

Microcystin detection

Liquid chromatography-MS analysis of sample A (Figure 3-4) showed multiple compounds present in the extract. One of these compounds had a similar retention time to that of MC-RR (Figure 3-4B), 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; Frias et al. 2006). 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; Ferranti et al. 2009). 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 3-2). This was most likely the known MC; [Asp^3, $ADMADDA^5$, Dhb^7] MC-RR (Figure 3-5), previously described in *Nostoc* (Beattie et al., 1998).

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 (Kleinteich et al., 2012) also 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 a PKS) 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 3-1). 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.

For the other four samples (B-E), negative for MC in the ELISA and LC-MS analysis, at least one gene involved in MC synthesis was amplified, sequenced and annotated in GenBank to a known gene involved in MC synthesis (Supplemental Table 3) with similarities ranging between 39 % and 99 %. In total eight sequences, annotated to genes involved in MC synthesis, were amplified. The genera corresponding to these genes were *Microcystis, Nostoc* and *Microcoleus* (Supplemental Table 3).

3.4.2 Potential toxin producers in Arctic cyanobacterial mats

A clone library was constructed for the samples that contained either MC (sample A) or STX (sample E) to identify the potential toxin producers. Species were identified using light microscopy. Molecular characterization of the 16S-ITS region demonstrated that the species present in the STX containing sample E were most similar to: *Nostoc puntiforme* (CP001037, 95 %), *Leptolyngbya (frigida)* (AY493573, 97 %), *Calothrix* sp. (JN385289, 92 %), *Snowella littoralis* (AJ781040, 98 %), and *Tolypothrix distorta* (GQ287651, 98 %). Few cyanobacterial species were identified in sample A, namely: *Aphanizomenon gracile* (FJ424575, 94 %), *Leptolyngbya* sp. (DQ431004, 94 %), and *Chroococcus* (FR798926, 97). Light microscopy showed that both samples had a similar appearance, with dominating Nostocales embedded in a firm mucilaginous matrix. Other orders, albeit in lower abundance, i.e. Oscillatoriales (e.g. *Tolypothrix, Leptolyngbya*) and Chroococcales were also present. The Nostocales observed had cells of 3 – 6 µm in diameter and contained heterocytes (Supplemental Figure 2). *Leptolyngbya* with a trichome width of approximately 1.7 µm was present in both samples. In Sample A *Tolypothrix* was characterized by dark brown coloured sheaths and a trichome width of approximately 15 µm.

3.5 Discussion

Cyanobacterial toxin production is a worldwide phenomenon with concomitant widespread adverse health effects in humans and wildlife of the temperate and tropical regions (Chorus and Bartram, 1999). Despite the high abundance of cyanobacteria in the Arctic (Vincent, 2000a), at present there is only a single recent report of MCs in Arctic cyanobacterial mats (Kleinteich et al., 2012). Although MC was below the limit of detection in most of the environmental samples analyzed by Kleinteich et al. (2012), MC concentrations increased dramatically when cultured under laboratory conditions and at higher ambient temperatures thus suggesting a high potential for toxin production in all of the samples. In an extension of the latter studies, the same cyanobacterial mat communities from the Canadian Arctic were analyzed for the presence of STX as well as to provide more detailed understanding of the molecular predisposition for toxin production of the cyanobacteria present and finally to identify the MC variants detected earlier.

3.5.1 Saxitoxins in Arctic cyanobacterial mats 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 ng / g dry weight) were one magnitude lower than those reported for pure cultures of benthic cyanobacteria of temperate regions (*Scytonema* cf. *crispum*, 66 mg / g dry weight in Smith et al. 2011; *Lyngbya wollei*, maximum 58 mg / g dry weight in Onodera et al. 1998). The latter difference is likely a result of the low abundance of the producer in a mixed environmental sample in contrast to a pure culture. Whether differences in species diversity, a higher temperature or the competition for light and nutrients, amongst many explanations also influenced STX levels, cannot be ascertained at the moment.

Lack of STX detection using HPLC-FLD may be explained by the fact that approximately 26 different variants of STXs are known to date (Codd, 2000). While all STX variants are indiscriminately detected in the ELISA, thus giving a sum value of STXs present in the respective sample analyzed, HPLC analysis relies on the detection of each individual STX variant. Thus if multiple variants are present in a sample, reliable HPLC detection depends entirely on the concentration level of each variant present, i.e. on the STX variant specific LOD of the HPLC method used.

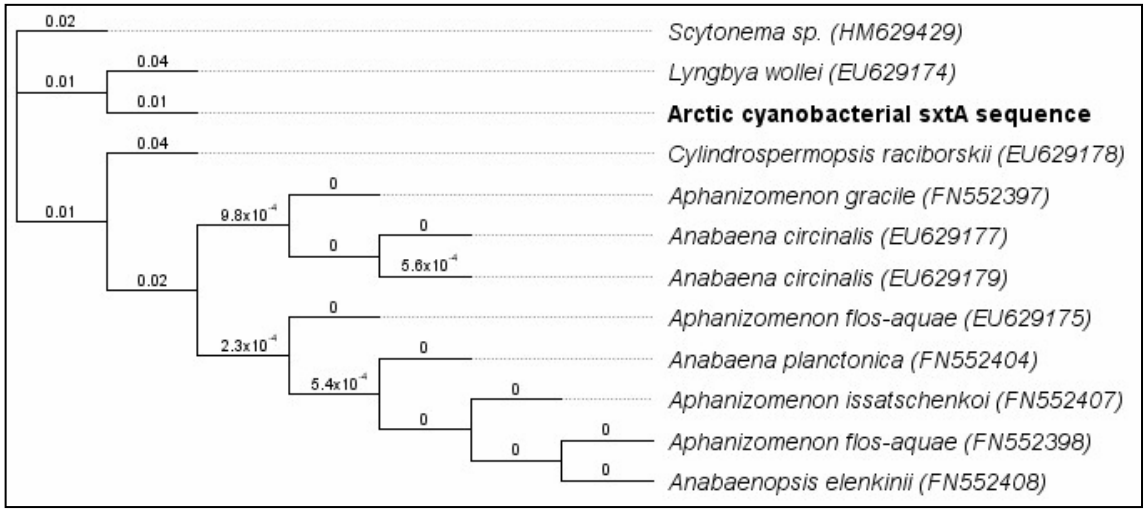


Figure 3-3: Phylogenetic analysis of sxtA.

The 657 bp long sequence obtained is given in boldface; other sequences were obtained from GenBank. Sequences were aligned and the tree constructed using Geneious™ software (version 5.5.6) as a Neighbour-Joining tree after the method of Jukes-Cantor. Substitutions per site are indicated next to the branches.

In contrast to MC which is widely distributed among cyanobacterial taxonomic groups (Pearson and Neilan, 2008), only a few cyanobacterial species have been reported to produce the STXs, including *Aphanizomenon*, *Planktothrix, Scytonema* and *Lyngbya* (Smith et al., 2011). The limited number of STX producing cyanobacteria may explain why STXs and the sxt genes were only detected in one out of five Arctic cyanobacterial mat samples analyzed. Consequently, the distribution of STX producing species in Arctic, at least in the area studied here, could be limited and solely dependent on the unique species composition of the respective cyanobacterial mats. Although the STX producing species could not be identified *Tolypothrix* or *Lyngbya* appeared likely candidates. Both genera seem to have a widespread distribution in the Arctic regions as they have been reported from the Canadian High Arctic (Sheath and Müller, 1997; Bonilla et al., 2005), as well as Spitsbergen (Matula et al., 2007). While the *sxtA* sequence obtained from this sample showed highest sequence similarity to *Lyngbya* and *Scytonema* sequences. *Tolypothrix* was identified in the mat sample by microscopy as well as in the 16S rRNA gene sequence library and belongs to the same family as *Scytonema* which has recently been identified as a STX producer (Smith et al., 2011).

3.5.2 Microcystins in Arctic cyanobacterial mats and their potential producers

In a previous study MC was detected in one of the five mat samples (Sample A) using an ELISA assay. In the present study the MC congener in Sample A was identified as [Asp^3, ADMADDA^5, Dhb^7] MC-RR. This variant has been reported before by Beattie et al. (1998) in a *Nostoc* strain (DUN901) isolated from brackish water in the United Kingdom and is reported here for the first time in the Arctic region. A MC with the same mass, [Asp^3, ADMADDA^5] MC-RR, was recently reported in lichen (*Peltigrea membranacea* / *Peltigrea hymenia*) associated cyanobacteria (most likely *Nostoc* or *Nodularia*) from Scotland (Kaasalainen et al., 2012). While Kaasalainen et al. (2012) reported the presence 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 of the continental Antarctic (McMurdo Ice Shelf, Bratina Island and Dry Valleys). More often than not 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 (Wood, Mountfort, et al., 2008) also observed in this study. Wood et al. (2008) identified the cyanobacterial genus Nostoc as an Antarctic MC producer based on microscopy and molecular methods. The latter corroborated the reports by Beattie et al. (1998) and Kaasalainen et al. (2012) suggesting that *Nostoc* could be the producer of the [Asp^3, ADMADDA^5, Dhb^7] MC-RR congener.

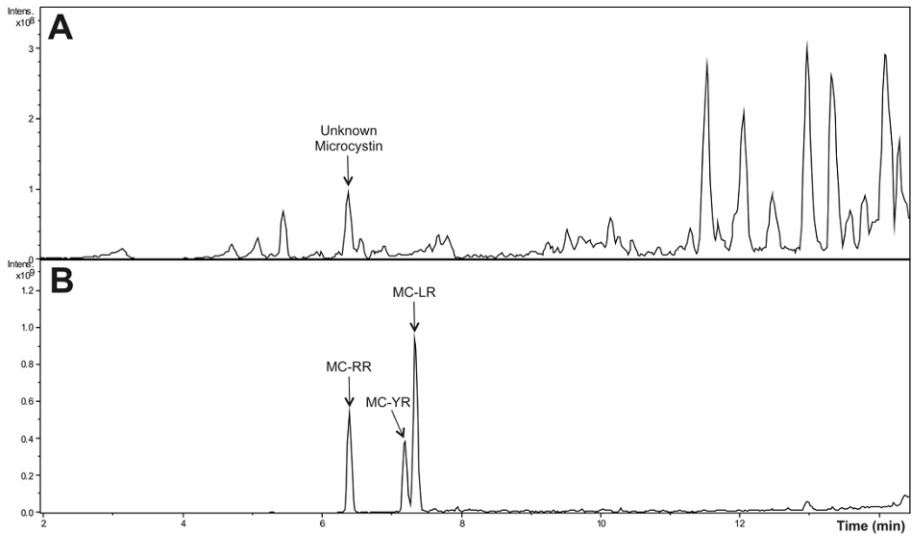
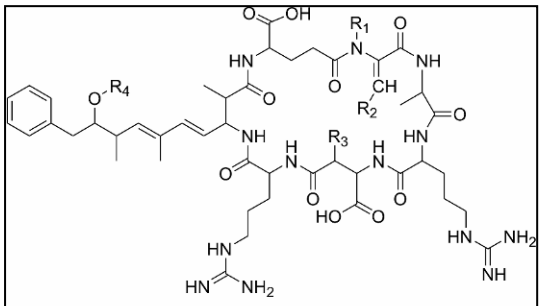


Figure 3-4: HPLC chromatogram of the MC in the Arctic cyanobacterial mat.

High pressure liquid chromatography – mass spectrometry basepeak chromatograms (m/z 100-2000) of a methanolic extract of Artic cyanobacterial Sample A (A) and of authentic standards of MC RR, MC YR and MC LR (B) separated on a C18 column.

Even though in this study all of the Arctic cyanobacterial mats were dominated by Nostocales, also other potential MC producing genera of cyanobacteria were observed, e.g. *Leptolyngbya* (Mohamed and Al Shehri, 2010). 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 of Beattie et al. (1998), Wood et al. (2008), and Kaasalainen et al. (2012), it is hypothesized that *Nostoc* is also the MC producer in the Arctic mat sample of this study. 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.



Microcystin (MC)	R_1	R_2	R_3	R_4
MC-RR	$-CH_3$	-H	$-CH_3$	$-CH_3$
[Asp^3,ADMADDA^5, Dhb^7] MC-RR	-H	$-CH_3$	-H	$-COCH_3$

Figure 3-5: Structures of MC-RR and [Asp^3, ADMADDA^5, Dhb^7] MC-RR.

Genetic analyses of the Arctic cyanobacterial mat samples revealed that at least some species within the mats have the ability to produce MCs. All of the samples contained at least one gene of the mcy gene cluster involved in MC production. These included the *mcyA*, the *mcyB* and in one case the *mcyE* region (Table 3-1, Supplemental Figure 1), encoding for the non-ribosomal peptide synthetases, and a hybrid enzyme (Börner and Dittmann, 2005). The signal intensity of *mcyA* was distinctly higher in sample A which tested positive for MC previously, suggesting a high abundance of this MC producer. Due to the low sequence similarities between orders and even genera of the genes in the cyanobacterial *mcy* operon (Christiansen et al., 2003) the lack of absolute identification of two gene products is not surprising. Moreover, low values for matches in the BLAST search (Supplementary Table 3-II) could thus be a result of low sequence similarities between known MC producing cyanobacterial species from temperate regions and Arctic species. However, for each of the samples at least one mcy sequence could be identified (Table 3-1; Supplementary Table 3-II), showing that each mat contains at least one potential MC producer.

Table 3-2: Fragment-ion pattern detected for [Asp3, ADMADDA5, Dhb7] MC-RR.

Observed by electrospray ionisation collision-induced dissociation. [a] *m/z* values in italics are deconvoluted from their respective $[M+2H]^{2+}$ ions.

$[M+H]^{+}$ [a]	$[M+2H]^{2+}$	Fragment Ion Assignment
155.0	-	Dhb-Ala
157.1	-	Arg
213.0	-	Glu-Dhb
265.1	-	ADMADDA – HOAc – NH_3
272.1	-	Asp-Arg
311.1	-	Dhb-Ala-Arg
426.2	-	Dhb-Ala-Arg-Asp
582.2	291.6	Dhb-Ala-Arg-Asp-Arg
613.2	-	Asp-Arg-ADMADDA
627.2	-	Arg-ADMADDA-Glu
710.3	-	Arg-ADMADDA-Glu-Dhb
711.3	-	Glu-Dhb-Ala-Arg-Asp-Arg
742.2	-	Asp-Arg-ADMADDA-Glu
937.4	469.2	M – Asp
981.4	491.2	M – Ala
992.4	496.7	M – HOAc
1008.2	504.6	M – CN_2H_2
1034.4	517.7	M – H_2O
1052.4	526.7	M

3.5.3 Ecological implications of toxins in Arctic cyanobacterial mats

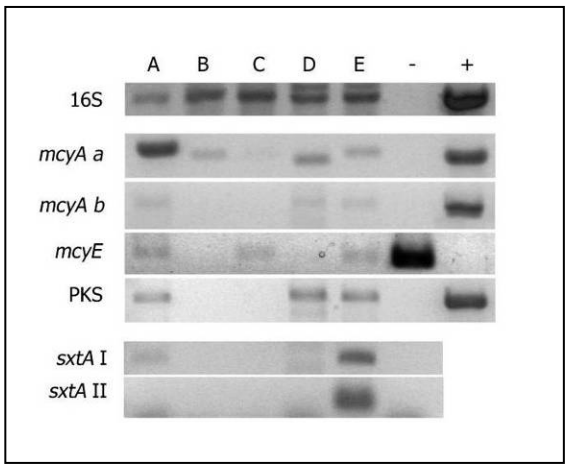
The concentrations of STX and MC detected (21 ng STX / g dry weight and 106 µg MC / kg dry weight) (Kleinteich et al., 2012) are low when compared to those found in planktonic cyanobacterial blooms of temperate and tropical regions (Carmichael et al., 1997; Chorus and Bartram, 1999; Smith et al., 2011) but are comparable to the levels detected in cyanobacterial mats of the Antarctic (1 to 15,900 µg / kg dry weight; Wood et al., 2008). Nevertheless, the presence of potent toxins, albeit in low concentrations, could have an adverse effect on the fauna living in and feeding on the mat communities. Saxitoxin and MC containing cyanobacteria have been reported to be toxic to nematodes, crustaceans and rotifers (Demott et al., 1991; Gilbert, 1996; Li et al., 2009; Ferrão-Filho et al., 2010; Garcia et al., 2010). These groups are also present in many cyanobacterial mats. Trophic interactions in these mat communities currently poorly understood and the effects of the toxins on the organisms present are largely unknown. Due to their close proximity in the microhabitat of a cyanobacterial mat, microorganisms and metazoans are unlikely to be able to avoid contact with the toxins. It has been suggested that rising temperatures could increase toxin concentrations in polar cyanobacterial communities (Dziallas and Grossart, 2011; Kleinteich et al., 2012) and thus exacerbate potential effects of toxins on metazoan organisms (Gilbert, 1996). In the cryosphere many physiological processes happen just above a minimum threshold level (Convey et al., 2003) and energy consuming production of secondary metabolites e.g. MC and STX may be limited (Kleinteich et al., 2012). Kaebernick

& Neilan (2001) postulated that MC synthesis is increased under optimal temperature and growth conditions, which are speculated to be around 20 °C for polar cyanobacteria (Tang et al., 1997). Thus a warmer climate as predicted for the Arctic in current climate change models (Trenberth et al., 2007) could elevate the temperature above the minimum threshold level for toxin synthesis, leading to an increased general metabolic activity and thus an increased level of toxin production (Convey, 2006; Velázquez et al., 2011; Kleinteich et al., 2012). If the latter hold true then long-term changes in the species diversity within cyanobacterial mat ecosystems of the Polar Regions should be expected. Monitoring toxins and changes in cyanobacterial diversity within mats could help in understanding climate change effects in the Polar Regions as well as assisting in evaluating the role of cyanobacterial second metabolites in these environments.

3.6 Acknowledgements

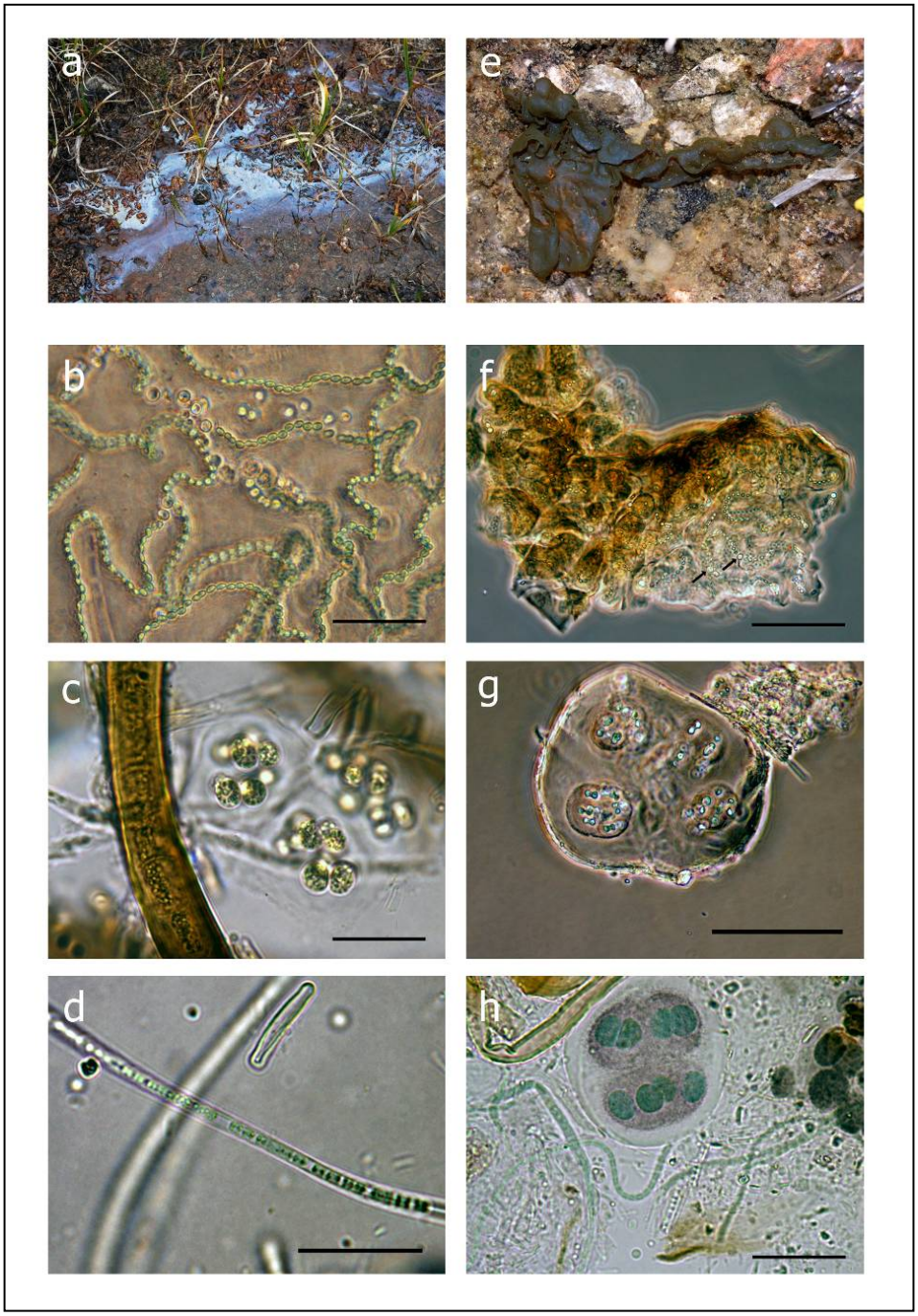
We would like to acknowledge the Deutsche Forschungsgemeinschaft (DFG)-funded project DI698/18-1 Dietrich. We are grateful to the TOTAL Foundation (Paris) and the UK Natural Environment Research Council (NERC, WP 4.3 of Oceans 2025 core funding to FCK at the Scottish Association for Marine Science) for funding the expedition to Baffin Island and within this context Olivier Dargent, Nice, France, and Dr. Pieter van West, University of Aberdeen, UK, for collecting and photographing Arctic cyanobacterial mats in Baffin Island. We would also like to thank the Carl Zeiss Stiftung and the Excellence Initiative of the University of Konstanz, Germany, for funding the PhD project of J.K. and APECS (Association of Polar Early Career Scientists) for their educational support. Furthermore, we acknowledge the support of the European Community research infrastructure action under the FP7 `capacities' specific program ASSEMBLE No.227788. For technical support and new ideas we are very grateful to Heinke Bastek, Kathrin Leinweber and Lisa Zimmermann from the University of Konstanz, Germany, Martina Sattler, University of Jena, Germany, and Dr. Anne Jungblut from the Natural History Museum, London, UK as well as Wendy Jackson (University of Waikato, New Zealand) for valued technical assistance.

3.7 Supplemental Information



Supplementary Figure 3-I: Genetic toxic potential of five Arctic cyanobacterial mat samples as evaluated by PCR amplification of specific toxin-biosynthesis genes.

Target genes are indicated on the left. Lanes 1 – 5 show PCR products derived of total DNA extracted of samples A – E, lanes 6 of the negative controls, and lanes 7 of the positive controls using DNA of *Microcystis aeruginosa* CCAP 1450/16 as template (with exception of *mcyE*, where lane 6 is from the positive control and lane 7 from the negative control)



Supplementary Figure 3-II: Photographic images of the cyanobacterial assemblages in the natural environment.

Photgraphs (a,e; Olivier Dargent, Dr. Pieter van West) and microscopic images (b-c, f-h) of representative species in the respective communities. Microcystin positive sample A is displayed on the left, saxitoxin positive sample E on the right.

Supplementary Table 3-I: GPS coordinates and sampling dates.

Sample	GPS coordinates	Sampling Date
A	n.d.	2009-08-20
B	N 72°28.028' W 79°49.385'	2009-08-21
C	N 72°28.081' W 79°49.092'	2009-08-21
D	N 72°28.038' W 79°50.508'	2009-08-21
E	N 72°28.038' W 79°50.508'	2009-08-21

Supplementary Table 3-II: Cyanobacterial 16S rRNA gene- and toxin-biosynthesis gene-specific primers used in this study.

The primer sequence, annealing temperature (Tm), target region, and the corresponding references, are shown. Reactions indicated with an asterisk (*) were amplified using the Fermentas MasterMix, all others with the Phusion™ Polymerase (see materials and methods section).

Primer	Sequence	Tm	Target	Reference
27F	AGAGTTTGATCCTGGCTCAG	50 °C*	16S rRNA	Jungblut and Neilan, 2006
359F	GGGGAATYTTCCGCAATGGG	-	16S rRNA	Nubel et al., 1997
809R	GCTTCGGCACGGCTCGGGTCGATA	50 °C*	16S rRNA	Jungblut and Neilan, 2006
23S30R	CHTCGCCTCTGTGTGCCWAGGT	61 °C	23S rRNA	Rueckert et al., 2007
mcyA-F mcyA-R	AAAAGTGTTTTATTAGCGGCTCAT AAAATTAAAAGCCGTATCAAA	53 °C*	*mcyA*	Hisbergues et al., 2003
HEPF HEPR	TTTGGGGTTAACTTTTTTGGGCATAGTC AATTCTTGAGGCTGTAAATCGGGTTT	46 °C	*mcyE*	Jungblut and Neilan, 2006
FAA RAA	CTATGTTATTTATACATCAGG CTCAGCTTAACTTGATTATC	43 °C*	*NRPS*	Neilan et al., 1999
MTF mod MTR mod	GCNGGDGGRGCNTAYGTNCC CCNCGAAATYTTRACYTG	55 °C	*NRPS*	after Neilan et al., 1999
sxtaF sxtaR	GCGTACATCCAAGCTGGACTCG GTAGTCCAGCTAAGGCACTTGC	68.5 °C	*sxtA*	Ballot et al., 2010
sxtAF sxtAR	GATGACGGAGTATTTGAAGC CTGCATCTTCTGGACGGTAA	59 °C	*sxtA*	Al-Tebrineh et al., 2010
DKF DKR	GTGCCGGTNCCRTGNGYYTC GCGATGGAYCCNCARCARMG	61.5 °C	PKS	Moffitt and Neilan, 2003

Supplementary Table 3-III: List of sequences obtained in this study of genes for second-metabolite (microcystin and saxitoxin) production and of their closets phylogenetic matches in the NCBI database.

Sample	Gene	Closest phylogenetic match in NCBI database	Accession no. of BLAST match	Lg (bp)	Sm (%)
A	*mcyA*	*Clostridium* AA Adenylation domain	ZP_09202841	744	65
B	*mcyA*	*Microcystis* sp. *mcyA* gene	FJ379558	161	96
B	*mcyB*	*Microcoleus* sp. NRPS	AY768451.2	326	75
C	*mcyA*	*Microcystis viridis mcyA* gene	EU203575.1	237	89
C	*mcyE*	Uncultured *Microcystis* sp. clone	JF739303.1	469	100
D	*mcyB*	*Anabaena sphaerica* NRPS	DQ439641	129	74
D	*mcyA*	*Microcystis* sp. *mcyA* gene	FJ379558.1	210	99
E	*mcyE*	*Nostoc punctiforme*	YP_001866903	240	60
E	*mcyB*	*Nostoc punctiforme* AA adenylation domain	YP_001865727	696	39
E	*sxtA*	*Aphanizomenon* sp.	HQ338481.1	128	99
E*	*sxtA*	*Scytonema* sp.	HM629429.1	657	97

* GenBank accession number: JX887897

Supplementary Table 3-IV: Sequences obtained in this study of 16S rRNA genes affiliated to cyanobacteria.

Analysis was done with the Geneious™ software and against the NCBI database.

Sample	Accession no.	Closest annotated hit in GenBank	Length (bp)	Sm (%)
A	JX887887	Cf. *Leptolyngbya* sp. Greenland - DQ431004	1380	94
A	JX887891 JX887894	*Leptolyngbya compacta* - HQ132933	1647	93
A	JX887882	*Aphanizomenon gracile* - FJ424575	431	94
A	JX887890	*Chroococcus* sp. - FR798926	1448	97
E	JX887896	*Leptolyngbya frigida* - AY493573	1462	97
E	JX887883	*Tolypothrix distorta* - GQ287651	828	98
E	JX887888	*Leptolyngbya* sp. - DQ786166	1738	89
E	JX887889	Cf. *Leptolyngbya* sp. Greenland - DQ431004	1332	94
E	JX887892	*Nostoc punctiforme* - CP001037	1969	95
E	JX887893	*Calothrix* sp. - JN385289	1783	92
E	JX887885	Cf. *Leptolyngbya* sp. Greenland - DQ431004	1338	93
E	JX887895	*Nostoc punctiforme* - CP001037	1916	94
E	JX887886	*Snowella littoralis* - AJ781040	1461	98

Supplementary Table 3-V: Nucleotide sequence of the 128 bp sxtA gene in sample E.

TCTTATGACGGAGTATTTGAAGCTACACAACGAGCAACGGCAGCTTCTAGATCCGATTGTG
GGGTTCCATGTGTCGGGGGGAGCCGAAATTAGGGGAATTATTGCTAATTACCGTCCAGAAG
ATGCAG

4 MANUSCRIPT 3

4.1 Widespread distribution of cyanobacterial toxins in Antarctica and implications of climate change

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4.2 Introduction

4.2.1 Cyanobacterial toxins and their ecological role

Toxin production by cyanobacteria is a worldwide phenomenon and adverse health effects due to consumption or contact with toxic cyanobacteria have been reported for humans and livestock in many temperate and tropical countries (Dietrich, Fischer, Michel, Hoeger, et al., 2008). In the Polar Regions, where extensive benthic mats of cyanobacteria dominate freshwater systems (for details see Quesada et al. 2008), toxin production has rarely been investigated (Hitzfeld et al., 2000; Jungblut et al., 2006; Wood, Mountfort, et al., 2008; Kleinteich et al., 2012). Whereas in temperate and tropical water bodies trophic interactions are complex, the simple setup of Antarctic freshwater habitats provide unique systems and research on these may assist in understanding the causes and consequences of cyanobacterial toxicity.

Different species across all orders of cyanobacteria have been reported to produce toxic second metabolites (Chorus and Bartram, 1999). The high structural and functional diversity of cyanobacterial toxins include cyclic polypeptides and alkaloids that act as hepatotoxins, neurotoxins, dermatotoxins or general cytotoxins (for review see Humpage 2008; Pearson et al. 2010).

Cylindrospermopsin (CYN) is a cytotoxic alkaloid (Figure 4-1). It functions via an irreversibly inhibition of cellular protein synthesis as well as glutathione synthesis and seems to be activated by Cyt P450. It has also been reported be genotoxic (Pegram et al., 2008). In contrary to MC to date few species have been reported to produce CYN: *Anabaena bergii* (Schembri et al., 2001) and *A.lapponica* (Spoof et al., 2006), *Aphanizomenon flos aquae* (Preussel et al., 2006) and *A.ovalisporum* (Banker et al., 1997; Shaw et al., 1999), *Cylindrospermopsis raciborskii* (Hawkins et al., 1997), *Lyngbya wollei* (Seifert et al., 2007)*, Oscillatoria sp.* (Mazmouz et al., 2010), *Raphidiopsis curvata* (Li et al., 2001) and *mediterranea* (McGregor et al., 2011), as well as *Umezakia natans* (Harada et al., 1994). More non-identified species are suspected to produce the toxic compound (Humpage, 2008; Mazmouz et al., 2010). CYN has a circumpolar distribution but has never been reported from the Polar Regions (Sinha et al., 2012).

Microcystins are cyclic heptapeptides of which more than 110 structural variants are known (Puddick, 2012). They act by an irreversible inhibition of eukaryotic serin/threonin specific protein phosphatases (Pegram et al., 2008). Microcystin production is mainly associated with bloom forming planktonic cyanobacteria but has also been reported from benthic and terrestrial species (Humpage, 2008), and from species that live symbiotically with lichen (Kaasalainen et al., 2012). Saxitoxins are alkaloids that block voltage-gated Na^+ channels and are primarily neurotoxic to all higher organisms. They are more commonly

known as paralytic shellfish poisons when produced by marine dinoflagellates (Pegram et al., 2008). Cyanobacterial STX production has been confirmed in planktonic and benthic species of seven different genera (Murray et al., 2011; Smith et al., 2011). In the Antarctic MCs have been reported from cyanobacterial mats (Hitzfeld et al., 2000; Wood, Mountfort, et al., 2008), however, no other cyanotoxins have been identified from this pristine environment. Recently, we provided the first evidence of MC as well as the neurotoxic saxitoxin (STX), in a freshwater pond in the Arctic (Kleinteich et al., 2012)(Kleinteich et al., this study Manuscript 2).

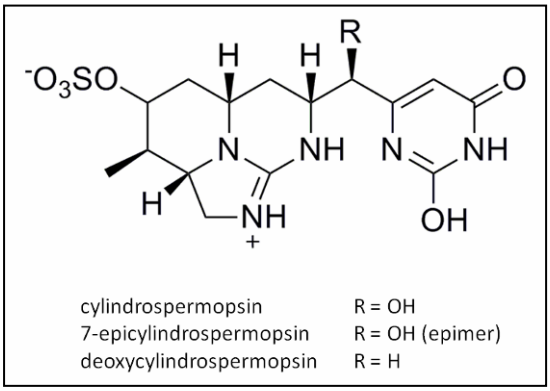


Figure 4-1: Chemical structure of cylindrospermopsin and known variants.

All of the above toxins are produced non-ribosomally by a complex and energy consuming pathway involving several enzymes including non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) (e.g. Neilan et al. 2008). The enzymes are encoded on large gene clusters containing multiple genes. The gene cluster for CYN production *cyr* (43 kb) has recently been sequenced in four different species (*Cylindrospermopsis raciborskii*, *Aphanizomenon* sp., *Oscillatoria* sp., and *Raphidiopsis curvata*) (Mihali et al., 2008; Mazmouz et al., 2010; Méjean et al., 2010; Stüken and Jakobsen, 2010; Jiang et al., 2012). The sequences of the *mcyA-J* (55 kb) and the *sxt* (> 35 kb) gene cluster for MC and STX synthesis respectively, are known from several species (Kellmann, Mihali, Michali, et al., 2008; Neilan et al., 2008; Murray et al., 2011). Phylogenetic analysis has shown that both gene clusters are very ancient and older than eukaryotic life (Rantala et al., 2004; Murray et al., 2011). In contrary to the *mcy* and *sxt* gene clusters which are variable between species (Christiansen et al., 2003; Rouhiainen et al., 2004; Mihali et al., 2009), the *cyr* gene cluster seems to have undergone little mutation (Stüken and Jakobsen, 2010) with the *Oscillatoria* sp. sequence the most distant from the other genera (Jiang et al., 2012). The number of variants known from each of the toxins, approximately 110 congeners of MC (Neilan et al., 2012; Puddick, 2012) and 30 of STX (Humpage, 2008) but only three for CYN (CYN, 7-epi-CYN, and 7-deoxy-CYN) (Jiang et al., 2012) have been identified to date, is partly due to

relaxed substrate specificity of the involved enzymes but is also a reflection of the described genetic diversity (Tanabe et al., 2004; Dittmann and Wiegand, 2006).

4.2.2 Climate Change

Globally toxic cyanobacteria are predicted to produce higher concentrations of toxins as a consequence of climate change (O'Neil et al., 2012) and some species, for example, CYN-producing *Cylindrospermopsis raciborskii*, will likely invade new habitats due to more favourable temperature conditions (Sinha et al., 2012). The Antarctic Peninsula is one of the few regions subjected to rapid regional climate change (Vaughan, Marshall, et al., 2003) with mean annual temperatures that have increased by 0.5 °C per decade over the past 50 years (Turner et al., 2005). Freshwater systems on the Antarctic have been reported to be especially sensitive to climate warming (Quayle et al., 2002; Pearce, 2005; Pearce and Laybourn-Parry, 2012) and are considered as one of the early indicators of climate change in Antarctica (Quayle et al., 2002). As a general result higher growth rates and changes in metabolism are expected (Vincent and Howard-Williams, 1989; Velázquez et al., 2011) and which will cause changes in microalgal colonization and community development (Wynn-Williams, 1996a; Pearce, 2005). We recently demonstrated that cyanobacterial toxin production in Antarctic cyanobacterial mats increased when cultured at elevated temperatures (Kleinteich et al., 2012). The consequences of increased levels of toxins in polar aquatic ecosystems are unknown. Monitoring toxins in Antarctic systems might help predict future changes in ecosystems of the temperate regions, where cyanobacterial toxicity is also expected to increase.

The recent detection of cyanobacterial toxins in the northern cryosphere (Kleinteich et al., Manuscript 2) as well as their increasing potential due to climate change in Antarctica (Kleinteich et al., 2012) and on a worldwide scale (O'Neil et al., 2012) requires a better understanding of the distribution of various cyanobacterial toxins in the Polar Regions. Several cyanobacterial mats from the Antarctic Peninsula were screened for the genes and the toxins CYN, MC and STX and report an unexpected high distribution of these toxins.

4.2.3 Material and Methods

4.2.4 Study sites and sampling

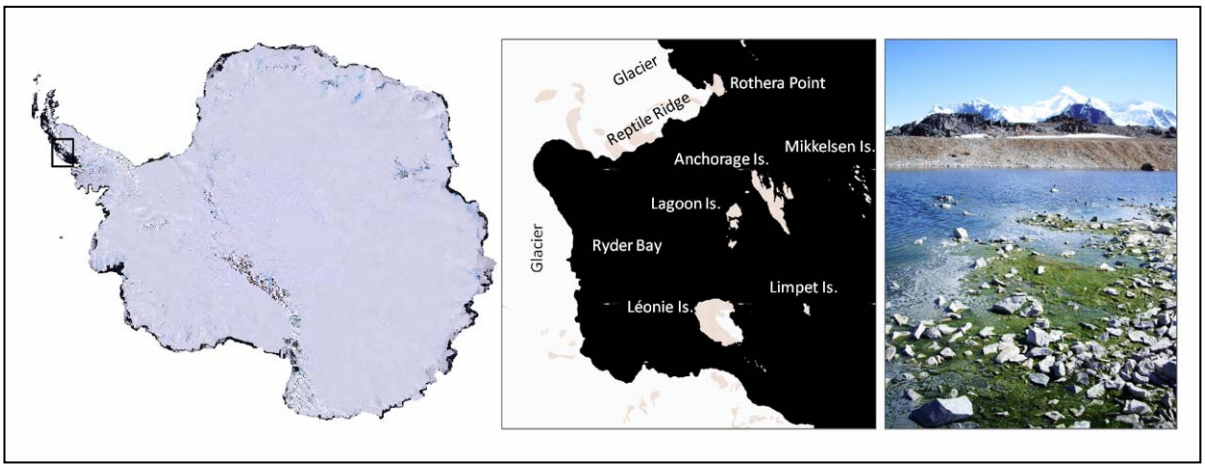


Figure 4-2: Map of sampling locations.

The sampling sites were located on the Antarctic Peninsula (left panel). All sites were located close to Rothera Research Station and on Anchorage, Lagoon and Léonie Island (middle panel). Images are adapted from Landsat Image Mosaic of Antarctic (LIMA, USGS). The photograph (right panel) was taken on Lagoon Island on 20th January 2011 (Courtesy of Julia Kleinteich).

Cyanobacterial mat samples were collected during an expedition to Rothera Research Station, Adelaide Island on the Antarctic Peninsula from December 2010 to February 2011. Sampling sites were located on Rothera Point and Anchorage, Léonie and Lagoon Islands, all within 20 km of Rothera (Figure 4-2). Samples from Rothera Point were collected from various melt water ponds and streams within variable distances from the sea (between 10 and 100 Meters) on East Beach, a flat gravel terrain surrounded by snow covered slopes. On Anchorage Island three main sites were sampled. An extensive mat close to the sea shore, probably impacted by the nutrient input of an elephant seal and bird colonies, a shallow but large melt water pond approximately 20 m in diameter that was used by Skuas and thus subjected to nutrient input as well as several mats along a melt water stream in an area less impacted by large animal. Léonie Island was generally less influenced by seals due to a steeper shore line, hosts however a large bird colony. The steep terrain allowed few melt water ponds and thus few cyanobacterial colonies. The mats sampled were located in shallow ponds app. 20 m from the seashore. Samples from Lagoon Island were derived from various small melt water ponds and streams of a flat and protected (inside the lagoon) southward coastal area that was located next to an elephant seal and bird colony as well as a larger lake of app. 30 meters in diameter.

Samples for DNA extraction and toxin analysis were sealed in sterile tubes or stored in RNAlater (Qiagen, Hilden, Germany) and frozen (-20 °C) within 24 h until further analysis.

Samples were usually collected between 1100 hr and 1600 hr when daily temperatures and radiation were highest. Temperature was recorded using a transportable thermometer (TFA Wertheim, Germany) at most sampling sites. Three independent mats (two on nd one on Anchorage Island) were logged for their temperature over several weeks using temperature loggers from iButton® (Maxim, CA, USA).

4.2.5 Screening for cylindrospermopsin, saxitoxin, and microcystin

Toxin extraction

Frozen cyanobacterial mat material was thawed, homogenized with a sterile glass spatula and lyophilized. From each sample, three replicate aliquots of lyophilized material were processed. Varying amounts of material were combined with 5 mL of 75 % methanol in a mortar and ground to a fine paste. The homogenate was filled up to 15 mL with 75 % methanol in a tube and placed in an ice-cold ultrasonic bath (30 min). Following centrifugation (30 min, 4,000 x *g*) the supernatant was collected for further processing. The latter extraction step was repeated three times on the pellet, whereby all supernatants were combined and dried under nitrogen. The resulting pellet was re-suspended in 15 mL H_2O (15 min in an ultrasonic bath for complete solubilisation) and cleaned using C18 cartridges (Sep-Pak, Waters, Dublin). The extract on the C18 cartridges was eluted with 15 mL methanol (100 %), the methanolic eluate dried under nitrogen and the dried extract re-dissolved in 3 mL methanol (20 %). The extract was centrifuged (20 min, 13,000 x *g*) Three replicate extracts were prepared per sample and combined before further analysis to minimize variations caused by the extraction method.

Determination of organic content

The samples were very heterogenic in composition and composed of a mixture of sand, stones and other inorganic material. To remove this source of variability toxin concentrations were calculated in relation to the organic percentage of the sample. For that purpose the three replicate subsamples) were lyophilized and their weight recorded. These were combusted at 600 °C for 7 h and the dry weight (inorganic weight) recorded immediately. The average organic part was calculated for the three replicate pellets in percent. This calculated organic percentage was applied on the dry weight initially used for extraction and all toxin concentrations normalized to this value.

Cylindrospermopsin analysis

Detection of CYN was performed using a CYN ELISA (Microtiter Plate) from ABRAXIS (Warminster, USA) according to the manufacturer's protocol. Absorption of the colour reaction was recorded at 450 nm using a TECAN infinite M200 plate reader. The assay has an LOD of 0.05 ng / mL. In a preliminary ELISA test all 30 samples were analysed for CYN

(data not shown). Based on this preliminary test samples that had a strong signal for CYN were selected for replicate analysis. The 12 selected extracts were tested in a minimum of 3 replicate CYN ELISA assays with each sample being measured in two technical replicates per ELISA plate. The presence of CYN and analogues was confirmed in a single positive sample using liquid chromatography-mass spectrometry as described in (Wood et al., 2007).

Microcystin analysis

All 30 samples were analysed for MCs using the microcystin-ADDA ELISA Microtiter Plate from ABRAXIS (Warminster, USA) with an LOD of 0.15 ng / ml. following the standard protocol. For each sample a minimum of 3 independent ELISA assays were conducted each in two technical replicates.

Saxitoxin analysis

A STX (PSP) ELISA (ABRAXIS, Warminster, USA) was employed to analyse for STX according to the manufacturer's protocol for all 30 extracted cyanobacterial samples. This STX (PSP) ELISA has an LOD of 0.0215 ng / mL. Due to negative results in the ELISA as well as negative results in the genetic analysis this assay was only performed in once in two technical replicates.

4.2.6 Screening for genes involved in toxin synthesis

DNA extraction

DNA was extracted from each sample in three individual extractions using three different extraction methods. The extracts were then combined for downstream applications. The MO BIO PowerSoil® DNA Isolation Kit was used to extract DNA from 5-10 mg of frozen mat material following the manufacturer's recommendations. In a second extraction DNA was isolated from 5-10 mg of sample using the hot-phenol method. Briefly the mat material was combined with 700 µL TES buffer (10 mM Tris HCL pH 8, 1 mM EDTA, 2 % SDS) and 70 µg Proteinase K (Qiagen, Hilden, Germany) and incubated at 50 °C for 1 h. The salt concentration of the solution was adjusted to 400 mM NaCl. An equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added and a DNAse free metal bead (Qiagen, Hilden, Germany) supplied to each tube. The samples were vortexed for 15 min and the phases separated by centrifugation (10.000 *x g*, 2 min). The aqueous phase was transferred to a new tube and the phenol extraction repeated. The DNA in the resulting aqueous phase was precipitated with 0.1 volumes of 3 M sodium acetate and 1 volume of cold isopropanol (incubation at -20 °C for 2 h) The precipitated DNA was recovered by centrifugation (13.000 *x g*, 15 min at 4 °C). The isopropanol was removed and the pellet washed with 70 % ethanol and air dried. The DNA pellet was re-dissolved in sterile, DNAse free water. In the third extraction DNA was extracted using xanthogenate following a

modified protocol after Saker et al. (2005). Subsamples (5-10 mg) of frozen mat material were combined with 1 mL XS buffer (0.1 M Tris HCL, 20 mM EDTA, 1 % w/v potassium-ethyl-xanthogenate, 1 % w/v SDS, 0.8 M NH_4OAc) in garnet-bead containing tubes (MO BIO). They were incubated at 65 °C for 3 h with a 30 min vortexing step after 1 h. Samples were subsequently placed on ice for 10 min and centrifuged (12.000 *x g*, 10min). The supernatant was collected in a clean tube and an equal volume of phenol/chloroform/isoamylalcohol was added and the tube vortexed. The suspension was centrifuged (10.000 *x g*, 3 min) and the aqueous fraction transferred into a new tube. The phenol extraction step was repeated and the DNA in the resulting aqueous fraction was precipitated and washed as described in the hot-phenol-protocol above.

All resulting DNA extracts were dissolved in sterile DNAse free water, their quality measured using NanoDrop (NanoDrop 3300 Fluorospectrometer, ThermoScientific) and stored at -20 °C. Whereas the hot-phenol-method and the xanthogenate-extraction generally resulted in better values in the Nanodrop, product amplification was usually more successful using the MoBio Kit. However, preliminary cyanobacterial analysis using RFLP revealed little to no difference between the three extraction-methods (data not shown). Before downstream application equal amounts of extracted DNA from each extraction method were combined, measured in NanoDrop and stored at -20 °C for long term storage and at 4 °C for further experiments. All steps were performed at room temperature if not stated differently. All chemicals were of highest quality and were derived from Sigma (Sigma-Aldrich, Seelze, Germany) if not stated differently.

Detection of genes involved in toxin synthesis

Several PCRs were performed on the *mcy, sxt, and cyr* operon involved in MC, STX, and CYN synthesis respectively. Primers and annealing temperatures are listed in Supplemental Table 1. For all reactions either a Master Mix™ (Fermentas, St. Leon-Rot, D) or the Phusion™ polymerase (NEB, Ipswich, USA) were applied as indicated in Supplemental Table 1, under the addition of BSA, DMSO and $MgCl_2$ (all Fermentas or NEB). Bands of interest were excised from a 1.5 % agarose-gel (TAE-buffered) using a sterile scalpel, purified with a Gel Extraction Kit (Fermentas, St. Leon-Rot) and sequenced bidirectionally using the primers in Supplemental Table 1 at Eurofins MWG Operon (Ebersberg, Germany). *Microcystis aeruginosa* CCAP 1450/16 served as a positive control for *mcy* genes and *Aphanizomenon ovalisporum UAM290* was used as a positive control for *cyr* genes. A cyanobacterial sample previously tested positive for *sxt* (Kleinteich et al. Manuscript 3) served as a positive control for *sxt* genes. The obtained sequences were analysed using Geneious Software (Geneious Pro 5.5.6) and the closest matches identified using the BLAST (megablast) search of GenBank. Accession numbers are given in Supplemental Table 2. Based on the two amplified sequences and the *cyrB* and *cyrJ* genes from cultured species

available in the GenBank database two phylogenetic trees were constructed. A Neighbour-Joining tree (Tamura-Nei) was constructed using Geneious based on the *cyrB* gene from this study and various *cyrB* genes from the GenBank database.

4.2.7 Diversity analysis

ARISA

The ITS regions (intragenic transcribed spacer) of the total community DNA was amplified in a PCR reaction as described before (Kleinteich et al. 2012) with a set of cyanobacteria-specific oligonucleotides (Wood, Rueckert, et al., 2008). Intergenic spacer lengths were detected and statistically analysed as described in detail previously (Wood, Rueckert, et al., 2008). A signal intensity of below 100 fluorescence units was considered to be background noise and those peaks were discarded. Only fragments of >180 base pairs were regarded to be true intragenic transcribed spacer signals and considered for the statistical analysis using the Primer-E 6 Software (PRIMER-E).

Isolation of organisms

Single filaments were isolated from selected samples in order to identify potential toxin producers. An aliquot of the defrosted sample was diluted in BG-11 medium and dispersed on a BG11-agar plate. Using a Nikon Eclipse TS 100 inverted Microscope single filaments were picked from the plate with an elongated Pasteur pipette and placed in 24 well plates with each well containing 500 µL of BG-11. The agar plates were subsequently partly covered with a lightproof shield to induce active movement of filaments towards the light. After visible growth on agar plates and 24 well plates (about one to two weeks) single filaments were picked and placed in BG-11 containing Erlenmeyer flasks. Aliquots of the isolated filaments were stored at 4 °C and used for DNA extraction.

Molecular identification of isolated organisms

DNA was extracted from the isolated culture material using the MO BIO PowerSoil® DNA Isolation Kit after manufacturers' recommendations. The DNA was eluted in water and stored at 4 °C or at -20 °C for long term storage. Amplification of a region of the 16S rRNA gene sequence and the 16S-23S ITS region was undertaken using cyanobacterial specific primers 27F and 23S30R (Supplemental Table 1) in a 50 µL PCR reaction containing the Fermentas (St. Leon-Rot, D) Master Mix, 2.5 mM $MgCl_2$, 0.2 µg / µL BSA, 3 % DMSO (all Fermentas or NEB) and 0.5 µM of each primer (Eurofins MWG Operon, Ebersberg, Germany). PCR cycling conditions were 4 min for 95 °C [92 °C 60 s, 55 °C 60 s, 72 °C 120 s] x 35, 72 °C for 10 min. The PCR products were run on a 1.5 % agarose gel (TAE buffered) to check for correct product amplification and then purified with the GeneJET™ PCR Extraction Kit (Fermentas, St. Leon-Rot, Germany). Sequencing was performed using the primers 27F,

359F, 781R, 809R and 23S30R (Supplemental Table 1) at Eurofins MWG Operon (Ebersberg, Germany). The contigs were assembled using Geneious software (Geneious Pro 5.5.6) and checked for sequence similarities in GenBank using BLAST (megablast). BLAST hits and accession numbers of obtained sequences are listed in Supplemental Table 2.

Morphological identification of isolated organisms

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 prepared 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). Identification to genera level was made with taxonomic guides of Komarek & Anagnostidis (2005) and Komarek & Anagnostidis (1982).

4.2.8 Data evaluation and software

Statistical evaluation and graphs were created using GraphPad Prism Version 5.04. Treatment of photographs was performed 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).

4.3 Results

4.3.1 Sampling sites

Several locations in the vicinity of Rothera research station (Figure 4-2) were sampled for cyanobacterial mats during the austral summer of December 2010 and January 2011. At the beginning of the growth season (end of December 2010) the rocky landscape was still covered with snow and only few phototrophic organisms could be detected (mostly red and green snow algae). No cyanobacterial growth was visible when snow cover was removed manually. In protected areas cyanobacteria was visible in liquid freshwater (sample 24.12.10-001). On Anchorage Island snow melt occurred earlier than on Rothera Point and mats could be sampled already by the end of December (samples 28.12.10-004 to -007). When snow melt occurred (beginning of January) cyanobacterial growth could be observed within few days. Growth occurred wherever liquid water was available. Due to fluctuation of the melt water streams, growth of cyanobacterial mats was highly variable. Larger melt water ponds (app. 2 m diameter) provided a more stable habitat than streams. During late January and February extensive cyanobacterial mats had established in many locations. Cyanobacterial mat growth was observed in all sampled areas (Rothera Point and attached islands Anchorage, Léonie and Lagoon). Appearance of the mats was highly diverse, displaying different colorations and texture (Figure 4-3). Whereas some were found bright green others were almost black or orange-red. The size varied from a few square centimetres to several square meters.

4.3.2 Temperature regime

The temperatures in shallow freshwater ponds and streams measured ranged from 4 °C to 16 °C at midday. The temperature on the direct surface of the mat was generally recorded to be 2-3 °C higher than the surrounding water. In the early morning and on cold days a thin layer of ice was observed on some mats. The temperature logging over several weeks revealed extreme variations between day and night that ranged from below freezing to almost 20 °C during midday. In general temperatures seemed to increase during the growth season from December to the beginning of February (Supplementary Figure 4-I).

Figure 4-3: Images of mats sampled.

(A) Panorama and (B) details of mats on Anchorage Island, (C) of the cyanobacterial mat habitat on Lagoon Island as well as details of mats (D) 20.1.11-002 (note oxygen pearls on mat surface) and (E) 20.1.11-007 (note shoe tips for scale). (F) The layering of sample 28.12.10-004 (scale bare 5 mm). Photograph courtesy of Julia Kleinteich.

4.3.3 Toxicity

Table 4-1: Toxicity data

The table indicates the geographical origin, the detected MC concentrations, CYN concentrations as well as the *mcy* and *cyr* genes detected in each sample. * Samples tested positive for CYL in a preliminary ELISA assay (*n*= 1) but concentrations were not determined.

Sample	Origin	MC [ng/g organic weight]	CYL [ng/g organic weight]	Genes mcy	Genes cyr	other genes
24.12.2010-001	Rothera Point	302,6	n.d.*	*mcyE, mcyA*	-	PKS
28.12.2010-004	Anchorage Island	0,00	n.d.	-	-	PKS
28.12.2010-006	Anchorage Island	47,5	n.d.	*mcyE, mcyA*	-	PKS
28.12.2010-007	Anchorage Island	89,3	n.d.*	-	-	PKS
30.12.2010-001	Rothera Point	109,3	n.d.	*mcyA*	-	PKS

Sample	Origin	MC [ng/g organic weight]	CYL [ng/g organic weight]	*Genes mcy*	*Genes cyr*	other genes
05.01.2011-005	Rothera Point	298,2	n.d.	-	-	PKS
07.01.2011-001	Rothera Point	169,0	n.d.*	*mcyE, mcyA*	-	PKS
09.01.2011-001	Rothera Point	125,9	n.d.	-	-	PKS
10.01.2011-001	Léonie Island	92,5	9,59	*mcyE, mcyA*	-	PKS
10.01.2011-005	Léonie Island	78,6	n.d.*	*mcyA*	-	PKS
10.01.2011-006	Léonie Island	230,5	156,76	*mcyE, mcyA*	*cyrA, cyrB, cyrJ*	PKS
11.01.2011-001	Rothera Point	11,7	n.d.	-	-	PKS
11.01.2011-006	Rothera Point	n.d.	n.d.	*mcyE*	-	PKS
12.01.2011-002	Rothera Point	n.d.	n.d.	*mcyA*	-	-
12.01.2011-006	Rothera Point	20,9	n.d.*	-	-	PKS
14.01.2011-001	Anchorage Island	153,8	n.d.*	-	-	PKS
14.01.2011-002	Anchorage Island	122,6	2,47	-	-	PKS
14.01.2011-003	Anchorage Island	115,3	2,87	*mcyE*	-	-
20.01.2011-001	Lagoon Island	153,6	4,19	*mcyE, mcyA*	-	PKS
20.01.2011-002	Lagoon Island	285,5	n.d.*	*mcyE, mcyA*	-	PKS
20.01.2011-003	Lagoon Island	163,4	n.d.	*mcyE*	-	PKS
20.01.2011-004	Lagoon Island	108,7	n.d.*	*mcyE*	-	PKS
20.01.2011-005	Lagoon Island	134,1	4,37	*mcyE*	-	PKS
20.01.2011-006	Lagoon Island	89,3	n.d.*	*mcyE, mcyA*	-	PKS
20.01.2011-007	Lagoon Island	185,5	5,83	*mcyE*	-	PKS
20.01.2011-009	Lagoon Island	107,0	1,96	*mcyE, mcyA*	-	PKS
20.01.2011-010	Lagoon Island	n.d.	n.d.*	*mcyA*	-	PKS
24.01.2011-1.2out	Rothera Point	207,5	3,81	*mcyE*	-	PKS
24.01.2011-2.3in	Rothera Point	125,6	2,33	-	-	PKS
25.01.2011-3.1in	Rothera Point	160,3	4,12	-	-	PKS

Cylindrospermopsin

The cyanobacterial toxins CYN was detected in a preliminary assay in 21 out of 30 cyanobacterial mat samples and its presence was confirmed in 11 of these samples (Table 4-1). Concentrations ranged between 2 and 10 ng CYN / g organic weight. In one sample (10.1.11-006 from Léonie Island) significantly higher levels of CYN (156 ng / g organic weight) were measured. This sample extract was additionally tested for CYN using LC-MS which confirmed the presence of CYN as well as its variant deoxy-CYN (data not shown). Levels of CYN in the other samples were too low to be detected using LC-MS.

To provide further evidence for the presence of a CYN producer in this sample, crude DNA extracts of this mat was screened for the presence of *cyr* genes involved in CYN production. A 478 bp product of the *cyrB* gene, a 584 bp product of *cyrJ* and finally a 1005 bp product of the *cyrA* gene, coding for a mixed NRPS / PKS, a putative sulfotransferase, and an amidinotransferase, respectively (Mazmouz et al., 2010) were successfully amplified and sequenced. The identity of the amplified sequences was verified by a BLAST search of the GenBank database.

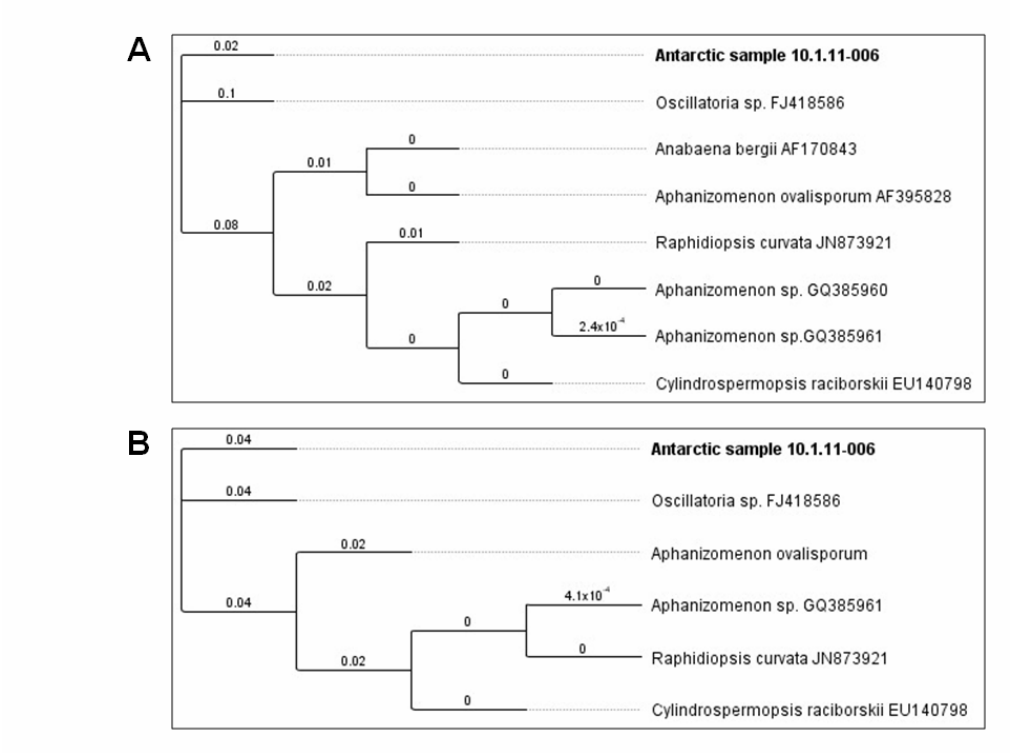


Figure 4-4: Phylogenetic analysis of the cyrB and cyrJ genes detected in this study.

Phylogenetic analysis of (A) the 478 bp long sequence of the *cyrB* gene, coding a mixed NRPS / PKS and (B) a 584 bp long sequence of *cyrJ* coding for a putative sulfotransferase detected in sample 10.1.11-006.

The amplified *cyrB* gene was found most similar to the *cyrB* gene of an uncultured cyanobacterium from Florida (89 %; HQ712110), the highest similarity to a cultured strain

was *Aphanizomenon ovalisporum* (89 %; AF395828). The *cyrJ* gene (584 bp) and the *cyrA* gene (1005 bp) showed highest similarity to the CYN biosynthetic gene cluster of *Oscillatoria sp.* (93 % for *cyrJ* and 96 % for *cyrA*; FJ418586). In the phylogenetic trees constructed, the *cyrB* and *cyrJ* sequences of this study, were located next to the *Oscillatoria sp.* (FJ418586) sequence and were rather distant to any other sequences available in the database (Figure 4-4).

Microcystin (MC)

Microcystins were detected using an Adda-ELISA assay in 26 of 27 samples tested (Table 4-1). MC levels varied between 10 and 300 ng / g organic weight. In 20 samples the presence of MC could be supported by the detection of genes involved in MC synthesis (*mcyE*, *mcyA,* bacterial PKS gene) (Table 4-1). The identity of the *mcyA* gene was verified by sequencing the products of two different samples (Supplementary Figure 4-II). Sample 24.12.10-001 had the highest MC concentration of 303 ng MC / g organic weight and bands of the correct size for two *mcy* genes (*mcyE*, *mcyA*) were amplified as well as a general bacterial PKS involved in secondary metabolite synthesis (Table 4-1). In sample 10.1.11-006, that contained high levels of CYN, MC was found in high levels (231 ng / g organic weight) and a band of the correct size for *mcyE* as well as *mcyA* were amplified. The amplified products of the *mcyA* gene of sample 20.1.11-006 as well as sample 20.1.11-001 were sequenced and found to be most similar to the amino acid adenylation domain of *Nostoc punctiforme* (both 80 %; CP001037.1) in a BLASTn search. The sequences were however was of poor quality, possibly due to the presence of several species capable of producing MCs been present in the sample resulting in a mixed signal, and therefore these were not deposited in GenBank or used for detailed phylogenetic analysis. Amplification of the *mcyH* gene coding for an ABC-transporter was not successful in any of the samples.

Saxitoxin

No SXT was detected using the ELISA assay from Abraxis. No genes involved in STX production were detected.

4.3.4 Diversity

To get an overview above the diversity of the individual samples an ARISA analysis, evaluating the number and length of the ITS region as a measure of community composition was performed. It could be revealed that the samples corresponded in their diversity to the sampling location. In detail, the samples collected from Lagoon and Anchorage Island clustered in the MDS blot, whereas the samples collected from Rothera Station grouped in two different clusters (Figure 4-5). Sample 20.1.11-009 was significantly different to the other samples. This sample was collected from a scum sample of a cyanobacterial bloom in a lake on Lagoon Island and therefore contained fewer and most likely planktonic species (Supplementary Figure 4-II). Samples from Léonie Island were heterogeneous. Diversity was furthermore correlated to the MC and CYN concentrations detected, but no correlation to these factors could be observed (data not shown).

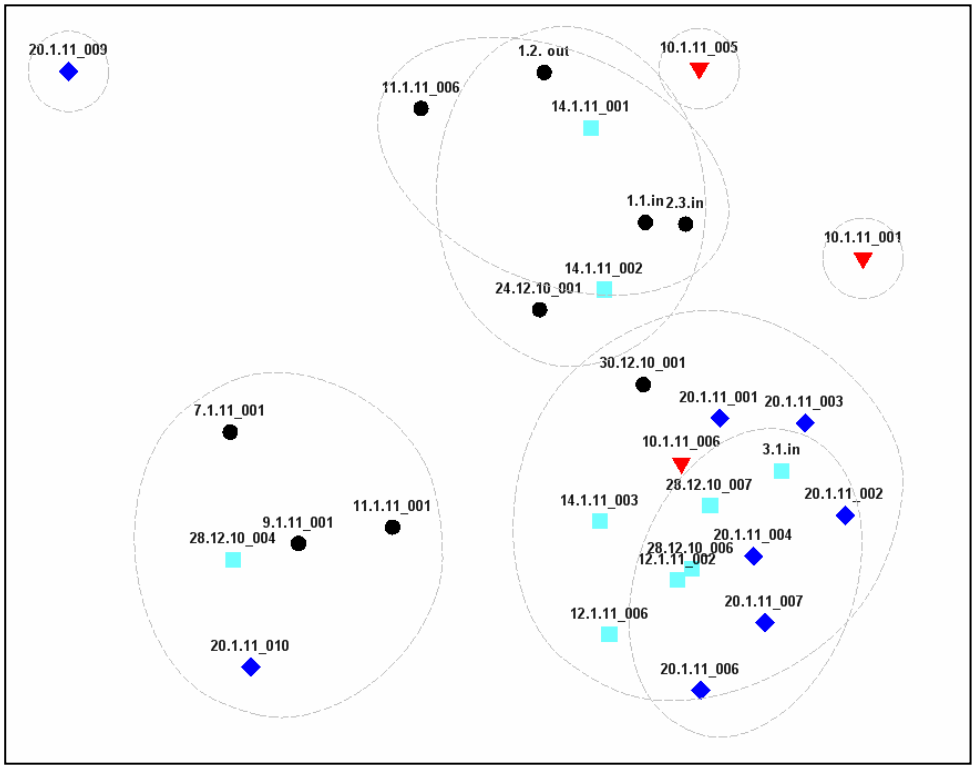


Figure 4-5: Community analysis of cyanobacterial mat samples from Rothera Research Station and near islands in relation to their geographic origin.

Community composition of cyanobacterial mats from Rothera Point (black circles), Lagoon Island (blue diamond), Anchorage Islands (blue squares), and Léonie (red triangles), shown as a two-dimensional non-metric multidimensional scaling ordination (stress value of 0.12) based on Bray–Curtis similarities of ARISA fingerprints. Points within a circle cluster at 40 % similarity.

4.3.5 Isolates

To identify potential toxin producers four strains of cyanobacteria were isolated from two samples containing CYN: 10.1.11-006 from Léonie Island and 20.1.11-009 from Lagoon Island. The isolated organisms were cultured in unialgal cultures and characterized. Three isolates from sample 10.1.11-006 could be annotated to the same organism. The filamentous species had a brown or green colouration, depending on the isolate. The trichome width was approximately 5-7 µm. No heterocysts were visible. The filaments were sometimes arranged in spirals. (Supplementary Figure 4-II). The 16S rRNA - ITS - 23S rRNA gene was most similar to *Anabaena variabilis* (100 %; CP000117.1) as well as *Camptylonemopsis sp*. (100 %; JN385292.1) in a megaBLAST search. The isolate of sample 20.1.11-009 was identified by its morphology as well as the 16S rRNA - ITS - 23S rRNA gene sequence as *Phormidium priestleyi*. The filamentous species contained no heterocysts and had a trichome width of approximately 7-9 µm. The cells had a bright green colouration (Supplementary Figure 4-II). The 16S rRNA - ITS - 23S rRNA sequences of the isolates can be found in GenBank under the accession numbers as stated in Supplemental Table 2. Unfortunately none of the isolates tested positive for the *cyr* genes.

4.4 Discussion

4.4.1 Biogeography of cyanobacterial mats on Rothera Point and Islands

The toxicity of a diverse range of cyanobacterial mats from Rothera Point and the close islands (Anchorage, Léonie and Lagoon), on the Antarctic Peninsula were assessed. Several biotic and abiotic factors seemed to influence a range of microhabitats in the landscape sustaining the diversity, integrity and structure of the cyanobacteria mats. At the beginning of the season, when snow melt occurred mats formed quickly in the melt water on empty bedrock and sand. The absence of visible cyanobacterial growth at the beginning of the season suggests that mats were seasonal and did not survive the winter under the snow cover as it has been described by Quesada et al. (2008). Streambed cyanobacterial mats from the Antarctic have also been reported to survive the winter in a freeze-dried manner, quickly recovering at the beginning of the next growth season (Vincent and Howard-Williams, 1986; Quesada et al., 2008), which could be the case for some of the larger mats in this study. Polar benthic communities have been described to be largely dependent on freshwater availability (Quesada et al., 2008), which was highly dynamic at the study site creating a dynamic system of moisturised and desiccated cyanobacterial mats. Larger and deeper ponds seemed to provide a more stable habitat and accumulated more biomass than the surrounding melt water streams. The quick formation of the sometimes up to two centimetres thick mats within few weeks suggests very high growth rates for the mats which could be an adaptation to the variable conditions in melt water streams. Growth rates of cultured Antarctic mats have been reported to be much lower, ranging between 2.3 and 5.3 mm / year (Buffan-Dubau et al., 2001).

Antarctic cyanobacteria are considered cryotolerant rather than cryophile with optimal growth temperatures around 20 °C (Tang et al., 1997). Moreover metabolic rates, nitrogen fixation, as well as photosynthesis of Antarctic cyanobacteria are described to be optimal around 15 °C (Vincent and Howard-Williams, 1989; Velázquez et al., 2011). Therefore highest growth rates may be expected during midday when most light is available and temperatures sustain a high biochemical turnover. Indeed the greatest oxygen production (indicated as visible oxygen pearls on the mat surface) was observed at midday (Figure 4-3) albeit we did not measure photosynthesis or metabolic rates.

The high variation in recorded temperatures (-1 °C - 18 °C) within short time periods, are likely to stress organisms living in this habitat. Upton and colleagues however reported a higher bacterial diversity under fluctuation temperature conditions than under stable average temperatures for artificially cultured Antarctic lake sediment (Upton et al., 1990). It can be hypothesized that during midday high metabolic activity could sustain species adapted to higher temperatures, whereas extreme cold temperatures during the

night could give advantage to cryophile species. Therefore the high temperature variation recorded could rather than to constrain it, result in high species diversity.

4.4.2 Cyanobacterial toxins in Antarctica

Cylindrospermopsin

First evidence of CYN in Antarctic cyanobacterial mats is provided in this study, as well as the first report of MC on the Antarctic Peninsula. Cylindrospermopsin and deoxy-CYN have to our knowledge never been recorded before in a cold environment and is more commonly associated with cyanobacterial blooms in tropical and temperate regions (Sinha et al., 2012). The concentration of CYN found in benthic species of these climatic zones is also higher when compared to those detected in this study (0 – 20 µg / g dry weight CYN and 0 – 547 µg / g dry weight deoxy-CYN (Seifert et al., 2007)).

With the exception of the genus of *Oscillatoria* none of the known CYN producers is to our knowledge abundant on the Antarctic continent (e.g. Jungblut et al. 2012b). This raises speculations about the species responsible for the CYN production recorded in this study. We were unable to successfully isolate and identify the CYN producer. However genes involved in CYN production were successfully amplified from sample 10.1.11-006 that contained highest concentrations of CYN). Based on the low genetic similarity of the *cyrB* and *cyrJ* gene sequences from this study compared to the best results of known *cyr* sequences we suspect that a cyanobacterial species previously unknown to produce CYN is present in the Antarctic mats. The *cyr* genes have been described to be highly conserved between species even though gene rearrangements must have occurred (Stüken and Jakobsen, 2010). The *cyrB* and the *cyrJ* gene of *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* for example have similarities of 99.7 % and 98.7 % respectively and >95 % for all orthologous *cyr* genes (Stüken and Jakobsen, 2010). They are thus higher than or in the range of the 16S rRNA gene similarities in these species (Stüken and Jakobsen, 2010). The high dissimilarity between our sequence and the sequences known to the database is thus surprising. It can be speculated that the CYN producer present the cyanobacterial mats could be endemic to Antarctica and has been separated from other CYN producers for a long time allowing mutations to occur. However, this would need to be verified by isolation of the producer.

Microcystin and Saxitoxin

Previous studies have reported the presence of MC on continental Antarctica on the McMurdo Ice Shelf (Hitzfeld et al., 2000; Jungblut et al., 2006) as well as on Bratina Island and the Dry Valleys (Wood, Mountfort, et al., 2008) but never from the Antarctic Peninsula or the maritime Antarctic. Whereas Jungblut et al. 2006 reported MC in only one microbial mat, Hitzfeld et al. (2000) as well as Wood et al. (2008) detected the toxin in a large number

of samples. Compared to the Continental Antarctic, the Antarctic Peninsula is a different biogeographic zone with higher average temperatures, more precipitation, strong seasonality and thus a different biodiversity (Huiskes et al., 2006). The high portion of MC positive cyanobacterial mat samples in this study suggests wide distribution of the toxin in this habitat. Although the concentrations of MC were low (11 - 303 ng MC / g organic weight) when compared to cyanobacterial blooms of the temperate regions (Chorus and Bartram, 1999) they lie in the range of those reported for other Antarctic (11.4 ng MC-LR / mg dry weight in Jungblut et al. 2008; 1 - 16 ng / mg dry weight in Wood et al. 2008) and Arctic habitats (106 ng MC / g dry weight, Kleinteich et al. Manuscript 2). The detection of MC in multiple mats of the Antarctic Peninsula in this study in concert with previous reports from the continental Antarctic as well as the Arctic suggests a widespread distribution of MC in the Polar Regions.

Many species have been reported to produce the heptapeptide MC (Chorus and Bartram, 1999), some of which are present in Antarctica. In the continental Antarctic *Nostoc sp.* has been described as a producer of MC (Wood, Mountfort, et al., 2008). Also in our previous study on cyanobacterial toxins in the Arctic we suspected a *Nostoc* species to be the MC producer (Kleinteich et al. Manuscript 2). However in the present study *Nostoc* was not very abundant in the mats by microscopic observation. Amplicons of the correct size for genes of the *mcy* cluster in 20 out of 30 cyanobacterial mat samples (Table 1) were amplified. Only the products of selected samples were sequenced. The sequences showed low similarity to known *mcy* gene sequences in the GenBank database (Supplemental Table 2). The amplified *mcyB* gene product was most similar to the amino acid adenylation domain of *Nostoc punctiforme*. Genes involved in MC and STX production are less strongly conserved than for CYN. Similarities have been reported to vary between 67 and 81 % for *mcy* genes of three different genera (Rouhiainen et al., 2004) and 53 to 99 % for *sxt* genes of *Aphanizomenon, Anabaena* and *Cylindrospermopsis* (Mihali et al., 2009). The low genetic similarity between the *mcy* sequences found in this study compared to the GenBank database could thus suggest a low phylogenetic relationship between the Antarctic MC producers and known MC producers from temperate regions. In the past we had difficulties to amplify *mcy* genes in Arctic and Antarctic cyanobacteria. We suspect that this is due to the low similarity of polar *mcy* genes to known sequences in the database. Most primers used for *mcy* gene detection have been developed based on *Microcystis aeruginosa* and other temperate climate cyanobacteria (e.g. Sivonen 2008). These primers are most likely poorly suitable for the amplification of Antarctic *mcy* genes and lead to limited or no amplification. A detailed study on the genetics and genomics of toxin producers in extreme and isolated ecosystems could improve the understanding of the evolution of the *mcy* genes and diversity of cyanobacterial toxin production. The full (pyro-)sequencing of the genome of an Antarctic MC producer might help to detect highly conserved gene regions on

the *mcy* cluster that could be the basis for molecular detection of a wide range of toxic cyanobacteria.

Growth optima of Antarctic cyanobacteria usually lie higher than what they usually experience in their natural habitat (Tang et al., 1997). The temperatures measured for the mats on Lagoon were generally high (8 - 16 °C) and we have previously shown that MC production was increased in cyanobacterial mats at these temperatures (Kleinteich et al., 2012). Elevated temperatures, closer to the biochemical growth optimum of cyanobacteria as persistent on warm and sunny days on Lagoon, might thus sustain toxin production in these mats. Due to the insufficient detection of the *mcy* gene cluster primers for the ketosynthase domain of polyketide synthases in various organisms (Moffitt and Neilan, 2003) were used. Polyketide synthases are enzymes involved the biosynthesis of a wide range of second metabolites including MC, STX, and CYN. A In 29 out of the 30 samples a product for a polyketide synthtase could be amplified suggesting the potential for second metabolite production is widespread in cyanobacterial mats of the Antarctic. We did not detect the *sxt* genes involved STX synthesis in the Antarctic cyanobacterial samples, nor did we find the toxin itself using the ELISA method. In the past we have detected STX in only one sample from the Arctic, which was the only report of STX in a polar environment. We therefore suspect that STX is not as widespread in the polar environments as CYN or MC. In this study we have only analyzed the more common cyanobacterial toxins (CYN, STX, MC) of which standardized detection methods are available. No other cyanobacterial toxins were screened for in this study, however, it cannot be excluded that other cyanobacterial second metabolites such as nodularin, anatoxin-a, anatoxin-a(S) or lyngbyatoxin are present in cyanobacterial mats of the Antarctic.

Potential function of cyanobacterial toxins in Antarctic mats

The biological role of cyanobacterial toxins is yet poorly understood and is the focus of ongoing research (for review see Kaplan et al. 2012). More commonly known from planktonic and benthic cyanobacterial blooms in temperate and tropical freshwaters (Chorus and Bartram, 1999), their widespread distribution in polar cyanobacterial mats is surprising. In an environment that allows life only at a minimum threshold level (Convey, 2006) the energy investment in the synthesis of the complex molecules must have good reason.

It has been postulated that cyanobacterial toxins act as a protection against grazing (e.g. Rohrlack et al. 1999). However, large predators are absent in the cyanobacterial mats (Quesada et al., 2008) and the ancient origin of the *mcy* and *sxt* genes contradict a primarily function of MC and STX as grazing deterrent (Kaplan et al., 2012). Recently more attention has focused on the potential function of cyanobacterial toxins involved in cellular communication (Kaplan et al., 2012; Neilan et al., 2012).

Cylindrospermopsin has been proposed to trigger segregation of alkaline phosphatases in other algae, resulting in increased extracellular inorganic phosphate (P) and thus a growth advantage for the cyanobacterial cell (Raven, 2010). If CYN is indeed excreted by the cyanobacterial cells in a low P environment CYN concentrations would be expected to correspond with P levels. *Cylindrospermopsis raciborskii* for example, a well described CYN producer from tropical and temperate regions, is known to thrive in low P environments (Sinha et al., 2012). No P levels are available for the water bodies in this study. However, Antarctic ecosystems are generally described as nutrient-poor (Pearce and Laybourn-Parry, 2012). As nitrogen input from marine sources (faeces of birds and seals) is high (102 mg N m^{-2} on Anchorage Island) even though spatial variations occur (Bokhorst et al., 2007; Yergeau, Bokhorst, et al., 2007) we would expect P rather than N to be the limiting growth factor. Cylindrospermopsin as a trigger of extracellular P in an otherwise Pi limited cyanobacterial mats thus seems plausible.

In a quorum-sensing like manner MC is released by during cell lyses and can be sensed by other cyanobacterial cells that then upregulate their *mcy* transcription (Schatz et al., 2007; Kaplan et al., 2012) and has been hypothesized to participate in colony formation of *Microcystis* cells (Zilliges, 2008). Antarctic cyanobacterial mats are highly diverse but very well structured and organized environments (de los Rios et al., 2004). Such a high degree of organization requires the communication of the interacting partners and MCs, as messenger molecules, could thus be involved the formation and function of Antarctic biofilms.

A putative role of MC in cellular protein modulation and protection during oxidative stress and extreme solar radiation has been proposed (Zilliges et al., 2011). Levels of solar radiation can be extreme during the polar summer (average maximum daily radiation$_{280 - 600\ nm}$ ~300 Wm^{-2} at Rothera Research Station in December and January, information by the British Antarctic Survey) and cyanobacteria are often only covered by a thin layer of water (few millimetres to centimetres) that would poorly protect them from UV radiation. High UV radiation as well as high temperature extremes in the polar summer and thus higher metabolic rates could explain the presence of MC in the mats and would imply a seasonal cycle of MC concentration in the mat. However, MC is a very stable molecule and once produced it is likely to be present in the cyanobacterial mat for several days. A daily and seasonal cycle of MC concentrations would therefore need to be tested by monitoring MC production in cyanobacterial mats over the whole growth season.

4.4.3 Global warming

Benthic cyanobacterial mats, the basis of the food web of many Antarctic freshwater systems are considered as ideal model systems to monitor the effects of climate change. Positive metabolic responses on Lake Fryxell stream communities have been

connected to elevated temperatures (Vincent and Howard-Williams, 1989). Velázques et al. (2011) report increased photosynthetic rates and nitrogen metabolism for phototrophic communities from Livingston Island at > 15 °C, whereas photosynthesis of artificially cultured Lake Fryxell communities was maximal at 10 °C but was completely inhibited at 15 °C (Pringault et al., 2001). We have recently shown that cyanobacterial toxin production is increased in cultured cyanobacterial mats at average temperatures of 8 - 16 °C (Kleinteich et al., 2012) and that MC and STX are also present in the Arctic (Kleinteich et al. Manuscript 2). The results of this study demonstrate that MC that has been detected in range of continental Antarctic habitats is also very abundant on the Antarctic Peninsula. Moreover CYN was detected for the first time in Antarctica in a large number of samples. The widespread distribution of the two toxins suggests that the potential for toxicity, even though expressed in low concentrations at the moment, is thus high in Antarctic cyanobacterial mats. With higher temperatures due to climate change and thus generally higher metabolic rates and cyanobacterial growth, concentrations of toxins could thus increase in many Antarctic habitats. Rates of colonization and community development of Antarctic microalgae are expected to change as a consequence of climate warming (Wynn-Williams, 1996a). Retreating glaciers and increased freshwater availability opens new habitats for cyanobacterial colonization (Convey, 2006) and could thus lead to a further increase of toxic cyanobacterial mats.

4.4.4 Final conclusions

In this study we detected the presence of MC and CYN in Antarctic cyanobacterial mats. The presence of the toxins was widespread, suggesting that cyanobacterial toxins are more widely distributed in Antarctica than previously thought. Although the function of the toxins is unclear, climate change could increase the presence of cyanobacterial toxins on a large scale in Antarctic freshwater habitats with yet unknown consequences on the ecosystem.

4.5 Acknowledgements

We would like to acknowledge the Deutsche Forschungsgemeinschaft (DFG)-funded project DI698/18-1 Dietrich and are grateful to the Carl Zeiss Stiftung as well as the Excellence Initiative of the University of Konstanz, Germany, for funding the PhD project of J.K.. We are especially grateful to NERC and BAS for funding the CGS-70 grant and the field-trip to Antarctica as well as all BAS staff for their great logistic and scientific support, especially the team of Rothera Research Station. For technical support and new ideas we are very grateful to Dr David Schleheck, Lisa Zimmermann and Julia Stifel, from the University of Konstanz, Germany, Martina Sattler, University of Jena, Germany as well as Dr. Anne Jungblut from the Natural History Museum, London, UK.

4.6 Supplemental Information

4.6.1 Supplemental Tables and Figures

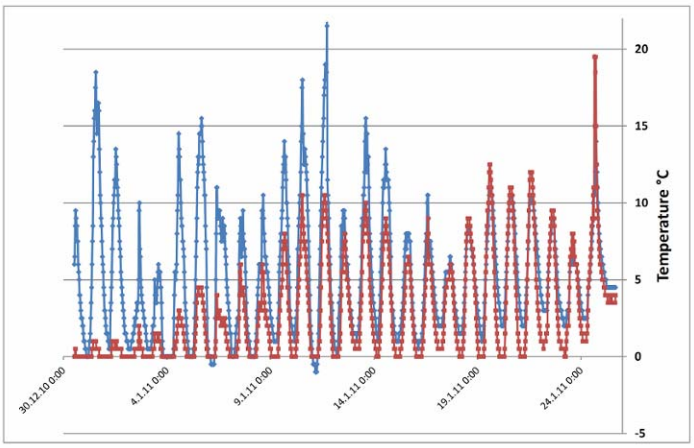
Supplementary Table 4-I: Primer used in this study.

Primer	Target gene	Sequence	Tm	Reference
27F	16S rRNA	AGAGTTTGATCCTGGCTCAG	50 °C* 61 °C	Jungblut and Neilan 2006
359F	16S rRNA	GGGGAATYTTCCGCAATGGG	60 °C*	after Nübel 1997
781R	16S rRNA	GACTACWGGGGTATCTAATCCCWTT		
809R	16S rRNA	GCTTCGGCACGGCTCGGGTCGATA	50 °C*	Jungblut & Neilan 2006
23S30R	23S rRNA	CHTCGCCTCTGTGTGCCWAGGT	61 °C	Rueckert et al. 2007
mcyA-F	*mcyA*	AAAAGTGTTTTATTAGCGGCTCAT	53 °C	Hisbergues et al. 2003
mcyA-R	*mcyA*	AAAATTAAAAGCCGTATCAAA		
HEPF	*mcyE*	TTTGGGGTTAACTTTTTTGGGCATAGTC	46 °C	Jungblut and Neilan 2006
HEPR	*mcyE*	AATTCTTGAGGCTGTAAATCGGGTTT		
FAA	*mcyA*	CTATGTTATTTATACATCAGG	43 °C*	Neilan et al. 1999
RAA	*mcyA*	CTCAGCTTAACTTGATTATC		
MTF mod	*mcyA*	GCNGGDGGRGCNTAYGTNCC	55.5 °C	modified after Neilan et al. 1999
MTR mod	*mcyA*	CCNCGAAATYTTRACYTG		
abcs F	*mcyH*	CAAACTCCATTTTTTCAACA	50 °C	Kaebernick et al. 2002
abcs R	*mcyH*	GGTGAAGTAGTAGTCATCGT		
Cyl M13	*cyrB*	GGCAAATTGTGATAGCCACGAGC	62 °C	Schembri et al., 2001
Cyl M14	*cyrB*	GATGGAACATCGCTCACTGGTG		
Cyl sulfF	*cyrJ*	ACTTCTCTCCTTTCCCTATC	60 °C	Mihali et al., 2008
Cyl namR	*cyrJ*	GAGTGAAAATGCGTAGAACTTG		
sxtaF	*sxtA*	GCGTACATCCAAGCTGGACTCG	68.5 °C	Ballot et al. 2010
sxtaR	*sxtA*	GTAGTCCAGCTAAGGCACTTGC		
sxtAF	*sxtA*	GATGACGGAGTATTTGAAGC	60 °C	Al-Tebrineh et al. 2010
sxtAR	*sxtA*	CTGCATCTTCTGGACGGTAA		
DKF	PKS	GTGCCGGTNCCRTGNGYYTC	61.5 °C	Moffitt and Neilan 2003
DKR	PKS	GCGATGGAYCCNCARCARMG		

*PCR using Fermentas Master Mix, all other reactions using Phusion polymerase

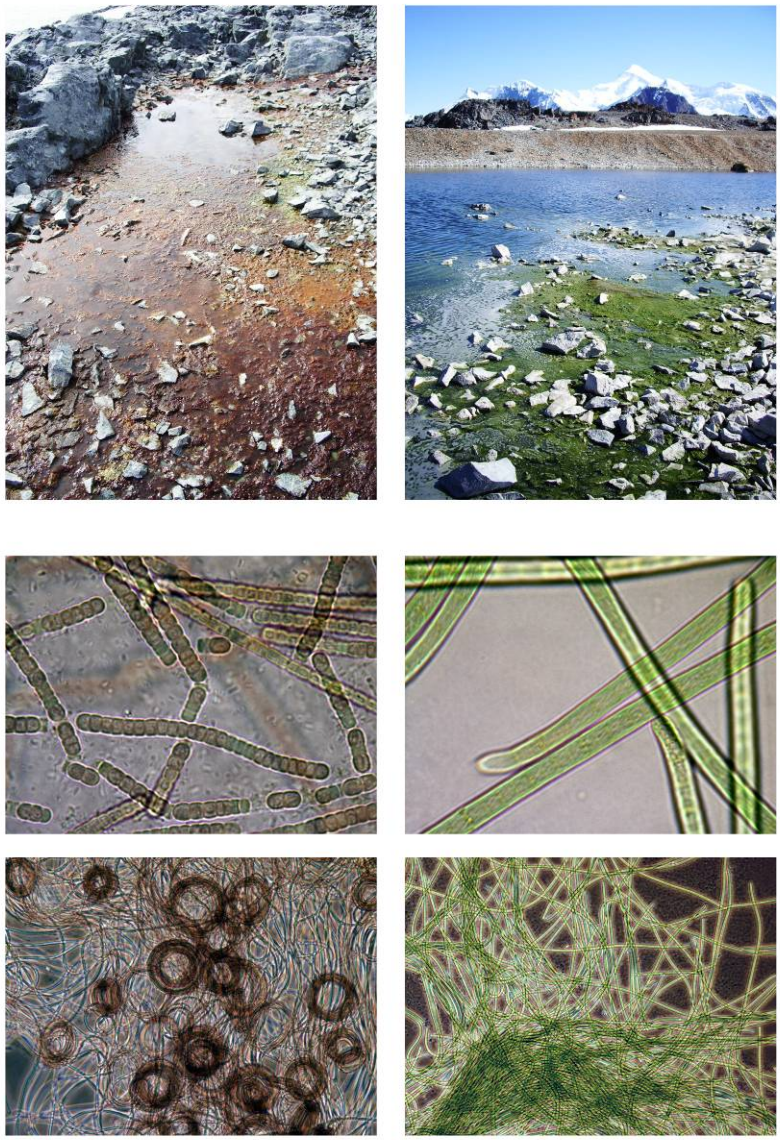
Supplementary Table 4-II: Accession numbers of sequences derived in this study from 16S rRNA - ITS 23S rRNA genes and cyr genes.

Sample	Gene	Accession number	First annotated hit in NCBI database	Accession no. of BLAST match	Length (bp)	Sm
Isolate 10.1.11-006	16SrRNA	n.d.	*Phormidium priestleyi*	AY493579	1465	93 %
			Leptolyngbya sp.	JX401929	1772	88 %
Isolate 20.1.11-009	16SrRNA	n.d.	*Phormidium pseudopriestleyi*	AY493600	1785	100 %
20.1.11-006	*mcyA*	n.d.	amino acid adenylation domain of *Nostoc punctiforme*	CP001037.1	903	80 %
20.1.11-001	*mcyA*	n.d.	amino acid adenylation domain of *Nostoc punctiforme*	CP001037.1	827	80 %
10.1.11-006	*cyrA*	n.d.	CYN biosynthetic gene cluster of *Oscillatoria* sp.	HQ712110	1005	89 %
10.1.11-006	*cyrB*	n.d.	*Aphanizomenon ovalisporum*	AF395828	478	89 %
10.1.11-006	*cyrJ*	n.d.	CYN biosynthetic gene cluster of *Oscillatoria sp.*	FJ418586	584	93 %



Supplementary Figure 4-I: Temperature records for two cyanobacterial mats.

Water temperature was recorded from the end of December 2010 to the end of January 2011 in two separate cyanobacterial mats (blue and red).



Supplementary Figure 4-II: Photographs and microscope images of filamentous isolates of samples 10.1.11-006 and 20.1.11-009 containing CYN. Photograph courtesy of Julia Kleinteich.

5 UNPUBLISHED DATA

Besides the results presented and published in the manuscripts as described above, several experiments have been performed that are not yet published or may serve as preliminary data for future studies. In this paragraph these data will be presented and their impact on future investigations be discussed.

5.1 Diversity analysis

Cyanobacterial mat samples from a range of Arctic and Antarctic regions were collected or provided by other scientists for this study. This included Arctic sites i.e. Northern Baffin Island, Canadian Arctic (as described in Manuscript 2 and 3) and Svalbard, collected in June 2012 (data not published), as well as Antarctic sites i.e. Byers Peninsula (Manuscript 1) and Rothera Point (Manuscript 3), both located on the Antarctic Peninsula. Albeit the diversity of these samples was partially analysed in detail, (Manuscript 1, 2, and 3) an in-depth and a comparative approach is yet missing. For that purpose a next-generation sequencing approach documenting the whole spectrum of genetic diversity within a single step was performed (Armougom and Raoult, 2009; Petrosino et al., 2009). This method is much more effective than conventional approaches such as cloning technologies or DGGE (Denaturing Gradient Gel Electrophoresis). The method allows the simultaneous amplification of several thousand genes in a sample, exceeding the sampling depth by at least a magnitude compared to a standard clone library that usually comprises only a few dozen clones per sample. The large amount of sequencing data thus allows also species with very low abundance to be recorded. This cost-intensive but highly effective experiment was possible by the funding of the Antarctic Science Bursary that was obtained especially for this purpose.

The DNA of 25 cyanobacterial mats samples of Rothera Point, as well as 5 samples from each of the other three locations as described above was extracted by three different extraction methods for a maximum percentage of species covered. The DNA was analyzed at the Research and Testing Laboratory, Texas, USA for its diversity by the 454 next generation sequencing technology using cyanobacteria specific DNA markers for the 16S

rRNA gene. One of the main tasks of next generation sequencing, the bioinformatic data preparation will be performed by collaborating bioinformatics specialists.

Unfortunately the data of the 454 sequencing were received from the Research and Testing Laboratory only a few days before the submission of this manuscript. Therefore it was not possible to include the data in the present version of the dissertation. The data will be prepared and published in the near future. The outcomes of this experiment may therefore be considered as part of the dissertation.

Next to a detailed analysis of the cyanobacterial diversity around Rothera Research Station, which will be included in Manuscript 3, another manuscript (Manuscript 4) will be prepared as a comparative approach between Arctic and Antarctic cyanobacterial mat diversity. As indicated in Manuscript 1, it can be expected that Antarctic cyanobacteria will be generally higher in diversity, compared to Arctic sites and may be dominated by different species. It will also allow identifying potential endemic- or toxin producing species.

In general it can be expected that an increasing number of future studies will include next generation sequencing as a standard method to record biodiversity. This correlates with a great drop of the costs of this method and simultaneously improving methodology. On a long term this method will thus simplify the identification of the complete diversity of a range of habitats. Barcoding organisms will help us in our understanding of many ecological processes and distribution patterns, and will assist in monitoring species transition as a consequence of climate change in the future.

5.2 Ecosystem response to cyanobacterial toxins

The cyanobacterial mats of the Arctic and the Antarctic are inhabited by a range of eukaryotic organisms including metazoans such as nematodes, rotifers and tardigrades (Cary et al., 2010). These organisms live within the mats and are very likely to feed on the cyanobacteria. In this study several cyanobacterial toxins were found within mats which also contained rotifers, tardigrades, and nematodes (data not shown). It is highly unlikely that the metazoans can avoid contact with the toxins in this confined habitat. Thus, it was hypothesized that the metazoans (i.e. rotifers and tardigrades) living in the cyanobacterial mats are to some extent resistant towards the toxins.

An experiment was designed to test the susceptibility of tardigrades and rotifers, isolated from Antarctic cyanobacterial mat material, towards MC. The organisms were isolated from thawed cyanobacterial mat material that had been collected on the Antarctic Peninsula (Rothera) and stored frozen (-20 °C) until the experiment was performed. Metazoans revitalized within a few hours after thawing of the material, showing their great adaptability towards long periods of freezing. Rotifers and tardigrades were cultured in different media (sterile tap water, ASM1 medium) and at variable temperatures (4 °C, 16 °C and RT). Cultures of rotifers and tardigrades were stable over several months at 4 °C and 16 °C but did not survive at RT. To test their sensitivity towards cyanobacterial toxins, in a preliminary experiment a defined number of tardigrades and rotifers were exposed to MC-LR within thawed mat material as their natural habitat and the survival rate was recorded after an exposure of 72 h. The results indicated that rotifers were indeed resistant towards very high concentrations of MC-LR (up to 10 ng / µl), whereby tardigrades reacted somewhat more sensitive towards the toxin (Figure 5-1).

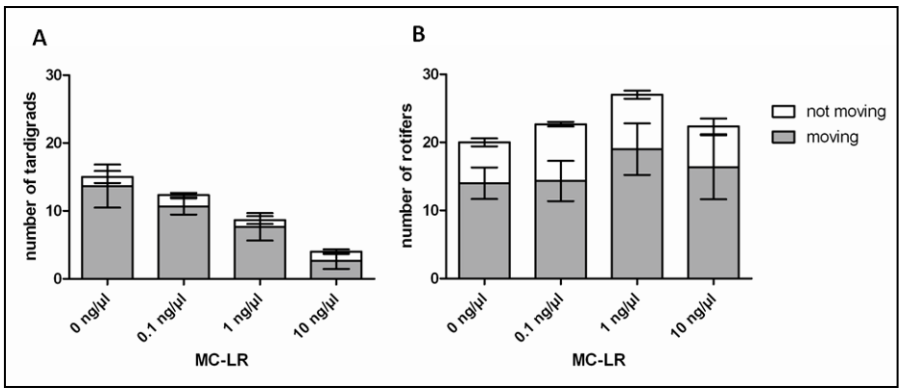


Figure 5-1: Toxic effect of MC-LR on Antarctic tardigrades and rotifers

Rotifers and tardigrades were isolated from a sample collected on Anchorage Island, Antarctic Peninsula. The experiment was performed at a stable temperature of 16 °C. A defined number of rotifers were exposed to MC-LR for 72 h and the absolute number of rotifers and tardigrades recorded. Mean and SEM. *n* = 3.

In a more detailed experiment the percentage of moving to non-moving animals as a measure of fitness and survival was evaluated for rotifers only. No significant decrease in rotifer activity was recorded in this experiment up to a MC-LR concentration of 10 ng / µl (Figure 5-2). These values lye in the range or are even one to two magnitudes higher than LD_{50} values of MC for *Daphnia* and copepods, respectively at a 24 h exposure (Reinikainen et al., 2002; Chen et al., 2005), whereas rotifers in general seem to show more resistance towards MC (Blom et al., 2006).

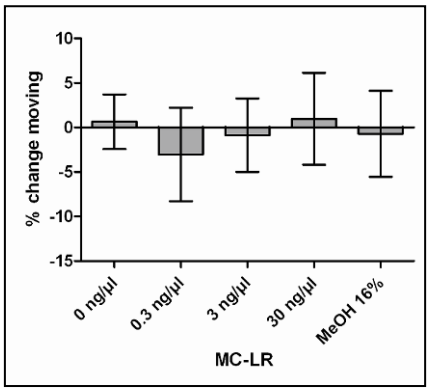


Figure 5-2: Toxic effect of MC-LR on Antarctic rotifers.

Rotifers were isolated from a sample collected on Anchorage Island, Antarctic Peninsula. The experiment was performed at a stable temperature of 4 °C. A defined number of rotifers were exposed to MC-LR for 72 h and the difference in percentage of moving to non moving rotifers recorded. Mean and SEM. *n* = 3.

Finally the quality of different food sources was analyzed on the growth of Antarctic rotifers. The algae *Chlorella vulgaris* and *Chlamydomonas reinhardtii* and the MC producing cyanobacterium *Microcystis aeruginosa* were supplied as a food source in equal cell numbers. Growth rates (in terms of the increase of the absolute number of moving rotifers) were recorded. Highest growth rates were recorded for *Chlorella vulgaris, Microcystis aeruginosa*, as well as a mix of all three food sources (Figure 5-3). Albeit, no significant results could be obtained in this experiment, there is a clear indication that the presence of the toxic cyanobacterium does not inhibit growth or activity of the rotifer.

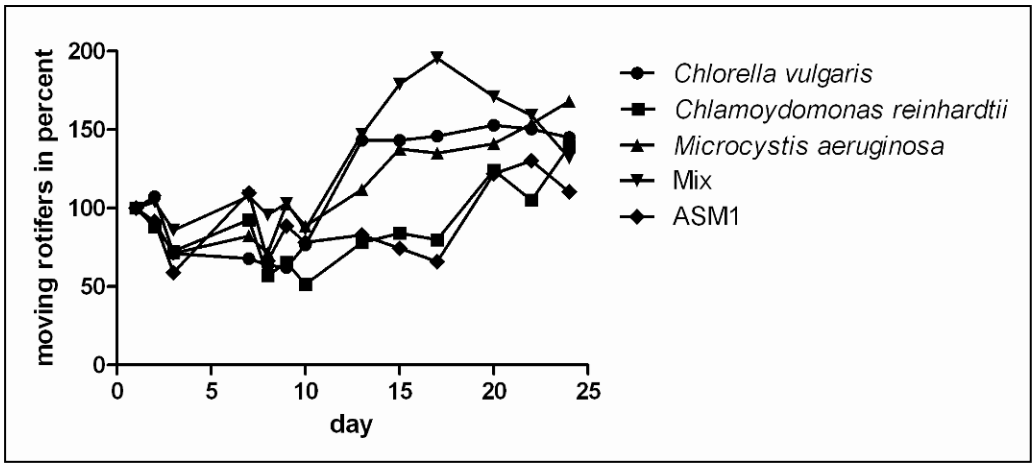


Figure 5-3: Growth experiment of Antarctic rotifer with different food sources.

A defined number of rotifers isolated from a sample collected on Anchorage Island, Antarctic Peninsula, were cultured at 16 °C on different food sources as indicated. The percentage of moving rotifers compared to day 0 is displayed over a time of 21 days. Mix is comprised of the two algal and the cyanobacterial species. ASM1 medium indicates no food source present. Mean, no SEM due to clear view of single data. *n* = 5.

It can be summarized that Antarctic metazoans seem to show reduced sensitivity towards MC-LR and potentially towards other cyanobacterial toxins. The reason for this is yet unclear. It may be hypothesized that Antarctic metazoans have evolved highly effective mechanisms to excrete the toxins. Moreover, a different structure of their protein phosphatases may protect them from the toxic molecular function of MC. Comparative analysis of the gene- and protein-sequences of protein phosphatases of various Antarctic and non-Antarctic metazoans may give an indication on the latter hypothesis and could be the topic of future investigation.

5.3 Cultivation of Cyanobacterial Mats

In this section laboratory based experiments were performed using specially developed incubation chambers for the growth of phototrophic biofilms (Figure 5-4) to characterize the effects of toxins and climate change-related variables on Antarctic cyanobacteria in a controlled environment. The incubation chambers were developed by Dr. David Schleheck, University of Konstanz, and have been successfully tested on biofilm formation of diatoms (Buhmann et al., 2012). In this study, the incubation chambers and the protocol were tested and adapted to the sterile growth of isolated strains of benthic cyanobacteria as well as Antarctic mat inoculums (data not shown). Biofilm formation was measured directly in these chambers over long incubation periods (weeks to months) by absorption of light without physically disturbing the biofilm. Visible changes in biofilm texture were recorded by photo-documentation as well as sterile analysis under the microscope. Cultivation of artificial benthic mats has been proven to be an adequate tool for studying physiological responses to environmental factors under controlled conditions (Fenchel and Kühl, 2000; Buffan-Dubau et al., 2001; Pringault et al., 2001). The chambers thus allowed the investigating of cyanobacterial biofilm formation under the influence of specific parameters in a stable and sterile environment.

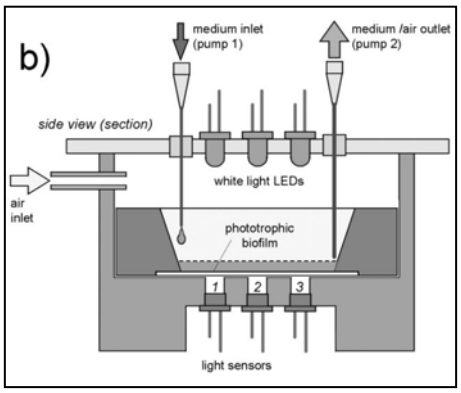


Figure 5-4: Incubation chamber for the growth of phototrophic biofilms, developed by Dr. David Schleheck of the University of Konstanz (Figure by Dr. David Schleheck).

Next to the adaptation of the chambers to the growth of cyanobacterial mats we tested the effect of toxins (i.e. MC-LR) on the formation of the biofilm. Unfortunately, during the course of the dissertation no effect could be observed. This may however be due to the experimental setup and time constraints. Long incubation periods of the biofilms only allowed a limited number of experiments. More experiments are needed based on the established protocol to analyze the effect of MC, under different environmental parameters i.e. temperature. Due to the promising preliminary results the chambers are included in the plans for follow-up experiments in the near future.

5.4 Field Experiment

Due to their simplicity, cyanobacterial mat communities are the ideal model system for studying the effects of global warming in cold biospheres. Cyanobacterial diversity and toxicity can be altered with changing environmental factors, (i.e. temperature) under laboratory conditions (Kleinteich et al. 2012, Manuscript 1). A field experiment to support these results was undertaken at Rothera Research Station on the Antarctic Peninsula in the season of 2010/2011. This area has a high toxic potential as indicated by the results of this study (Manuscript 3) and is also being affected by climate warming (Vaughan, Mulvaney, et al., 2003; Trenberth et al., 2007). To simulate and study the effects of climate change Open Top Chambers were deployed on cyanobacterial assemblages in their natural habitat (Figure 5-5). Within these chambers the temperature in and above the mats was increased by approx. 2 °C over a period of six weeks (data not shown) mimicking the warming effects of climate change proposed for this area (Trenberth et al., 2007). The cyanobacterial mat samples of this experiment were tested for any alteration in their diversity and toxicity in connection to increased temperatures. With this experiment we hoped to demonstrate that rising temperatures resulted in increasing toxin concentrations in the field.

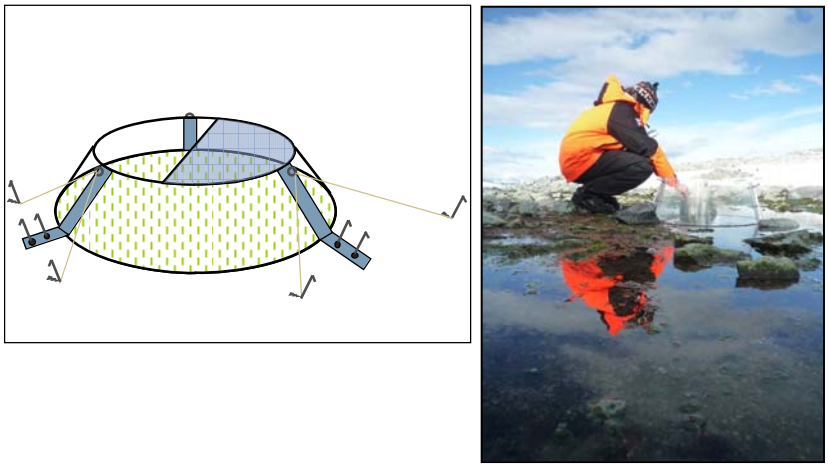


Figure 5-5: Deploying Open Top Chambers (left, approx. 60 cm in diameter) on a cyanobacterial mat on Anchorage Island (left), Antarctic Peninsula. Photograph courtesy of Frithjof Küpper.

However, toxin analysis did not reveal any change in MC concentration over the study period when compared to the control mats (data not shown). This may be explained be the relatively short incubation period (maximum of three weeks) as well as several factors which biased the experiments. The design of the chambers permitted too much water flow, so that the water and mats would not warm significantly within the chambers. In another approach, too much water was retained within the chamber, so that the water volume was elevated and the current reduced compared to the control mats. Moreover,

the disturbance by birds was significant, which moved the chambers and destroyed much of the surrounding cyanobacterial mats.

Nevertheless, such a field experiment is crucial to carry the results obtained from the laboratory based experiments of this study into the field. The design of the chambers should be changed, so that the water can flow at equal rates through the chamber. A pre-warming of the water by 2-3 °C may also help in increasing the effect on the mats. Furthermore it is strongly recommended to install the chambers for a much longer incubation period, at least for the whole growth season and ideally over a time frame of two to three years. Similar experiments have been performed on terrestrial Antarctic fellfield sites and give valuable information of the effect of warming on the ecosystem as discussed in paragraph 1.2.7. Similar experiments are needed on Antarctic freshwater systems, to evaluate and predict the effects of climate warming.

6 DISCUSSION

Toxic cyanobacteria are a global hazard to human health. Climate change may intensify this problem in the near future, by simultaneously limiting available freshwater resources and favouring toxic phytoplankton blooms. Studies are needed to understand the ecological background of toxic cyanobacteria, monitor existing ecosystem changes, and predict future developments. Three main topics were addressed in this work:

(1) **Diversity.** Few studies have dealt with the diversity of cyanobacteria from the Polar Regions. At the same time diversity is a measure of ecosystem response towards climate change. Therefore a detailed comparative approach on cyanobacterial diversity from the Arctic and the Antarctic was undertaken in this study to achieve baseline information on current polar cyanobacteria species distribution to be able to monitor future ecosystem changes.

(2) **Toxicity.** Toxic cyanobacteria are known on a worldwide scale, but have never been described in the Arctic and to a large extent in the Antarctic. Both regions remain understudied in terms of cyanobacterial toxicity. Yet toxic cyanobacteria are proposed to increase as a consequence of climate warming on a global scale. The latter may also be the case for polar ecosystems dominated by cyanobacteria.

(3) **Climate Change.** As freshwater systems of the Arctic and the Antarctic have been described as early sentinels of climate change, and are dominated by cyanobacteria, they were considered in this study as the ideal model system to study the effects of climate change on cyanobacteria dominated ecosystems.

Detailed information and discussion of the results of this study can be found in the respective manuscripts. In the following paragraphs a short summary will be given on the results of each of the core topics (1) diversity, (2) toxicity and (3) climate change, and the individual manuscripts put in a common context.

6.1 Diversity

Cyanobacterial mats originating from two locations each in the Arctic and the Antarctic were analysed for their diversity by different molecular and morphological methods. Several cyanobacterial species were identified in the mats and some were isolated into pure cultures. The diversity differed among the Arctic and the Antarctic, with the Antarctic samples being generally higher in species number and dominated by Oscillatoriales, while the Arctic was not as rich in species number and was dominated by Nostocales (Manuscript 1, 2, and 3). Besides circumpolar species occurring both in the Arctic as well as in the Antarctic, also cyanobacterial sequences that seemed to be confined to only one of the regions were detected (Manuscript 1). The latter supports the theory of endemism in the Antarctic (paragraph 1.2.3) at least to a certain extent.

In this study, the primarily method of identification was molecular, using the 16 rRNA gene and adjacent intergenic spacer region. Less focus was laid upon morphological description, which is extremely time intensive and requires high taxonomic expertise usually acquired only after several years of experience. As discussed in paragraph 1.2.3, only a combination of both, molecular and morphological description is required to describe the full biodiversity of Arctic / Antarctic cyanobacteria. This was however not the focus of this study and would have exceeded the time frame of the thesis. Modern techniques, such as the 454 sequencing technology, may however simplify the evaluation of biodiversity at least on molecular level as discussed in paragraph 5.1. No such experiment has been undertaken for cyanobacteria of the Arctic and the Antarctic. In the frame of this dissertation such an experiment using the 454 technology to explore the 16S rRNA gene diversity of cyanobacterial samples from the Arctic (Baffin Island and Spitzbergen) as well as the Antarctic (Byers Peninsula and Rothera) was performed (paragraph 5.1). Unfortunately, until the point of submitting this manuscript, the analysis of the data was not available. It will however be included in publications in the near future, which may also be considered as an outcome of this work. The results will compare the cyanobacterial diversity of Arctic and Antarctic cyanobacteria to a yet unknown extent.

6.2 Toxicity

Until this study no data on the toxicity of cyanobacteria in the Arctic and very few for the Antarctic were available. This study demonstrated that the potential for toxicity is high in cyanobacterial mats of the cryosphere. Several cyanobacterial toxins could be detected in both Polar Regions, most of which were described for the first time in this environment. The genetic basis for toxin production was described in several samples and was very different from those known of the temperate and tropical regions.

6.2.1 Microcystin

The cyanobacterial toxin MC has a circumpolar distribution and has been detected in fresh- and brackish waters worldwide with the exception of the high Arctic (Chorus and Bartram, 1999). In this study we report for the first time the presence of MC in this environment i.e. in a freshwater cyanobacterial mat on Northern Baffin Island of the Canadian Arctic (Manuscript 1). In the Antarctic, MC has been reported in three studies from meltwater ponds of the continental Antarctic (Hitzfeld, 2000; Jungblut, 2006; Wood, 2008) but is now identified for the first time in several freshwater locations of the Antarctic Peninsula (Manuscript 1, 2, and 3), suggesting a wide distribution of this group of toxins in the southern continent. Microcystin is more commonly known from the temperate and tropical regions associated with cyanobacterial bloom events (de Figueiredo et al., 2004). The widespread distribution of these toxins in the cryosphere is therefore surprising.

The MCs were detected on two levels. Firstly the toxins were detected, via specific antibodies in an ELISA assay as well as via LC-MS, and secondly on genetic level, i.e. the gene operon *mcyS* responsible for MC synthesis. The *mcy* genes were detected in a range of samples originating from the Arctic as well as the Antarctic (Manuscript 2 and 3) confirming a widespread presence of cyanobacterial toxin producers in these habitats. The identified sequences however, showed low phylogenetic similarity to *mcy* sequences originating from the temperate regions and available in public databases (Manuscript 2 and 3). The high spatial separation of the polar cyanobacterial communities and therefore a low genetic exchange with toxic species from the temperate and tropical regions may be a reason for this low level of genetic relationship and indicates a high mutation rate in these genes. In a recent study, Kaasalainen et al. (2012) described lichen originating from Svalbard to contain unusual MC variants and identified the corresponding *mcyE* genes. They hypothesized lichen to be a source of worldwide MC and *mcy* variability, due to very efficient dispersal rates of lichen and a resulting genetic separation via the bottle-neck effect (Kaasalainen et al., 2012). As lichen are very abundant in the Polar Regions, they could therefore serve as a source for *mcy* diversity in the Arctic and possibly in the Antarctic. The bottle neck effect

could here be especially prominent due to high spatial separation and low species competition. The level of genetic exchange between the Polar Regions (i.e. the Antarctic) and the temperate and tropical regions is not yet clear, but may be resolved by future sequencing projects as discussed in paragraph 6.1. Albeit the data in this study indicate a distant phylogeny between polar and temperate MC producers, no specific conclusion can be drawn on the exact phylogeny. In the Antarctic endemism among cyanobacterial species is supposed to be present at least to a certain extent, whereas in the Arctic genetic exchange with the surrounding continents seems more likely. Therefore cyanobacterial toxin producers from the Arctic can be suspected to be more closely related to those in the temperate regions compared to those in the Antarctic.

The genes for MC synthesis have been postulated to be very ancient and have a common ancestor in most cyanobacteria (Rantala et al., 2004). In that study though the *mcy* sequences of only 4 different species known from the temperate regions at that time are included. Therefore a study, analysing the to-date much more numerous *mcy* gene sequences available in public databases, including sequences from the Arctic and the Antarctic as well as other extreme habitats worldwide, is yet missing. The sequences obtained in this work may help to conduct such a study, which would help to describe distribution patterns of toxic cyanobacteria and to clarify the evolution and age of the *mcy* genes.

The amplification and sequencing of the *mcy* genes proved to be difficult for most of the samples and several sequences amplified had to be discarded due to poor quality. Other molecular-biological reactions performed on the DNA extracts, including amplification of the *sxt* and *cyr* genes, were however successful, so that poor quality DNA can be excluded as a limiting factor. It may be suspected that low sequence similarities between known and polar MC producers may lead to insufficient binding of primers that have been developed on known (and therefore originating from temperate and tropical regions) sequences. More studies will be needed to complete sequence information of *mcy* genes of Arctic and Antarctic origin.

The high variety of MC with more than 110 congeners known (Puddick, 2012) is a result of low substrate specificity of involved enzymes but also of variable genetic information of the *mcy* gene (Dittmann and Wiegand, 2006). Low genetic relationships of polar *mcy* sequences to those known from lower latitudes could therefore indicate the presence of unusual MC variants. In this study, the unusual MC variant [Asp^{3}, $ADMAdda^{5}$, Dhb^{7}] MC-RR was detected an Arctic cyanobacterial sample. This variant has been reported before from the genus of *Nostoc* (Beattie et al., 1998). The *Nostoc* genus has a circumpolar distribution and is generally known to produce various MC variants (Sivonen et al., 1992). Correspondingly, the single identified MC producer origination from the Antarctic is assumed to be *Nostoc* sp., most likely synthesising an ADMAdda substituted MC variant

(Wood, et al., 2008). MC variants containing ADMAdda are commonly found in lichen associated cyanobacteria i.e. *Nostoc* and a very similar variant [Asp^3, $ADMAdda^5$] MC-RR to the one identified in this study was found in cyanolichen from Scotland produced by symbiotic *Nostoc* (Kaasalainen et al., 2012). The data indicate that *Nostoc* may be a common producer of MC in the cryosphere.

Many other cyanobacteria are known to produce MCs including *Microcystis*, *Synechococcus* and *Snowella* (e.g. O'Neil et al. 2012). *Snowella* sp. was identified by molecular as well as by morphological methods in an Arctic sample of this study, whereas some of the *mcy* genes identified were most similar to *Microcystis* sequences (Manuscript 1). Therefore more than one species (i.e. *Nostoc*) in the cyanobacterial mats may be a potential MC producer. This has already been indicated by Wood et al. (2008) who identified MCs in samples in which *Nostoc* was absent. An exact determination of the MC producers in Arctic and Antarctic cyanobacterial mats can not be made based on the results of this study. However, detailed phylogenetic studies of Arctic and Antarctic *mcy* genes could not only help in identifying MC producing species but provide data to investigate the dispersal and evolutionary history of cyanobacterial toxicity and therefore the potential biological function of this toxin.

No data are available on the toxicity of ADMAdda-substituted MC variants as well as the particular variant [Asp^3, $ADMAdda^5$, Dhb^7] MC-RR detected in the Arctic in this study. Different MC congeners have however been reported to vary in their toxicity as well as in their potential to inhibit serin / threonine specific protein phosphatases (Chen et al., 2006; Fischer et al., 2010; Feurstein et al., 2011). For the inhibitory potential of MCs the Adda side chain is essential. This amino acid attaches to the active centre of serin / threonine specific protein phosphatases and thereby inhibits their activity (Craig et al., 1996; Taylor et al., 1996). Modifications of this amino acid thus lead to decreased toxicity (Taylor et al., 1996; Gulledge et al., 2003; Chen et al., 2006). Based on the latter findings, it is likely that the detected ADMAdda-containing MCs are reduced in their toxicity compared to non-modified variants such as MC-LR or MC-RR. The variant detected in this study was identified to have an exchange of the Mdha amino acid for Dhb (Manuscript 2). The Mdha amino acid is responsible for the covalent binding of MC to a cysteine-residue of the protein phosphatases (Cys^{273} of PP1) (MacKintosh et al., 1995; Runnegar et al., 1995). An exchange of the Mdha to Dhb is therefore likely to result in a non-covalent and therefore reversible binding of MCs with protein phosphatases and thus reduced toxicity (Chen et al., 2006).

The effects of the toxin in the cyanobacterial mat habitat are yet unclear. Preliminary results suggest that eukaryotic organisms (i.e. rotifers and tardigrades) living in the mats are rather tolerant towards MC-LR (see paragraph 5.2) compared to *Daphnia* and other metazoans from temperate regions. Rotifers were significantly more resistant towards the toxin than tardigrades, suggesting different levels of susceptibility. The latter

results indicate that the organisms living and feeding on the cyanobacterial mat have evolved mechanism of resistance against cyanobacterial toxins. Among other possibilities this could be due to insufficient uptake of the toxin into cell, increased excretion, or reduced sensitivity of protein phosphatases. As most toxicity studies of MCs are based on mammalians (i.e. mice) and only the more common congeners (e.g. MC-LR, MC-RR, MC-LF) have been evaluated. Other MC congeners could have different effects on the here described organisms. More studies are required to elucidate the toxicity of standard and unusual MC variants on the organisms living in cyanobacterial mats. Potential mechanisms of resistance in the eukaryotes may be an interesting topic in the light of intoxication treatment or medical applications.

6.2.2 Saxitoxin and Cylindrospermopsin

Saxitoxins and CYN were detected for the first time in the Arctic and the Antarctic, respectively. Both toxins are more commonly distributed in tropical and temperate marine and freshwater habitats (Kinnear, 2010; Wiese et al., 2010) and were recorded for the first time in a cold environment.

While in the marine environment STXs are generally produced by dinoflagellates, an increasing number of freshwater cyanobacteria are known to produce the toxin (Onodera et al., 1998; Wiese et al., 2010; Smith et al., 2011). This includes pelagic as well as benthic species (Onodera et al., 1998; Lagos et al., 1999; Velzeboer et al., 2000; Smith et al., 2011; Wörmer et al., 2011). Type and concentration of STXs produced varies in different species (Wiese et al., 2010). Neither the exact STX variant nor the producer of the STX could be elucidated in this study. Based on the genetic information of the *stxA* gene however, a low phylogenetic relationship with known STX producers form warmer climates can be anticipated. In this study, STX was detected in low concentrations in only one Arctic cyanobacterial mat, none of the other Arctic nor the Antarctic samples were tested positive for STX (Manuscript 1, 2, and 3). Therefore it can be suspected that STXs or STX producers are not very abundant in the cryosphere. The biological reason for the cyanobacteria to produce this compound in the Arctic environment is unclear. The effects of the toxin on the Arctic mat invertebrates, and the higher food chain, are unknown. Whereas bivalves seemed resistant towards its toxic effects (Pearson et al., 2010), *Daphnia* is inhibited by STX producing cyanobacterial strains (Ferrão-Filho et al., 2010). Due to the low concentrations of the toxin in the Arctic mat and the limited distribution acute toxic effects are unlikely in this habitat, whereas chronic low dose effects cannot be excluded.

While STX had a rather limited distribution in this study, CYN was widespread in the Antarctic cyanobacterial mats tested. The detection and confirmation of the toxin in 11 out of 30 cyanobacterial mat samples from the Antarctic Peninsula, suggests a widespread distribution of the toxin in that area (Manuscript 3). In various cyanobacterial mats of other

Antarctic locations however, the toxin could not be detected (Prof. Antonio Quesada, personal communication). As this is the first report of the toxin in any polar environment and Arctic samples were not tested for CYN, no assumption can be made upon its distribution in the northern cryosphere. Cylindrospermopsin is more commonly known from tropical and sub-tropical regions but is increasingly recorded also in the temperate regions. This has been suspected to be a consequence of the invasion of the cyanobacterial species *C. raciborskii* to temperate latitudes as a consequence of climate warming (Kinnear, 2010; O'Neil et al., 2012). Simultaneously, increased scientific and public attention as well as improved analytical methods may contribute to and therefore bias the number of CYN records. Nevertheless, the widespread presence of the toxin in a cold environment is surprising. The energy investment for the production of second metabolites is high, thus there has to be a definite biological benefit for the cyanobacterial cell. The biological function of CYN is yet unclear but has been discussed as an inducer of alkaline phosphatase excretion in other algal cells, in a low phosphate environment (discussed in paragraph 1.1.8). In general, the intestinal water of benthic communities in Antarctic freshwater habitats is thought to be high in nutrients (Quesada et al., 2008), but may experience nutrient limitation at the end of the growth season (Hawes, Safi, Webster-Brown, et al., 2011). Therefore the presence of CYN, especially in low concentrations, may assist the growth of cyanobacteria in these mats. The phylogenetic relationship of the CYN producer in this study, based on *cyr* genes sequences, was very distant to CYN producers known from the temperate and tropical regions (Manuscript 3). As for the producer of MC and STX, this high genetic divergence may be a result of the spatial separation of Antarctic species. More data on the *cyr* sequences are needed to elucidate exact phylogenies and the origin and arrangement of *cyr* gene clusters in these species.

In summary several cyanobacterial toxins (MC, CYN, STX) were detected in a large number of cyanobacterial mats from the Arctic as well as the Antarctic. Albeit the concentrations of the toxins were low when compared to toxic cyanobacterial blooms from the temperate regions, the wide distribution suggests that cyanobacterial toxins are a common feature in Arctic and Antarctic freshwater systems. The low similarity between the *mcy, sxt,* and *cyr* sequence detected and the known sequences from lower latitudes suggests a low phylogenetic relationship possibly due to high spatial separation. Unfortunately, neither the producer of CYN, STX, nor MC could be identified in this work. Identifying the species and sequencing its genome will, however, provide valuable information on the distribution and phylogenies of toxic cyanobacteria and may be the topic of future investigation. Preliminary experiments suggest low sensitivity of present eukaryotic organisms towards MC-LR, however further studies are required to confirm these results.

6.3 Climate Change

In this study the question was addressed how the small but highly specialised ecosystems of Arctic and Antarctic cyanobacterial mats react when subjected to the increasing pressures resulting of climate change.

A shift of average temperatures as a consequence of global climate change is likely to alter the cyanobacterial community composition within the microbial mats. An extreme range of temperatures may be experienced in the daily cycle of many moist terrestrial Antarctic habitats from below freezing to up to 20 °C (Manuscript 3; Quesada and Vincent 2012). As many Antarctic cyanobacterial species are considered rather cryotolerant than cryophile, with temperature optima of around 20 °C (Tang et al., 1997), extremely cold temperatures (below 4 °C) and especially regular occurring freeze-thaw cycles may be a strong selection pressure in the present ecosystem. The latter may be diminished as a consequence of climate warming in the near future, favouring warm adapted species over cryophile species.

Many studies evaluating the response of cyanobacteria to variable temperatures, are based on isolated strains and do not consider all variables given in the natural environment. It was for example hypothesized that low temperatures are beneficial for cyanobacterial biomass accumulation as bacterial decomposition is inhibited (Vincent and Howard-Williams, 1986). Similarly Upton et al. (1990) showed that fluctuating temperatures (between 0 °C and 16 °C) sustained a more diverse community in cultured Antarctic microbes than constant average temperatures (Upton et al., 1990). It may therefore be suspected that the high fluctuation in temperature of many High Arctic and Antarctic habitats are increasing the microbial diversity rather than constraining it. The high temperature range may support the growth of a wide range of organisms with different temperature adaptations. This is supported by the data presented in this study (Manuscript 1). Here, cultures of Arctic and Antarctic cyanobacterial mat inocula evolved different community compositions at individual temperatures, with the highest diversity at intermediate temperatures (8-16 °C). In contrary, diversity was clearly decreased at very high temperatures (23 °C) implicating the presence of few warm adapted species. In this experiment temperature seemed to be are more relevant factor for community composition selection than the initial species inoculum, suggesting that species with different temperature adaptations are present within one mat system.

In Manuscript 1 it was furthermore demonstrated that the concentration of MCs was increased at elevated temperatures between 8 °C and 16 °C, concomitant with the change in the community composition. This increase in toxicity may be explained by two possible scenarios:

(**A**) Toxin production may favour the growth of a certain cyanobacterial species, resulting in its increased reproduction and dominance, a change in the community composition, and therefore increased toxin concentrations.

(**B**) Generally higher metabolic rates, as a response to increased temperatures, may induce a higher rate of toxin production in the species already present.

In this study significant species shifts at 8-16 °C were recorded that would support scenario (A) however none of these species could be connected to toxin production. In the cryosphere, many physiological processes happen just above a minimum threshold level (Convey et al., 2003), thereby potentially limiting the highly energetic production of secondary metabolites. This could explain the low to non-detectable concentrations of toxins in both Arctic and Antarctic cyanobacterial mats in the field (Manuscript 1, 2, and 3). Kaebernick and Neilan (2001) postulate that MC synthesis is increased under optimal temperature and growth conditions, which are speculated to be around 15 - 20 °C for polar cyanobacteria (Tang et al., 1997; Velázquez et al., 2011), supporting the latter theory that toxin production is in general elevated at 8 - 16 °C (Kleinteich et al., 2012). A warmer climate as predicted for the Arctic as well as the Antarctic Peninsula in current climate change models (Trenberth et al., 2007) could therefore elevate the temperature above the minimum threshold level for toxin synthesis, leading to an increased general metabolic activity (Convey, 2006; Velázquez et al., 2011) and thus an increased level of toxin production as presented in this study (Kleinteich et al. 2012). With increasing temperatures the diversity and complexity of the ecosystem is also expected to increase (Yergeau, Newsham, et al., 2007). According to Convey et al. (2006), in a more complex ecosystem the importance of competitive behaviour and abilities is increased. Higher temperatures could thus selectively favour the fitness and abundance of toxin producing cyanobacterial species due to their higher resistance against grazing pressure. The system used in this study though has several limitations. Certainly a laboratory based experiment cannot be transferred directly to natural conditions. Many more variable may influence the system in the field. As such, the increase of toxicity in Arctic and Antarctic habitats on a long term is uncertain. Increasing temperatures and human activity will most likely result in a shift of vegetation zones with mosses and grass originating invading cyanobacterial dominated habitats and most likely outcompeting them.

A long term field based experiment is therefore crucially needed to this hypothesis. In the frame of this dissertation, such an experiment was performed at Rothera Research Station on the Antarctic Peninsula in December and January 2010 / 2011 as described in paragraph 5.4. Open top chamber were used to increase the ambient temperature of cyanobacterial mats by 2-3 °C over a time period of several weeks. The experiment however, did not deliver any conclusive outcomes due to several reasons (short incubation times, design of chambers, as discussed in paragraph 5.4). Therefore a long term field

experiment, artificially increasing the temperature of cyanobacterial mats over at least a whole growth season and ideally for 2-3 years, is still required to clearly link temperature increase with changing cyanobacterial diversity and increased toxicity.

In addition to the scenarios discussed above, a third scenario in relation to climate change should be considered.

(**C**) As toxin production is generally a feature of cyanobacterial species from warmer regions, a shift in temperature may favour the invasion and establishment of toxin-producers to higher latitudes.

The continued dispersal of toxic cyanobacterial species is a worldwide phenomenon and is thought to be concomitant with more suitable growth conditions due to climate change (Wiedner et al., 2007; O'Neil et al., 2012). Toxic strains of cyanobacteria seem to have a growth advantage over non-toxic strains under elevated temperatures and higher light intensities (Dziallas and Grossart, 2011; Zilliges et al., 2011). Increased human activity has furthermore lead to the introduction of a diverse range of invasive species to the Antarctic continent (Convey et al., 2006; Frenot et al., 2007), and is thought to be a main factor in the global spread-out of toxic cyanobacterial species (Neilan et al., 2012). In the Polar Regions invasive cyanobacteria have not yet been reported, mainly due to the lack of reliable base-line information. The detection of cyanobacterial toxins in the southern and northern cryosphere (Manuscript 1, 2, and 3) as well as their increasing toxic potential due to climate change in these regions (Kleinteich et al., 2012) require a better understanding of the distribution of toxic cyanobacteria worldwide. Large scale diversity analyses based on modern high throughput sequencing methods are required to document the total biodiversity of Arctic and Antarctic as well as other freshwater habitats worldwide. This will help to predict future trends in community composition and to track the potential spread-out of toxic cyanobacterial species. The increased presence of toxins might have a profound effect on other species living in cyanobacterial mats such as nematodes or rotifers and could play a key role in shaping food webs and microbial community structure.

6.4 Outlook

This study demonstrated for the first time toxic second metabolites (i.e. MC) produced by cyanobacteria are a common phenomenon not only in the temperate and tropical regions but as well in many Arctic and Antarctic freshwater habitats. It was revealed that the toxins STX and CYN are not confined to warm latitudes but do also occur in the cold regions of the Arctic and the Antarctic. Albeit more studies are needed, the finding on the distribution pattern of toxic cyanobacteria and especially their genetic background, that was partially resolved, is valuable information for the understanding of the evolutionary history of cyanobacterial toxicity and thus the biological function of the toxins. Concurrently, the finding of Antarctic metazoans being resistant towards cyanobacterial toxicity is relevant for general ecosystem functioning and, if the mechanism of resistance can be resolved, it may represent important new knowledge when applied for medical and human health purposes.

On a global scale toxic cyanobacterial blooms are expected to increase as a consequence of climate change. Simultaneously, an intensified exploration of available freshwater resources will intensify the potential health hazards for humans caused by toxic cyanobacteria. Understanding the processes controlling cyanobacterial toxin production is therefore crucial. In this study polar cyanobacteria have been proven to be a useful model to study the effects of climate change in a cyanobacterial dominated ecosystem. It was demonstrated that increasing temperatures result in elevated toxin concentrations in cultured cyanobacteria. Despite the limitations of the experimental setup of this study, the latter findings can be transferred to other cyanobacteria dominated environments in lower latitudes which are more relevant to human affairs. Several studies support the idea of increased cyanobacterial toxicity with increasing temperatures in other climatic zones. This study therefore serves as additional hint in a chain of evidence which will help us to predict future developments of toxic cyanobacterial occurrence.

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IX. Eigenabgrenzung

Die vorliegende Arbeit wurde ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Weitere Personen, insbesondere Promotionsbetreuer, waren an der inhaltlich materiellen Erstellung dieser Arbeit nicht beteiligt. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt. Die Beteiligung von mir selbst und den Co-Autoren an den einzelnen Manuskripten verhält sich wie folgt:

Manuskript 1: J.K. plante und führte alle präsentierten Experimente aus. Die Daten, sowie Abbildungen und das Manuskript wurden von JK erarbeitet. S.A.W. assistierte in den experimentellen Abläufen und der Analyse der ARISA Daten. F.C.K. organisierte und sammelte Proben auf der Arktis Expedition. Die Antarktis-Expedition wurde von A.Q. und A.C. organisiert, die auch für das Sammeln der Proben zuständig waren. T.F. führte die bioinformative Analyse der Sequenzdaten durch. D.R.D. war der Projektkoordinator und Betreuer der Experimente, plante und führte einige der Experimente durch und bearbeitete das Manuskript. Alle Autoren beteiligten sich zu gleichem Anteil an der kritischen Überarbeitung des Manuskripts und der Diskussion der Ergebnisse.

Manuskript 2: J.K. plante und führte alle präsentierten Experimente aus. Die Daten, sowie Abbildungen und das Manuskript wurden von J.K. erarbeitet. S.A.W. assistierte in den experimentellen Abläufen, im speziellen der HPLC und LC-MS Analyse der Toxine und beteiligte sich an der Planung der Experimente. F.C.K. organisierte und sammelte Proben auf der Arktis Expedition, J.P. ermittelte die genaue Variante des detektierten MC. D.S. beteiligte sich an der Planung der Experimente, im Speziellen der Klon-Bibliothek. D.R.D. war der Projektkoordinator und Betreuer der Experimente und bearbeitete das Manuskript. Alle Autoren beteiligten sich zu gleichem Anteil an der kritischen Überarbeitung des Manuskripts und der Diskussion der Ergebnisse.

Manuskript 3: J.K. plante und führte alle präsentierten Experimente aus. Die Daten, sowie Abbildungen und das Manuskript wurden von J.K. erarbeitet. S.A.W. assistierte in den experimentellen Abläufen, im speziellen der Isolation der Cyanobakterien, der Analyse des detektierten CYN und beteiligte sich an der Planung der Experimente. S.C. beteiligte sich an der Planung und Durchführung der molekularbiologischen Analyse. A.Q. beteiligte sich an der Planung der Experimente und fungiert als Betreuer der Arbeit. D.P. und P.C. sind die Organisatoren der Antarktis-Expedition auf Seiten von BAS und beteiligten sich an der Planung der Expedition. F.C.K. war an der Organisation und dem Sammeln der Proben der

Antarktis Expedition beteiligt. D.R.D. war der Projektkoordinator und Betreuer und bearbeitete das Manuskript. Alle Autoren beteiligten sich zu gleichem Anteil an der kritischen Überarbeitung des Manuskripts und der Diskussion der Ergebnisse.

Einige der Experimente wurden unter meiner Betreuung und Anleitung von den Bachelor- und MasterstudentInnen Heinke Bastek, Martina Sattler, Katrin Leinweber, Jan Fischer, Lisa Zimmermann und Julia Stifel durchgeführt. Dies trifft insbesondere auf die Ergebnisse aus Paragraph 5.2 und 5.3 zu, deren Inhalt Teil der Bachelorarbeiten von Nele Hunsche und Heinke Bastek war.

Konstanz, Dezember 2012

Julia Kleinteich