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Cyanobacteria and microalgae associated with mosses in wet meadows (High Arctic)

Bachelor's thesis

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Annotation

Associations of nitrogen fixing cyanobacteria with mosses in wet meadow habitats of central Svalbard are of significant ecological importance as they represent a dominant nitrogen source. The first aim of this thesis was to find a suitable method to mechanically dislodge cyanobacteria from the moss stems, which would allow subsequent cyanobacterial identification and quantification. The second aim was to perform pilot Sanger sequencing which would allow to obtain another assessment of the cyanobacterial community for future studies of the wet meadow.

Affirmation

I hereby declare that I have worked on my bachelor thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Anna Polášková

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Abstract

Polar regions are characterised by extreme environmental conditions such as short summer season, cold temperatures or low levels of available mineral nutrients. These conditions in consequence limit development of life. Nevertheless, mosses and cyanobacteria seem to respond well to these conditions and are one of the main primary producers in the High Arctic. Wet meadow habitats dominated by mosses and cyanobacteria thus exhibit one of the highest biological productivity in the Arctic and act as a carbon sink. Moreover, mosses serve as hosts for heterocystous cyanobacteria which can fix atmospheric nitrogen. These associations thus represent an important nitrogen input into the wet meadow habitats. Despite their ecological importance, only little is known about details and mechanisms of these associations. The aim of this thesis was to find a suitable method which would allow to mechanically dislodge cyanobacteria from the mosses and which would allow their further quantification. Therefore, three different methods were tested. Air-dried moss samples were: A) frozen in liquid nitrogen, grinded to homogenous powder and diluted in water; B) sonicated in water, and C) vortexed in water. These procedures were followed by cyanobacterial biovolume estimation using light and epifluorescence microscopy. Freezing of the moss sample with liquid nitrogen was revealed to be too destructive and not suitable for the cyanobacterial quantification. Moreover, the results suggest that sonication and vortexing don't dislodge all cyanobacteria from the mosses. Nitrogen-fixing cyanobacteria of the Nostocales order were found to be predominant (from 82 to 95 % relative abundance). Furthermore, another assessment of the moss-associated cyanobacteria was performed through Sanger pilot sequencing and the 16S rRNA gene was amplified with different specific cyanobacterial primers. The primer specificity was improved by pre-treatment of the moss sample by either sonication or vortexing and 26 cyanobacterial sequences were obtained.

Abbreviations

ADP	adenosine diphosphate
ANOVA	analysis of variance
ARA	acetylene reduction assay
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
bp	base pair
CTAB	cetyltrimethylammonium bromide
ddNTP	dideoxynucleotide triphosphate
DNA	deoxyribonucleic acid
GTR model	general; time; reversible model
HIF	hormogonia inducing factor
HPLC	high-performance liquid chromatography
HSD	honest significant difference
IPTG	isopropyl β-D-1-thiogalactopyranoside
MAFFT	multiple alignment using Fast Fourier transform
NNI	nearest neighbour interchange
NPP	net primary production
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PhyML	phylogeny; maximum likelihood
P_i	inorganic phosphate
qPCR	quantitative PCR
RNA	ribonucleic acid
Rpm	revolutions per minute
rRNA	ribosomal RNA
RuBisCO	ribulose-1,5-bisphosphate carboxylase oxygenase
SD	standard deviation
SOC	super optimal broth
TAE buffer	tris base; acetic acid; EDTA buffer
TBE buffer	tris base; borate; EDTA buffer
T-REX	tree and reticulogram reconstruction
UV-B	ultraviolet B
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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1 Introduction

1.1 Polar regions

In the last decades, polar regions attracted attention of many researchers from diverse study fields. This can be explained by the increasing concern about the contemporary global climate change, which have the biggest impact on the high-latitude environments. Within the last century and especially within the last three decades, the polar regions have undergone the most pronounced changes of all ecosystems in the world. Mainly in temperature changes, precipitation, and an increase of UV-B radiation caused by the ozone depletion. They are therefore considered as a possible indicator of the speed and intensity of global climate changes. And although they are remote, they are still an important part of the global element and energy cycles with several induced feedback processes. In fact, the polar processes influenced by climate change can further significantly alter the global climate (ACIA, 2005; Chapin III et al., 1992; Robinson et al., 2003; Stocker et al., 2013; Thomas et al., 2008). Studying of polar ecosystems is therefore of great importance and can offer valuable information.

Many possibilities exist to define polar regions (see Figure 1). The simplest would be the geographical definition based on the Arctic and Antarctic Circles at 66°32' north and south, which represent the boarder of midnight sun. Such a definition is rather simplistic since it doesn't reflect any ecological characteristics. Another possibility would be to define polar regions as areas above the 10°C isotherm (mean temperature under 10°C during the warmest month), which in some places corresponds to the tree line boundary. In general, the Arctic and Antarctic regions differ from each other in several aspects such as geography, biodiversity as well as the energy transport and balance (AMAP, 1998; Elster & Benson, 2004; Pichrtová, 2014; Thomas et al., 2008).

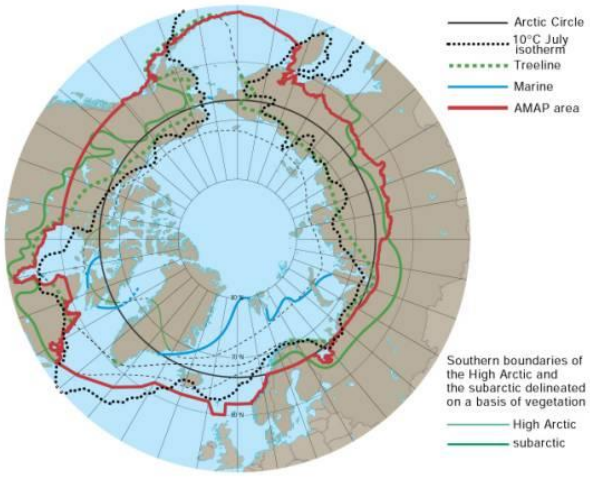

Figure 1: The Arctic boundaries from (AMAP, 1998)

The climate of polar regions is influenced by the low solar angles in respect to the Earth (Callaghan et al., 2005). Energy coming from the sun is spread over a wider region and the

light has to penetrate through a greater depth of atmosphere. Therefore, less energy, compared to the equator, reaches the high latitude regions. Concerning the general atmospheric circulation, these regions receive cold descending air masses. Because of the descending cold air, only little precipitation is released, making out of high-latitude regions polar deserts or semi-deserts. Nevertheless, due to ocean currents and atmospheric circulation, the western parts of Svalbard and the Antarctic peninsula can be characterised as Maritime Arctic with higher precipitation (Moore, 2006).

One of the main characteristics necessarily influencing living organisms of both regions, the Arctic and Antarctic, is a short vegetative season. During that time, otherwise frozen water is found in liquid state. In contrast to winter months, which are characterized by absence of light, continuous intense light ("midnight sun") dominates together with high levels of UV-radiation. Summer air temperatures, although low, are above zero, whereas winter air temperatures are often constantly below zero. Nevertheless, both polar regions are variable and influenced by seasonal and diurnal environmental fluctuations in temperature, water or mineral nutrients availability and light intensity (Elster et al., 2002; Tashyreva & Elster, 2012; Thomas et al., 2008; Vincent, 2000). The degree of stress therefore differs among distinct habitats. Hence, polar regions can be conventionally classified according to the water amount and its availability, which affects survival strategies of various organisms. According to this classification, three different categories were defined: aquatic (water in liquid state is always available), hydro-terrestrial (liquid water is present only in summer months) and terrestrial (availability of water in liquid state is impermanent and changes with diurnal oscillations) (Elster et al., 2002).

Another important characteristic of polar regions is the occurrence of permafrost. It is defined as soil or organic matter which has been subjected to temperature below 0°C for at least two consecutive years. Permafrost plays an important role in soil development. During summer months, the upper layer thaws and forms a so called active layer. The depth of this layer varies according to temperature, plant cover, material of the ground and its moisture (AMAP, 1998; Elster & Benson, 2004; Thomas et al., 2008). The permafrost is predominantly continuous in polar deserts and tundra soils in contrast to the forest tundra, where it becomes discontinuous and the depth of active layer deeper (Callaghan et al., 2005).

1.2 Wetlands

Although polar regions are “deserts” in terms of precipitation, at some locations in the High Arctic, small wetlands up to 10 m^2 can be found. Rarely also extensive wetlands of areas greater than 1 km^2 occur (Woo & Young, 2006). They are supplied with water from meltwater in spring. Since evaporation of water is slow due to the cold temperatures, the soils are saturated, and water tends to accumulate. Due to permafrost serving as an impermeable barrier, meltwater is not drained away (Moore, 2006; Pielou, 1994; Woo & Young, 2006; Wrona et al., 2005).

During the arctic winters, the wetlands remain frozen. The vegetation growth is connected to ground thaw which is limited to approximately 3 months in a year. Snowmelt starts from mid-May to beginning of July. During this time, large quantities of water are released (Woo & Young, 2006). Locally, wet meadows represent the most biologically productive ecosystem. Moreover, the wetlands represent an important storage of carbon and act as a carbon sink. The photosynthesis processes, the plant’s growth, as well as the decomposition, are slowed down by the cold temperatures. Due to slow decay of dead plant matter, peat is accumulated. Large quantities of carbon absorbed by plants through photosynthesis are therefore locked within it (Pielou, 1994). Furthermore, due to the slow decomposition, also mineral nutrients are locked within the peat. The amount of mineral nutrients available in the soil is therefore significantly reduced. Shortage in nitrogen supply represent another reason for the slow plant’s growth (Edwards et al., 2006; Pielou, 1994). More generally, it is estimated that wetlands in boreal and Arctic regions can store carbon 10 to 20 times faster than terrestrial systems. About 40 % of global soil carbon is estimated to be stored in wetlands, even though they represent only 3 % of the total land area (Beilman et al., 2009; van der Valk, 2006).

1.3 Mosses

Bryophytes, together with lichens, are the most abundant plants in high latitude ecosystems and play an important role in the local ecosystem functioning. In fact, with increasing latitude, also the relative bryophyte and lichen abundancy increases, while the vascular plant species richness decreases. It is estimated, that they represent more than half of all autotrophic species in high latitudes. This can be explained by several key traits characteristic for bryophytes. They tolerate desiccation, wide range of temperatures and can suspend their metabolism if needed. Unlike the vascular homoiohydric plants which avoid

desiccation by active water content regulation through morphological features such as stomata, roots or cuticles, bryophytes exhibit poikilohydry and lack such morphological adaptations. They simply take up water as well as nutrients over the whole plant surface like a sponge. Since they have no roots, they have also no troubles colonizing hard rocky substrates (Jägerbrand et al., 2006; Proctor, 2000; Street et al., 2012; Turetsky, 2003; Turetsky et al., 2012). Mosses are also better adapted to low light in comparison to vascular plants (Glime, 2017).

In the last years, mosses have become more and more recognized for their significant role in carbon sequestration, nutrient fluxes and vascular plant establishment (Turetsky et al., 2012). First of all, they fix carbon dioxide through photosynthesis and since they decompose at relatively slow speed, significant amount of global organic carbon is accumulated (Lang et al., 2009; Lindo & Gonzalez, 2010; Turetsky, 2003). NPP[1] of bryophytes in the polar regions was quite surprisingly estimated to be comparable to those in boreal and temperate ecosystems (Turetsky, 2003). Furthermore, mosses were revealed to host cyanobacteria capable of fixing atmospheric nitrogen (Arróniz-Crespo et al., 2014; Bay et al., 2013; DeLuca et al., 2002; Gentili et al., 2005; Ininbergs et al., 2011; Lindo et al., 2013), as it will be discussed later in detail. Therefore, cyanobacteria represent a nitrogen source of significant importance, especially in the pristine high latitude environments (Lang et al., 2009; Lindo et al., 2013; Turetsky, 2003). Moreover, mosses have insulating properties and preserve permafrost from air temperature fluctuation. They are also key players in soil stabilization and water retention (Lang et al., 2009; Turetsky et al., 2012).

1.1 Cyanobacteria

Cyanobacteria are the oldest living organisms. According to fossil records, they appeared on Earth around 3.5 billion years ago, and are considered to be responsible for the Great Oxygenation Event (Schopf, 2012). They possess many pigments (including chlorophyll a, carotenoids, phycoerythrin, phycocyanin) that enable them to perform oxygenic photosynthesis. Oxygenic photosynthesis is a process during which received light energy is converted into chemical energy. This chemical energy is essential to sustain physiological needs and photosynthesis plays an important role in the carbon cycle. The oxygen that cyanobacteria release as a byproduct of photosynthesis is thought to have altered the reducing atmosphere into an oxidizing one, and therefore to have enabled the aerobic mode of

[1] Net primary production (NPP) is defined as the difference between the gross primary production and plant's respiration (Turetsky, 2003).

heterotrophic organisms to develop. Nowadays, 20 to 30 % of the world photosynthetic activity is attributed to cyanobacteria. They are also sometimes called blue-green algae after their pigment coloration, however that is somewhat incorrect, since cyanobacteria are prokaryotes and algae eukaryotes (Fay, 1992; Schopf, 2012; Whitton & Potts, 2002).

Thanks to their amazing physical and biological plasticity, they belong to omnipresent groups of bacteria on Earth, inhibiting nearly all aquatic and terrestrial ecosystems from the poles to warm arid areas. That is why these organisms are ideal primary colonizers for various environments (Komárek, 2014; Vincent, 2000; Whitton, 2012). In polar regions, they represent one of the most dominant phototrophs and primary producers. The success of cyanobacteria in polar regions is mainly related to the ability to grow at temperatures below their optima (>20°C) and their tolerance to numerous abiotic environmental stresses (Elster et al., 2012; Vincent, 2000).

1.1.1 Classification and characteristics

Cyanobacteria are rather simple unicellular organisms with cells or filaments often grouped together through mucilaginous envelopes or sheaths (Madigan et al., 2012). They are also known for their morphological diversity. Traditionally, cyanobacteria have been classified into five morphological subgroups (see Table 1) (Rippka et al., 1979). Nevertheless, as molecular analysis advanced, monophyly of individual groups was often not observed and the taxonomy of cyanobacteria got very complex. (Komárek, 2006) therefore points at the need of revision of cyanobacterial classifications through combination of morphological, molecular and ecophysiological data. The last criterion is of significant importance because the morphology and metabolism of cyanobacteria may be influenced by the habitat they live in (Komárek, 2006).

Table 1
Subgroups of cyanobacteria according to (Rippka et al., 1979) modified by (Bay, 2013)

Subgroup	Organisation of cells	Mode of reproduction	Representative genera	Ability of N-fixation
I	unicellular	binary fission of budding	*Synechococcus* spp.	No[1]
			Cyanothece spp.	Yes
II	unicellular	multiple fission	*Dermocarpa* spp.	No
			*Chroococcidiopsi*s spp.	Yes
III	filamentous	filament random breaking; division in one plane	*Pseudoanabaena* spp.	Yes
			Spirulina spp.	No
IV[2]	filamentous with heterocysts	filament random breaking; division in one plane	*Nostoc* spp.	Yes
			Calothrix spp.	Yes
V[2]	filamentous with heterocysts	filament random breaking; division in more than one plane	*Stigonema* spp.	Yes
			Chlorogloeopsis spp.	Yes

[1] Although one strain (*Synechococcus* PCC7335) was reported to be able to fix nitrogen.
[2] (Gugger & Hoffmann, 2004) described these two subgroups as a uniform cluster Nostocineae.

Cyanobacteria are also known to form symbiotic associations with many organisms such as vascular plants, bryophytes (liverworts, hornworts, mosses), fungi or sponges (Adams et al., 2013). Concerning the associations with bryophytes, cyanobacteria form so called true-symbiosis with hornworts and liverworts, where specific symbiotic structures were described. Such true symbiosis of cyanobacteria with mosses were not reported, even though cyanobacteria-moss associations do occur (Solheim & Zielke, 2002).

1.1.2 Nitrogen fixation

Nitrogen is an essential element for life and related physiological processes as it is an important structural part of for instance nucleic acids, proteins, DNA, RNA or in case of plants of chlorophyll or RuBisCo (Bay, 2013; Burgess & Lowe, 1996). Preferentially, cyanobacteria take up nitrogen in easily available forms from the environment, such as amino acids, nitrate or urea (Fay, 1992). Even though the elemental dinitrogen is the principle constituent of our atmosphere (79 %), it is unfortunately not freely available for the organisms (Berman-Frank et al., 2003). However, in case of lack of easily available nitrogen in the environment, certain prokaryotes, including cyanobacteria, can use it as their main nitrogen source through its fixation. Both heterocystous and non-heterocystous forms of cyanobacteria, namely all cyanobacterial genera from groups IV and V and some from groups I to III (see Table 1) can fix atmospheric nitrogen, although till 1960 it was though that non-heterocystous cyanobacteria are unable to do so (Fay, 1992).

The process of nitrogen fixation, often referred to as diazotrophy, is extremely energetically demanding. To break down the triple bond of atmospheric nitrogen requires the hydrolysis of 16 ATP (the energy of a nitrogen triple bond N≡N is about 940 kJ mol^{-1}; Turetsky, 2003). The reaction which goes as follows, is catalysed by a nitrogenase enzyme complex (Berman-Frank et al., 2003).

$$N_2 + 8\,e^- + 16\,ATP + 10\,H^+ \rightarrow 2\,NH_4^+ + 16\,ADP + 16\,P_i + 8\,H_2$$

The nitrogenase complex is composed of several proteins whereas two of them are specific for the nitrogenase reaction. Firstly, the dinitrogenase reductase, also called iron-sulphur (Fe-S) protein (Campbell & Farell, 2005), which is encoded by *nifH* gene. Secondly it is dinitrogenase, also called iron-molybdenum protein (Fe-Mo), encoded by *nifD* and *nifK* genes (Burgess & Lowe, 1996). Both proteins are contained within an iron-molybdenum cofactor (FeMo-co), where the nitrogen reduction occurs (Madigan et al., 2012). The nitrogenase enzyme, and thus the process of nitrogen fixation, can be irreversibly inhibited by oxygen or some reactive oxygen species (Berman-Frank et al., 2003). To protect the oxygen sensitive nitrogenase from both, atmospheric and intracellular oxygen, cyanobacteria developed several strategies. They are able to perform two antagonistic processes within the same organism at the same time. At one hand to fix atmospheric nitrogen and on the other hand to generate oxygen through photosynthesis. For instance, they are known to increase their respiration to consume more oxygen, to use detoxifying enzymes to remove reactive oxygen species or to separate the two opposing processes of nitrogen fixation and photosynthesis temporally or spatially. In the case of temporal separation, nitrogen fixation occurs only in darkness, i.e. when no photosynthesis is performed and thus no oxygen released (Fay, 1992). Concerning the spatial separation, specialized cells, known as heterocysts, are formed. Such an ability was described only in cyanobacteria of group IV and V (see Table 1) (Bergman et al., 1997).

Heterocysts ensure a suitable anoxic environment to which the nitrogen fixation is strictly restricted (Bergman et al., 1997). The anoxic environment in heterocysts is maintained by several strategies. By an oxygen impermeable barrier made of polysaccharides and glycolipids, degradation of photosystem II, polar nodule production or induced increased respiration which removes remaining oxygen traces (Fay, 1992; Meeks et al., 2002). Moreover, heterocysts are intracellularly connected with vegetative cells so that the mutual exchange of molecules is facilitated (see Figure 2). The fixed nitrogen in the form of glutamine

is transported from heterocysts to vegetative cells in exchange for the product of photosynthesis, providing needed energy for nitrogen fixation (Madigan et al., 2012). Heterocysts are found either at the end of the filament or are distributed along the filament in semiregular manner - approximately one heterocyst for every ten to fifteen vegetative cells (Risser et al., 2012). In average, five to ten percent of vegetative cells are differentiated into heterocysts, however the frequency is species dependent (Bergman et al., 1997). Furthermore, it was hypothesised that only certain cells can induce the differentiation itself. Namely those that exhibit zero concentration of PatN membrane protein, which is considered to supress heterocyst's differentiation (Risser et al., 2012).

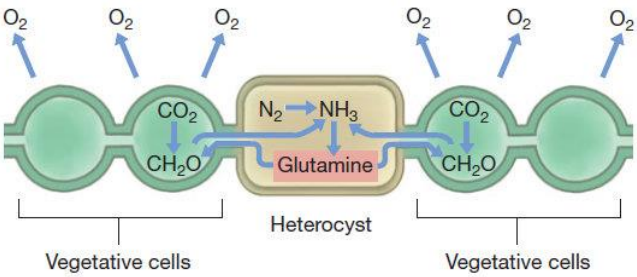

Figure 2: Function of heterocysts from (Madigan et al., 2012)

1.1.3 Hormogonia

Filamentous cyanobacteria of the orders Nostocales, Stigonematales and Oscillatoriales can fragment into small pieces called hormogonia. These are nongrowing motile filaments lacking heterocysts, which break away from the main filament and serve as dispersal units (Adams et al., 2013; Madigan et al., 2012; Tashyreva & Elster, 2016). The hormogonia formation is triggered by several environmental stimuli such as exposure to red light or hormogonia inducing factors (HIFs) that are secreted by plants. Afterwards, hormogonia are attracted towards their symbiotic partner by chemotaxis. The mechanism by which hormogonia move is still not fully understood (Adams, 2001).

1.2 Moss-cyanobacteria associations

The current knowledge about cyanobacteria-moss associations is mainly originating from studies in polar and boreal ecosystems (Lindo et al., 2013; Solheim et al., 1996). In contrast to sub-arctic regions, in the High Arctic either zero or limited nitrogen fixation by heterotrophic soil bacteria was reported because of the low nutrient availability and soil temperature. This suggests that moss-cyanobacteria associations represent the dominant nitrogen source (Solheim & Zielke, 2002). As already mentioned, the primary production in the High Arctic is often limited by nitrogen, making these associations to become of even bigger ecological importance (Rousk et al., 2017). However, despite this fact, only very little is known about the nature of these associations (Lindo et al., 2013; Warshan et al., 2017).

Up to now, several mosses were shown to be associated with following heterocystous cyanobacteria: *Nostoc* sp., *Calothrix* sp., *Anabaena* sp., *Stigonema* sp., *Cylindrospermum* sp., *Scytonema* sp. Cyanobacteria were described to be associated with the mosses mainly epiphytically (DeLuca et al., 2002, 2007; Gentili et al., 2005; Houle et al., 2006; Ininbergs et al., 2011; Jean et al., 2012; Lindo & Whiteley, 2011; Solheim & Zielke, 2002), but also intracellularly in moss hyaline cells filled with water. The determining factor of the association manner seems to be the pH of the environment (Solheim & Zielke, 2002). Cyanobacteria prefer a neutral or slightly alkaline environment (Hoffmann, 1973). That is why at acidic pH, the cyanobacteria escape into less acidic environment of hyaline cells and at higher pH, i.e. neutral or alkaline, they are found on mosses epiphytically. However, at high acidic pH below 3.8, no moss-associated cyanobacteria were observed anymore (Granhall & Selander, 1973; Solheim & Zielke, 2002).

The mechanism and factors which would regulate the capacity of the moss to host cyanobacteria and the possible benefits of such associations still remain unknown (Bay, 2013). Current knowledge about symbioses of bryophytes with cyanobacteria is mainly related to hornworts (e.g. *Anthoceros punctatus*; Campbell & Meeks, 1989) and liverworts (e.g. *Blasila pusilla*; Knight & Adams, 1996). The infection of hornworts and liverworts is induced via chemical signalling. To induce the hormogonia formation in cyanobacteria, plants release chemical compounds of unknown structure, HIFs, and subsequently a chemoattractant which guide hormogonia towards the symbiotic structure of the plant. In case of *Blasila*, these are spherical structures (so called auricles), and for *Anthoceros*, these are slime cavities within the thallus. Moreover, such a mechanism was observed only under nitrogen starvation (Adams, 2002; Adams et al., 2013). In fact, it is recognized that cyanobacteria provide bryophytes with significant amount of nitrogen (80 to 90 % of the fixed nitrogen) (Meeks, 2009). The nitrogen is in easily accessible forms, such as amino acids, and thus have positive influence on the bryophyte growth. In the symbioses between cyanobacteria with *Gunnera*, *Blasia* and *Anthoceros*, morphological and physiological changes were observed in cyanobacteria, such as irregularities in shape or cell enlargement. Moreover, repression of hormogonia development, reduction of photosynthetic activity and increase of heterocyst frequency with nitrogen fixation was observed. In fact, cyanobacteria underwent shift from photo-autotrophic to heterotrophic metabolism, being provided with organic carbon by the host to sustain the energetically demanding nitrogen fixation (Adams & Duggan, 2012;

Berg et al., 2013; Meeks et al., 2002). Furthermore, bryophytes provide the cyanobacteria with a safe environment (Lindo et al., 2013; Rousk et al., 2013; Solheim & Zielke, 2002).

Concerning associations of cyanobacteria with mosses, (Ininbergs et al., 2011) proposed that moss-associated cyanobacteria are highly host specific with mosses having the predominant role of choosing their cyanobacterial partner. This was in accordance with results of (Opelt et al., 2007), who showed moss-associated bacteria to be host specific, regardless of their location. But it was in contradiction to (Marshall & Chalmers, 1997), who suggested random phenomena such as wind dispersal, to be responsible for the cyanobacterial colonization of mosses. This hypothesis was further confirmed by results of (Gordon et al., 2000). Nevertheless, findings of (Ininbergs et al., 2011) lead to a hypothesis, that mosses secrete chemical compounds acting as "chemo-attractants", being responsible for the cyanobacterial establishment on the mosses, as it has been shown in other plant-cyanobacteria symbioses discussed above (Bay et al., 2013). Through an experimental set-up, where different moss species were physically separated (although not isolated) through a porous membrane from *Nostoc* culture, (Bay et al., 2013) proved that mosses are responsible for hormogonia differentiation of *Nostoc* sp. and thus also secrete chemical signals (HIFs). Interestingly, in that experiment only mosses *H. splendens* and *P. schreberi*, in contrast to *D. polysetum* and *P. commune*, were colonized by *Nostoc* sp., which could be explained by host-specific chemical compounds inducing chemotactic responses of cyanobacteria. Therefore, it seems that for successful cyanobacterial colonisation of host mosses, besides trigger of hormogonia differentiation, mosses must also attract and guide these hormogonia. The stimulus for hormogonia differentiation was furthermore confirmed to happen only under limited availability of nitrogen, as it is the case in other plant-cyanobacteria symbioses. Moreover, high-resolution secondary ion mass spectrometry confirmed transfer of fixed nitrogen from cyanobacteria towards moss partner (Bay et al., 2013).

1.3 Molecular approach

The idea that evolutional changes are encoded within the nucleic acids of organisms opened new possibilities for taxonomical studies in general, but also for cyanobacteria. The modern approach of sequencing allowed better definition of cyanobacterial diversity and to an important extent changed its taxonomy.

1.3.1 Sequencing techniques

Within the last 50 years, three different sequencing generations were developed. In the first-generation sequencing, Sanger chemistry (sometimes also "chain termination" technique) is applied. Radioactively or later fluorescently labelled dideoxynucleotide triphosphates (ddNTPs), which lack 3'-OH groups necessary to link two nucleotides through the phosphodiester bond, stop the DNA replication at random moments. Therefore, DNA copies of different lengths, all having labelled ddNTP at the end, are produced. The DNA sequence is then deduced through electrophoresis, whereby DNA fragments migrate at different speed according to their molecular weight (Sanger et al., 1977). Modern automated sequencing machines make use of fluorometric detection and capillary electrophoresis and produce reads slightly under 1000 bp in length that are of high quality (Heather & Chain, 2016).

Second generation sequencing no longer uses Sanger chemistry and electrophoresis. It is massively parallel, whereby from a single run a considerable amount of sequences is produced at the same time. The sequencing is based either on "sequencing by synthesis" (e.g. pyrosequencing or sequencing by reversible termination (Illumina)) or "sequencing by hybridization and ligation" (SOLiD platform). Although they differ in technical details and therefore also in the read lengths, error rates and costs, they share a few common features. The sample preparation requires first extraction of nucleic acids followed by library preparation where isolated DNA is randomly broken into smaller fragments. These are then clonally amplified. DNA fragments are amplified into many template fragments through bridge or emulsion PCR, dependent on the solid surface (platform) (Ambardar et al., 2016).

Pyrosequencing is based on the fact, that pyrophosphate is released upon incorporation of a new nucleotide during the DNA elongation. As a result of an enzymatic reaction cascade which pyrophosphate undergoes, visible light is produced, and its intensity is proportional to the amount of newly incorporated bases (Ronaghi, 2001). The downside of pyrosequencing is the inability to deal with identification of five and more identical consecutive bases. Moreover, the costs are relatively high in comparison to other second generation techniques (Ambardar et al., 2016).

In sequencing by reversible termination (Illumina, sometimes also called Solexa), bridge PCR is used to clonally amplify DNA fragments attached to the flow cells. Reversible terminator (RT) nucleotides, being fluorescently labelled and protected at 3'-OH groups, are

then used for subsequent sequencing by incorporation into the newly synthetized DNA strand by DNA polymerase. Fluorescent signal, different for the four bases, is detected and finally the free 3'OH group is restored by cleavage of the terminating moiety. In each cycle, contrarily to pyrosequencing, four nucleotides are competing for its incorporation. And because the terminator must be removed first before another base nucleotide can be incorporated, only one nucleotide is added in one cycle. This in turn reduces the sequencing error present in pyrosequencing (Ambardar et al., 2016; Heather & Chain, 2016).

Third-generation sequencing represents still a rather new and promising approach for the future. Instead of DNA fragment's amplification, a molecule of DNA can be sequenced in real time. Contrarily to the second-generation techniques, the nucleotides are in general integrated at the speed of DNA polymerase and no or simpler labelling of the nucleotides is required. Moreover, the whole process is done in fewer reactions and less material is used (Munroe & Harris, 2010). The average read can vary from 10 000 bp to 100 000 bp. Single Molecule Real Time (SMRT) sequencing (from the PacBio platform) is nowadays the most established platform. Other third-generation technologies are for instance Illumina Tru-seq Synthetic Long-Read technology and Nanopore DNA Sequencing (Lee et al., 2016).

1.3.2 16S rRNA gene

For the reconstruction of phylogenetic relationships, different genes can be studied. One of the most popular is the gene coding for RNA small ribosomal subunit (16S rRNA), but also protein coding genes can be used. For instance genes coding for nitrogenase complexes *nifD*, *nifK*, *nifH* (Gugger et al., 2005) or RubisCO large subunit and chaperonin-like protein X - *rbcL* and *rbcX* (Singh et al., 2015). Sequencing of 16S rRNA is considered to be a good choice for studying microbial evolutionary relationships, since it is a universal gene and contains both conserved and variable regions necessary for primer binding and species characterization, respectively (Johansen et al., 2017). Moreover, sequencing of 16S rRNA gene is routinely used and thus many public gene bank entries enabling good comparison are available. Unfortunately, 16S rRNA has also several downsides. Closely related taxa often cannot be discriminated because of limited 16S rRNA resolution. Furthermore, errors in sequencing, problems with OTU's assessment or relatively short length of obtained sequences limit the method (Poretsky et al., 2014).

2 Aims

The aim of this thesis raised after the work which was done by (Lesniak, 2012). He tried to describe the impact of cyanobacteria on the Arctic wet meadow in Petuniabukta, in the central part of Svalbard. Among other aspects, his aim was to describe the diversity and abundance of moss associated cyanobacteria. Due to the large surface of moss leaves it was impossible to examine the cyanobacteria-moss association under the microscope without any preceding changes. Due to the tight association of cyanobacteria and mosses, he encountered difficulties to separate cyanobacteria from the moss and finally described only microalgae and cyanobacterial diversity and abundance based on water originating from wrung moss samples. He also assessed the nitrogen fixation activity of mosses by Acetylene Reduction Assay (ARA), but couldn't correlate it anymore to the cyanobacterial biomass (Lesniak, 2012).

Attempts to dislodge moss associated cyanobacteria have been tried by either simple vigorous shaking of the moss in water (Arróniz-Crespo et al., 2014; Jean et al., 2012) or by putting moss stems onto cultivation medium BG11 and subsequent culturing of cyanobacteria according to (Rippka et al., 1979) (see Gentili et al., 2005; Ininbergs et al., 2011). Nevertheless, both methods have some limitations. Not all cyanobacteria can be cultivated and can grow on BG11 medium, and even though some cyanobacteria are dislodged from the moss by vigorous shaking, many might still stay on the moss. A trustworthy method to dislodge all cyanobacterial communities would be therefore extremely helpful. It would not only allow to correlate nitrogen fixation activity to the cyanobacterial biomass, but it would also bring us closer to understanding of wet meadows at the ecological level, which is extremely important as wet meadows play an important and maybe underestimated role in the ecosystem functioning as already mentioned earlier.

Therefore, the aims of this thesis were to test and compare different methods to mechanically dislodge cyanobacteria from the mosses, which would allow their identification and quantification and finally to perform a pilot study on the molecular sequencing of the moss-associated cyanobacteria as a basis for further detailed studies of the wet meadow.

2 Methods

2.1 Sampling

The study locality - the wet hummock meadow (N 78°43’49’’ E 16°26’41’’, 15 m a.s.l.) is situated in Petuniabukta, Central Svalbard, High Norwegian Arctic. The wet meadow undergoes pronounced water content fluctuation. Firstly, it is saturated with water at the vegetative start of the season due to extensive snowmelt. During summer and early fall it is subjected to continuous desiccation, being occasionally rehydrated by short rain events (Kvíderová et al., 2011). The vegetation is dominated by vascular plants (*Dryas octopetala*, *Carex rupestris*, *Salix polaris*), mosses (*Scorpidium* sp. and *Orthothecium chryseon*) and *Nostoc commune* (Prach et al., 2012). Based on visible contrasts in colour, shape and water content, the meadow was divided into following five major micro-environments: green, water, red, orange and grey (Lesniak, 2012).

The sampling was performed in August 2016 as follows. Three randomly distributed moss samples in each of the five major micro-environments of the wet hummock meadow were collected with a plexiglass corer (5 cm inner diameter; 34 cm length) equipped with an inner piston (for photo documentation see Appendix A). The vegetation was separated from the soil. The length of the moss was measured as well as the temperature, pH and conductivity of the water from wrung moss samples. Furthermore, the depth of active layer was assessed with a metal rod for each micro-environment (with three repetitions). Afterwards, the moss samples were brought to the station in polyethylene bags. The green parts of the moss were cut from the brown parts and mosses were let to air-dry on paper towels (for photo documentation see Appendix A). Later, only the green parts of the mosses were analysed. The sampled moss was identified as *Scorpidium sp.* Per each zone, also the percentage vegetation surface cover within 1 m^2 was estimated by eye (with three repetitions).

2.2 Mechanical dislodging tests

The air-dried moss samples were brought from Svalbard to the Institute of Botany in Třeboň, Czech Republic where following dislodging methods were tested (each method with three repetitions). Always the green parts of the moss samples coming from the red zone of the wet meadow were analysed. For better visualisation, see Figure 3.

1) Freezing with liquid nitrogen

50 mg of the air-dried moss sample was introduced into ceramic mortar and frozen by liquid nitrogen. It was grinded with a pestle till a homogenous powder was obtained. The resulting powder was diluted in 4 mL of water.

2) Sonication

50 mg of air-dried moss sample was introduced into a 50 mL plastic vial with 4 mL of water and the sample was sonicated with an ultrasonic processor Hielscher UP100H with micro tip MS7 (tip diameter of 7 mm; maximal amplitude of 125 µm). Different amplitudes (20 %; 60 %) and different sonication times (1 min; 2 min) with cycle of 0.5 and with 30 s intervals to avoid overheating of the solution were used. The sonication uses the principle of longitudinal mechanical vibrations which are generated by the ultrasonic processor.

3) Vortex

50 mg of air-dried moss sample was introduced into a 50 mL plastic vial with 4 ml of water and the sample was vortexed for 10 min at maximum speed (Arróniz-Crespo et al., 2014).

2.2.1 Cyanobacterial/microalgae biovolume

From all the above described procedures, cyanobacterial/microalgae biovolume as well as the proportion of cyanobacterial groups was estimated by using light and epifluorescence microscopy (Olympus BX 51, Japan). This non-stained protocol works on the principle of chlorophyll autofluorescence. 0.2 mL of samples resulting from different pre-treatment methods was pipetted onto a microscope slide and covered by 22x22 mm coverslip. The microscope magnification was set to 400x. Based on the cell morphology of observed cyanobacteria and microalgae, four different groups were recognized according to (Kaštovská et al., 2005): Nostocales, Chroococcales, Oscillatoriales/Synechoccales and green coccoid algae. The groups Oscillatoriales and Synechoccales were merged together for simplicity as Pseudanabaenaceae and Leptolyngbyaceae were moved to the order of Synechococcales in recent taxonomical classification of cyanobacteria by (Komárek et al., 2014). Green coccoid algae might have included Chlorophyceae and Tribophyceae algae classes, since those two are not distinguishable when using the epifluorescence microscopy (Kaštovská et al., 2005). The cyanobacterial and microalgal abundance was expressed as biovolume [μm^3 mg^{-1} of dry weight of moss] based on basic geometric equations (Olenina et al., 2006).

2.2.2 Postfreezing with liquid nitrogen (control treatment)

To prove the efficiency of the above described methods, once the biovolume was assessed, the sonicated and vortexed samples were washed under running water, let air-dry and later were frozen with liquid nitrogen as described above. Subsequently, the cyanobacterial biovolume was again estimated.

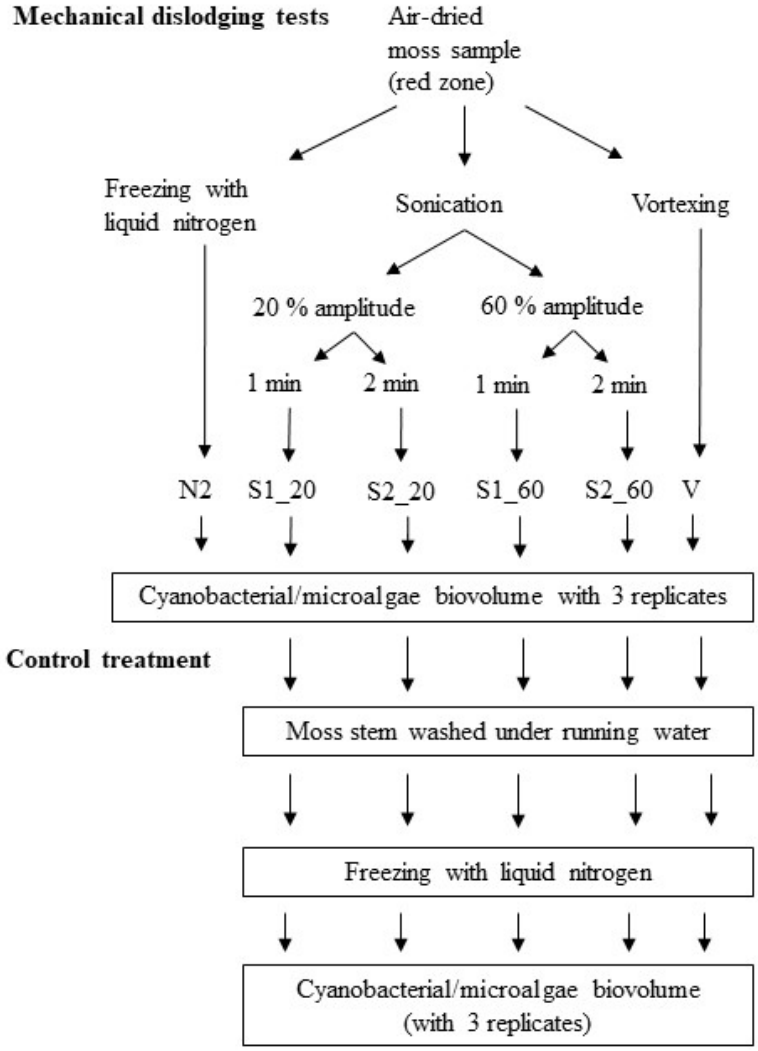

Figure 3: Scheme of mechanical dislodging tests including control treatment.

2.2.3 Comparison of cyanobacterial/microalgae abundance within the studied meadow

To compare cyanobacterial/microalgae abundance within the studied meadow, three moss samples per micro-environment of the wet meadow were studied using sonication (amplitude of 60 %, 0.5 cycle for 2 min) as it was revealed to be the most efficient of the tested methods. Due to time limit, only red, green and water micro-environments were investigated.

Differences in biovolume were examined by analysis of variance (ANOVA) and in case of significant differences ($p<0.05$), the Post-hoc comparison (Tukey Honest Significant Differences) was further performed. Analyses were performed in R software 3.4.4.

2.3 Pilot Sanger sequencing

2.3.1 DNA isolation

2.3.1.1 First try

As a pilot test, first one water sample and two moss samples from the green and red zones were tested and corresponding cyanobacterial DNA was isolated according to a readapted protocol by (Koutecký et al., 2010) from (Doyle & Doyle, 1987) as follows.

The water sample was centrifuged at 5000 rpm for 3 minutes. The supernatant was replaced with 500 µL of 2 % CTAB extraction buffer. The moss samples (approximately 40 mg) with 3 mL of 2 % CTAB were grinded with the use of mortar and pestle. Afterwards, all samples were transferred into 2 mL Eppendorf tubes and 5 sterile stainless-steel balls (diameter of 2 mm) with sterile sand were added to each of them. The samples were then subjected to mechanical cell lysis treatment in the mixer mill (Retsch MM 400) at 24 Hz for 15 min for the moss samples and 5 min for the water sample. Samples were incubated at 50°C for 30 min with gentle shaking in a thermoblock with 700 µL of the CTAB stock solution and 10 µL of 2-mercaptoethanol. The remaining moss tissue was removed by centrifugation at 13800 rpm for 3 min, whereby the supernatant was transferred to a new 1.5 mL sterile microtube. The samples were extracted with 500 µL of chloroform:isoamylalcohol (24:1 v/v), gently mixed and let stand for 5 min. The upper aqueous phase was transferred into new sterile microtubes. The DNA was precipitated with 500 µL of chilled isopropanol, mixed gently and let stand at -20°C for 30 min. The DNA pellet was first washed with 400 µL of 96 % cooled ethanol, incubated at 37°C for 15 min in a thermoblock and centrifuged at 13 800 rpm for 5 min. For the second time, the DNA pellet was washed with 200 µL of 70 % chilled ethanol, mixed gently, let stand for 5 min and centrifuged at 13 800 rpm for 5 min. Finally, the DNA pellet was air-dried (for approximately 15 min), resuspended in 100 µL of sterile molecular grade water and stored at -20°C.

2.3.1.2 Second try

As discussed later in result section 3.4.1, another approach to isolate DNA of moss associated cyanobacteria was set up. Each moss sample was subjected to following three different treatments from all of which the DNA was isolated. For better visualization, see Figure 4.

1) Vortex

About 40 mg of airdried moss was introduced into 25 mL falcon tube with 20 mL of distilled water and was vortexed at maximum speed for 10 min (Arróniz-Crespo et al., 2014).

2) Sonication

The moss was introduced into another falcon tube with 20 mL of distilled water and the sample was sonicated (for 2 min with 30 s intervals at 60 % amplitude, 0.5 cycle). The vortexed and sonicated solutions were concentrated by centrifugation at 8596 rpm for 5 min. The supernatant was poured out and the remaining 2 mL were pipetted into 2 mL microtubes and centrifuged at 13400 rpm for 5 min. The supernatant was replaced with 1 mL of 2 % CTAB and 5 sterile stainless-steel balls with sterile sand were added.

3) Moss smashing

The remaining moss together with 1 mL of 2 % CTAB solution was grinded with mortar and pestle to obtain a homogenous powder. The suspension was introduced into a 2 mL microtube together with sterile stainless-steal balls and sterile sand.

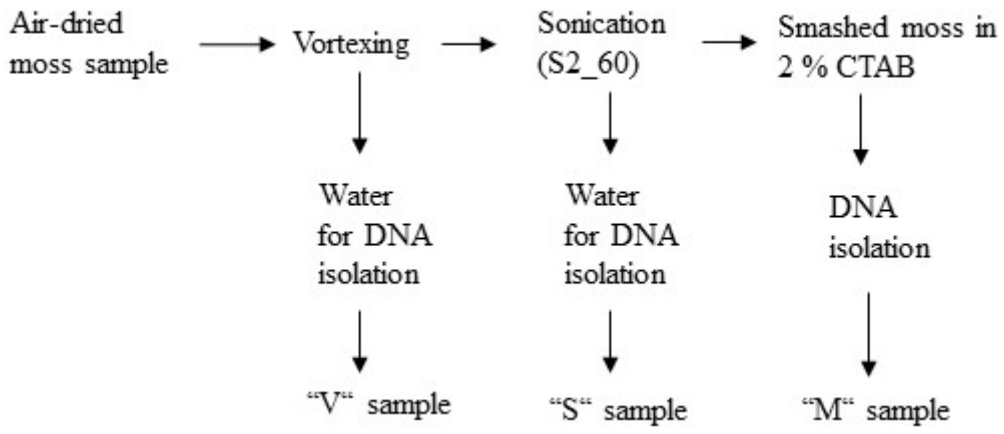

Figure 4: Scheme of DNA isolation approach.

Afterwards, all the three samples were subjected to the mechanical cell lysis in the mixer mill as described in 2.3.1.1 and from that on, the same procedure was carried out.

2.3.2 Polymerase chain reaction (PCR)

The 16S rRNA gene was amplified using specific cyanobacteria primers (see Table 2) purchased from IDT (Integrated DNA Technologies). All other reaction components were purchased from Top-Bio ('Top-Bio'). PCR reactions were run in Bioer XP cycler and a negative control was used in each run. The PCR reaction mixture reagents are summarized in Table 3, the temperature profile in Table 4.

Table 2
List of applied primers with corresponding sequence

Primer[a]	Sequence
CYA106F	5' - CGG ACG GGT GAG TAA CGC GTG A - 3'
CYA359F[d]	5' - GGG GAA TYT TCC GCA ATG GG - 3'
CYA781Ra[c]	5' - AAT GGG ATT AGA TAC CCC AGT AGT C - 3'
CYA781Rb[c]	5' - AAA GGG ATT AGA TAC CCC AGT AGT C - 3'
CYA1444R[b]	5' - GTG GCT GGA TCA CCT CCT TT - 3'

where F stands for forward and R for reverse primer orientation in relation to the rRNA
[a] from (Nübel et al., 1997)
[b] from professor Ólafur Andrésson (personal communication, September 2016)
[c] for CYA781R, an equimolar mixture of "a" and "b" primers was used
[d] Y stands for C/T nucleotide degeneracy

Table 3
PCR reaction mixture

Reagents	Volume [μL]	Final concentration
PCR water	3.1	-
Forward primer (0.005mM)	0.6	0.3 μM
Reverse primer (0.005mM)	0.6	0.3 μM
2x Plain Combi Master Mix	5	1x
DNA[a]	0.7	-

[a] The DNA dilution ratio ranged from 1:20 to 1:100 (DNA:molecular grade water)

Table 4
PCR temperature profile

Repetition	Temperature	Time	Process
1x	94°C	3 min	initial denaturation
45x	94°C	45 s	cycle denaturation
	60°C	45 s	specific primers annealing
	72°C	x_1	cycle elongation
1x	72°C	10 min	final elongation
1x	15°C	hold	mixture cooling

x_1…45 s for CYA781R; 1 min 30 s for CYA1444R

2.3.3 Agarose gel electrophoresis

The PCR amplifications were verified by agarose gel electrophoresis. 2 µL of the PCR product mixed with 1 µL of loading dye (30 % glycerol, 373 mM bromophenol blue, 40x GelRed), were loaded onto 1.5 % agarose gel (prepared in 1x TBE buffer). For size reference, 6 µL of 100 bp ladder was loaded onto the same gel. The electrophoresis was run at 140 V for approximately 20 min. The fragment separation was observed under UV transillumination and photographs were taken. An example is shown in Figure 5.

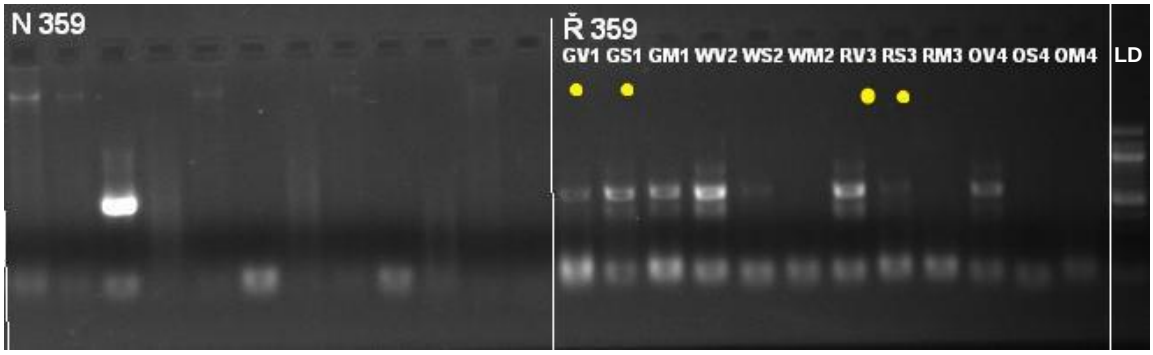

Figure 5: Agarose gel electrophoresis results.
"N" stands for nondiluted, "Ř" for diluted samples; "LD" for 100 bp DNA ladder; yellow dots represent chosen PCR products for Sanger sequencing.
The first index in the sample name stands for the wet meadow zone: "G" for green, "W" for water, "R" for red, "O" for orange. The second index refers to the used method: "V" stands for vortex, "S" for sonication, "M" for smashed moss.
Negative control band is not shown here, since it just didn't fit into the row and was run in the second one.

2.3.4 Cloning

The nicely amplified samples were chosen for subsequent cloning following readapted procedure from (Sambrook & Russell, 2006) using pGEM-T vector kit. First, 6 µL of PCR product were mixed with 2 µL of loading dye (30 % glycerol, 373 mM bromophenol blue, 20x SYBR Green), incubated for 10 min and loaded onto 1.5 % low melting agarose gel (prepared in 1x TAE buffer). The electrophoresis was performed at 120 V for approximately 20 min. The target DNA bands were cut off with a scalpel and the help of orange goggles under blue light, transferred into 0.5 mL microtubes and incubated at 65°C for 10 min in a thermoblock. Afterwards, 1.25 µL of the sample were mixed with 1.75 µL of sterile water and 0.25 µL of the pGEM-T vector and the mixed suspension was incubated for another 10 min at 65°C. The ligation mixture was prepared on ice from 2.5 µL vortexed ligation buffer with 0.25 µL T4 ligase per one sample and transferred into strip microtubes. 2.5 µL of the melted DNA was cooled for 3 s and mixed with the ligation mixture. The samples were subjected to ligation at 16°C for 14 hours in a cycler. 100 µL of *E. coli* competent cells, strain K12, mod. DHα, were let thaw on ice. For each sample, 33 µL of cells and 8.25 µL of ligation

mixture were pipetted into a 1.5 mL sterile microtube, gently mixed and incubated for 20 min on ice. Transformation was done through temperature shock. The samples were incubated at 42°C for 45 s and immediately put on ice for 2 min. 300 µL of SOC (super optimal broth) medium was added, samples were incubated in horizontal position on a shaking thermostat for 45 min at 37°C, 150 rpm. Per one Petri-dish, 64 µL of X-Gal were mixed with 3.5 µL of IPTG and spread onto Petri-dish with agar. Then 150 µL of the sample suspension was spread onto Petri dish and incubated upside-down in thermostat overnight at 37°C.

2.3.5 Direct PCR

The next day, 8 colonies per sample were taken and incubated at 94°C for 5 min to release the plasmids from bacteria through thermal denaturation. The inserted DNA was amplified using specific primers SP6 and T7 (see Table 5), which attach right next to the insert. The PCR reaction mixture is shown in Table 6 and the temperature profile in Table 7.

Table 5
List of applied primers

primer	sequence
S56	5' - TAC GAT TTA GGT GAC ACT ATA G - 3'
T7	5' - TAA TAC GAC TCA CTA TAG GG - 3'

Table 6
PCR reaction mixture

reagents	Volume [µL]
PCR water	1.3
SP6 primer (5 pmol μL^{-1})	0.4
T7 primer (5 pmol μL^{-1})	0.4
2x Plain Combi Master Mix	2.5
DNA	0.4

Table 7
Temperature profile

Repetition	Temperature	Time
1x	95°C	3 min
45x	95°C	30 s
	50°C	x_1
	72°C	x_2
1x	72°C	10 min
1x	15°C	hold

x_1…1 min for CYA781R; 50 s for CYA1444R
x_2…40 s for CYA781R; 1 min 30 s for CYA1444R

2.3.6 Sanger sequencing

The PCR products were verified by agarose gel electrophoresis according to the protocol described in 2.3.3. Four of the visually nicely amplified clones per sample were directly used for Sanger sequencing. An example of the gel is shown in Figure 6. The sequencing reaction consisted of 0.5 µL of PCR product mixed with 5 µL of either SP6 or T7 primer (final concentration of 5 pmol μL^{-1}) and 4.5 µL of PCR water in a 1.5 mL Eppendorf tube. Finally, the samples were sent to GATC Biotech company (Kostantz, Germany), where Sanger sequencing was performed.

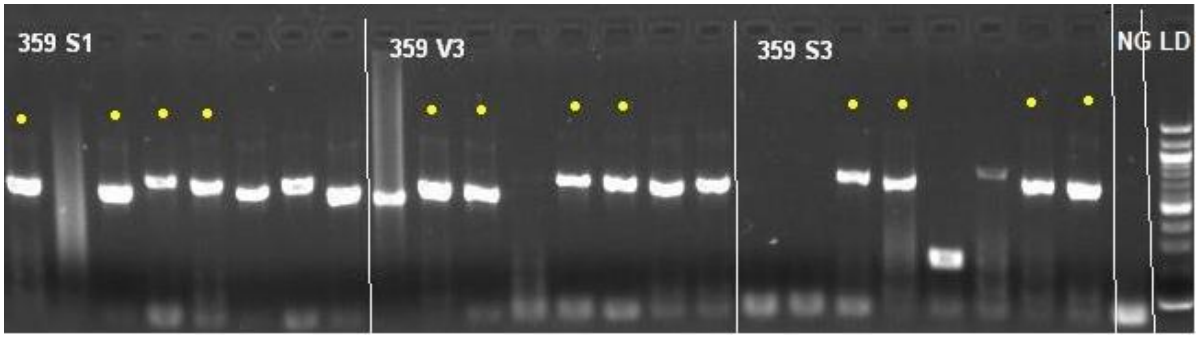

Figure 6: Agarose gel electrophoresis results after cloning.
"359" stands for the used forward primer CYA359F; "S1", "V3" and "S3" for the name of the sample referring to the cyanobacteria separation method used as discussed earlier; "NG" stands for negative control; "LD" for 100 bp DNA ladder; yellow dots represent chosen PCR products for Sanger sequencing.

2.3.7 Processing of molecular data

The quality of the obtained sequences was checked by visual inspection of chromatograms in FinchTV software 1.4.0. (Geospiza, 2015). The target parts between forward and reverse primers of good quality sequences (with distinct peaks indicating the individual bases) were stored in Bioedit 7.2. software (Hall, 1991) for further processing. For ambiguous bases, "N" was used instead. BLAST search (Basic Local Alignment Search Tool; available under https://blast.ncbi.nlm.nih.gov/Blast.cgi) against Nucleotide collection (nr/nt) of the NCBI database was used to verify whether the obtained sequences belonged to the phylum of Cyanobacteria. Cyanobacterial sequences were subsequently aligned with MAFFT version 7 (Katoh et al., 2017) using default settings. Afterwards, the alignment was visually checked in Bioedit 7.2. (Hall, 1991). Reads were cut to the same length of 380 bp and were clustered into operational taxonomic units (OTUs) at 97 % similarity using UPARSE (Edgar, 2013). Chimeric sequences were discarded and the most abundant sequence for each OTU was selected. These sequences were than blast-searched against nucleotide collection and the best isolate hits were recorded (hits from uncultured samples were omitted). Furthermore, phylogenetic reconstruction was inferred by maximum likelihood method

performed in PhyML (Guindon & Gascuel, 2003) (available online in T-REX at http://www.trex.uqam.ca/index.php?action=phyml2&project=trex; Boc et al., 2012) using GTR substitution model, BioNJ method for construction of the starting tree, and NNI algorithm for tree topology search. Branch support was tested using bootstrapping with 500 replicates.

3 Results and discussion

3.1 Micro-environment's characterisation

Basic parameters within each micro-environment of the wet meadow are depicted in Table 8. Moss length varied from 5 to 9 cm, active layer thickness from 64 to 124 cm. The pH of the water from the wrung mosses was neutral for the red zone and basic for green, water, orange and grey zones. The estimation of percentage vegetative surface cover is shown in Table 9. The genera diversity per micro-environment is low. Only five species of vascular plants (*Salix* sp., *Equisetum* sp., *Persicaria* sp., *Carex* sp., *Saxifraga oppositifolia*), *Nostoc commune* and mosses were observed. All these genera, except of *Persicaria* sp., were also described by (Lesniak, 2012; Prach et al., 2012). In each micro-environment, mosses are the dominant constituents.

Table 8
Average length of sampled moss and active layer; pH, conductivity and temperature of the water from the wrung mosses

Zone	Moss length ± SD [cm]	Active layer ± SD [cm]	pH	Conductivity [µS]	Temperature [°C]
Green	9 ± 3	106 ± 4	8.1	824	7.9
Water	5 ± 1	100 ± 10	8.25	866	7.8
Red	7 ± 2	64 ± 1	6.93	869	5.1
Orange	7 ± 1	85 ± 8	7.70	827	8.7
Grey	6 ± 1	124 ± 3	7.96	960	7.4

SD…standard deviation (n=3)

Table 9
Estimated percentage vegetation surface cover in different micro-environments of the studied wet meadow

Zone		Vegetation surface cover ± SD [%]				
		Green	Water	Red	Orange	Grey
Vascular plants	*Salix* sp.	11 ± 4	6 ± 4	0 ± 1	3 ± 3	15 ± 13
	Equisetum sp.	7 ± 3	1 ± 2	0 ± 0	0 ± 0	19 ± 10
	Persicaria sp.	2 ± 2	0 ± 0	0 ± 0	0 ± 1	1 ± 1
	Carex sp.	10 ± 9	14 ± 6	6 ± 2	18 ± 15	4 ± 5
	Saxifraga oppositifolia	1 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 1
Nostoc commune		0 ± 0	13 ± 16	0 ± 0	3 ± 2	4 ± 1
Mosses		70 ± 5	62 ± 9	94 ± 2	76 ± 20	57 ± 19
No cover		0 ± 0	4 ±7	0 ± 0	0 ± 0	0 ± 0

SD…standard deviation (n=3)

3.2 Comparison of dislodging methods based on estimated cyanobacterial/microalgae biovolume

Different dislodging methods were compared based on the estimation of cyanobacterial/microalgae biovolume (see Figure 7 and for detailed results Annex B). The highest cyanobacterial biovolume was obtained when using liquid nitrogen which would logically favour this method. However, freezing of moss with liquid nitrogen turned out to be too destructive since individual cyanobacterial filaments were mostly teared off into small pieces and colonies were often destroyed. Moreover, high background noise of the picture under light microscope due to the grounded moss powder was present. This all made the cyanobacterial identification difficult. In that respect, sonication and vortex were revealed to be more suitable methods, although the cells differentiation remained still difficult since cyanobacterial filaments and colonies were to a small extent destroyed as well. Nevertheless, to further compare cyanobacterial abundance within the three wet meadow zones, sonication for 2 min at 60 % amplitude (S2_60) was used as it yielded the highest cyanobacterial biovolume after the freezing method.

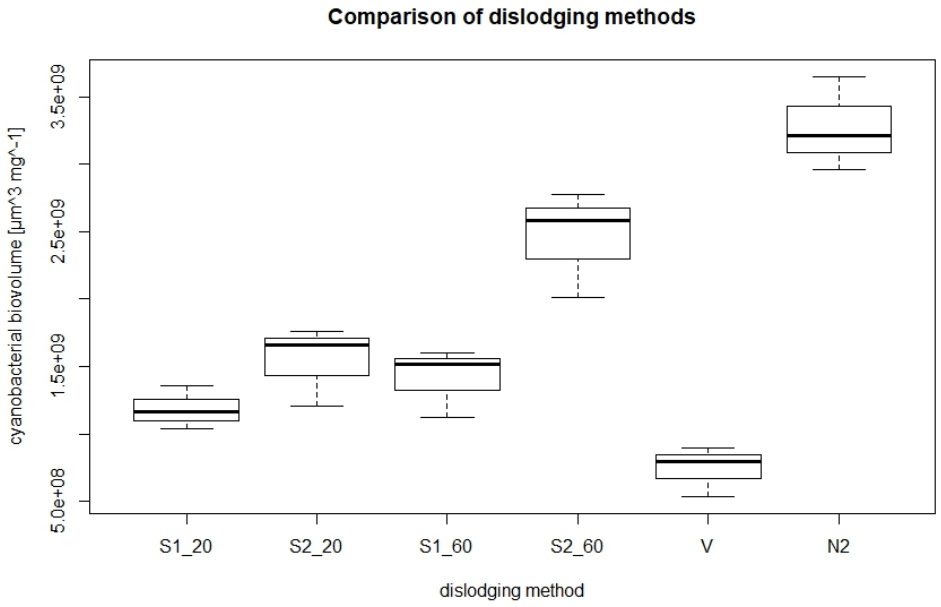

Figure 7: Boxplot comparing cyanobacterial biovolume resulting from different dislodging methods.
In "Sx_y", "S" refers to sonication, "x" to time (in minutes), "y" to amplitude; "V" stands for vortex, "N2" for treatment with liquid nitrogen.

3.2.1 Efficiency assessment by post-freezing (control treatment)

The efficiency of sonication and vortex was studied by freezing all analysed moss samples with liquid nitrogen after the initial treatment with either sonication or vortex. From these post-frozen samples, cyanobacterial biovolume was again estimated. Cyanobacteria were found after all treatments, indicating that neither sonication and vortex didn't allow to dislodge all moss-associated cyanobacteria. Concerning the estimated cyanobacterial biovolume after this post-freezing, somewhat strange results were obtained (see Table 10). The highest percentage of initial cyanobacterial biovolume was found in vortexed sample, which would be in accordance with Figure 7, as the least cyanobacterial biovolume was found in that sample. Also, in sonicated sample S2_60 (2 min, 60 % amplitude), the percentage of initial cyanobacterial abundance was lower than in sonicated sample S1_60 (1 min, 60 % amplitude), following the trend of Figure 7. However, in S1_20 (1 min, 20 % amplitude), lower percentage of initial cyanobacterial biovolume than in S2_20 (2 min, 20 % amplitude) was found, which is in contradiction to Figure 7. It could be explained by a small number of analysed samples (only three replicates). For a more robust analysis, at least ten sample replicates should be analysed. This was unfortunately not possible in the time frame of this bachelor thesis.

Table 10
Results of post-freezing cyanobacterial biovolume estimation

Initial dislodging method		S1_20	S2_20	S1_60	S2_60	V
Cyanobacterial biovolume [μm^3 mg^{-1}]	mean	1.21E+07	9.24E+07	4.67E+07	3.03E+07	1.29E+08
	SD	4.76E+06	2.77E+07	1.14E+07	7.00E+06	2.06E+07
Relative percentage of initial cyanobacterial biovolume [%]	mean	1.2	6.0	3.3	1.0	17.4
	SD	0.4	1.9	0.9	0.5	2.2
Chroococales biovolume [μm^3 mg^{-1}]	mean	0	0	2.20E+06	0	6.87E+06
	SD	0	0	1.91E+06	0	1.04E+07
Nostocales biovolume [μm^3 mg^{-1}]	mean	1.21E+07	9.06E+07	4.45E+07	3.03E+07	1.22E+08
	SD	4.76E+06	2.49E+07	1.02E+07	7.00E+06	3.01E+07

SD…standard deviation, n=3

3.2.2 Problems related to cyanobacterial/microalgae quantification

A fully precise method to quantify abundance of moss-associated cyanobacteria (neither free living cyanobacteria) unfortunately doesn't exist. As all other different approaches, also biovolume estimation has its limitations. Besides being time consuming, the method itself is rather subjective, since one must estimate the size of observed cells under microscope. This

makes it globally hardly comparable. Relative comparison within one study, performed by one person, is nevertheless possible. Moreover, the size of cyanobacterial cells in our case might have been affected by the moss desiccation. In our case also the uncertainty whether all cyanobacteria were successfully separated from the moss was present. Furthermore, cyanobacterial colonies tend to clamp together, which contributes to the inaccuracy as it is almost impossible to count all the individual cells. The distribution of these colonies on the moss is also most probably unequal. Another thing is the dispersion of the cyanobacteria on the moss, which was not taken into account and it was simply assumed, that cyanobacteria are equally distributed on the moss. Even though the approach of biovolume estimation was till now not applied to study the moss-associated cyanobacteria, it has been used for cyanobacterial abundance estimation for example in soil crusts and sediments (Pushkareva et al., 2017, 2018; Řeháková et al., 2010; Stibal et al., 2006). After all, other approaches are also problematic. (Arróniz-Crespo et al., 2014) tried to estimate abundance of moss-associated cyanobacteria by determining the amount of cyanobacterial pigment echinenone via HPLC. However pigment concentrations are in general influenced by several environmental factors and their relative concentration varies with light, temperature or availability of nutrients (Thrane et al., 2015). In case of carotenoids, where echinenone belongs to, the relative concentration is believed to be influenced by cellular stress (Zavrel et al., 2015). Therefore, the cyanobacterial abundance estimation based on echinenone measurement is also questionable. Another possibility would be to use quantitative real-time PCR (qPCR) to quantify for example *nifH* gene coding for the dinitrogenase reductase, component of the nitrogenase complex, as done by e.g. (Warshan et al., 2016). A rather time efficient method, however with only somewhat relative results. The quantification of target cyanobacteria can be estimated based on the concentration of target gene, in this case *nifH*, and the outcome is thus dependent on the accuracy of the target gene content measurement. Such an approach works relatively well in pure cultures, but can give inaccurate results for environmental samples (Kim et al., 2013).

3.3 Comparison of the cyanobacterial/microalgae biovolume between the wet meadow's micro-habitats

The estimated microalgae biovolume between different zones of the wet meadow was compared from sonicated moss samples (2 min, 60 % amplitude). The results are summarized in Table 11. Primarily, only cyanobacteria were found except of water zone, where green coccoid algae accounted for 3 % of the total cyanobacterial/microalgae biovolume (see Figure 9). Cyanobacterial biovolume was significantly bigger in water zone than in green and

red zones (see Figure 8). This trend was also valid for all identified cyanobacterial orders except of Chroococcales, which biovolume was significantly higher in water zone than in red one only. For all the zones, cyanobacteria of order Nostocales were the most abundant (see Figure 9), whereby *Nostoc* sp. genera and filamentous cyanobacteria, most probably belonging to *Tolypothrix* sp., were observed (for photo documentation see Appendix A). Concerning Oscillatoriales/Synechoccales order, primarily most likely *Leptolyngbya* sp. were observed. Within the Chroococcales order, it was most probably *Chroococcus* sp. and *Aphanothece* sp. However, as already mentioned earlier, the identification was difficult due to the broken cyanobacterial filaments after sonication and therefore rather provides an estimation.

Table 11
Summary of cyanobacterial/microalgae biovolume within the wet meadow zones

Zone		Green	Red	Water	ANOVA results
Total cyanobacterial/microalgae biovolume [μm^3 mg^{-1}]	Mean	3.31E+09	2.85E+09	6.37E+09	$F_{(2,6)} = 23.22$ $p = 0.0015$
	SD	6.20E+08	6.43E+08	7.89E+08	
	Tukey	a	a	b	
Cyanobacterial biovolume [μm^3 mg^{-1}]	Mean	3.31E+09	2.85E+09	6.18E+09	$F_{(2,6)} = 20.38$ $p = 0.00211$
	SD	6.20E+08	6.43E+08	8.02E+08	
	Tukey	a	a	b	
Chroococcales biovolume [μm^3 mg^{-1}]	Mean	1.65E+07	3.07E+07	1.60E+08	$F_{(2,6)} = 6.254$ $p = 0.0341$
	SD	1.29E+07	1.50E+07	9.30E+07	
	Tukey	a	ab	b	
Oscillatoriales/Synechoccales biovolume [μm^3 mg^{-1}]	Mean	2.50E+08	4.00E+07	7.89E+08	$F_{(2,6)} = 17.93$ $p = 0.00295$
	SD	1.74E+08	4.17E+07	2.07E+08	
	Tukey	a	a	b	
Nostocales biovolume [μm^3 mg^{-1}]	Mean	3.04E+09	2.77E+09	5.23E+09	$F_{(2,6)} = 14.82$ $p = 0.00477$
	SD	4.51E+08	6.14E+08	7.28E+08	
	Tukey	a	a	b	
Microalgae biovolume [μm^3 mg^{-1}]	Mean	0	0	1.89E+08	$F_{(2,6)} = 292$ $p = 1.05e-06$
	SD	0	0	1.95E+07	
	Tukey	a	a	b	

SD… standard deviation; significant differences in Tuckey HSD test are indicated by different letters, n=3.

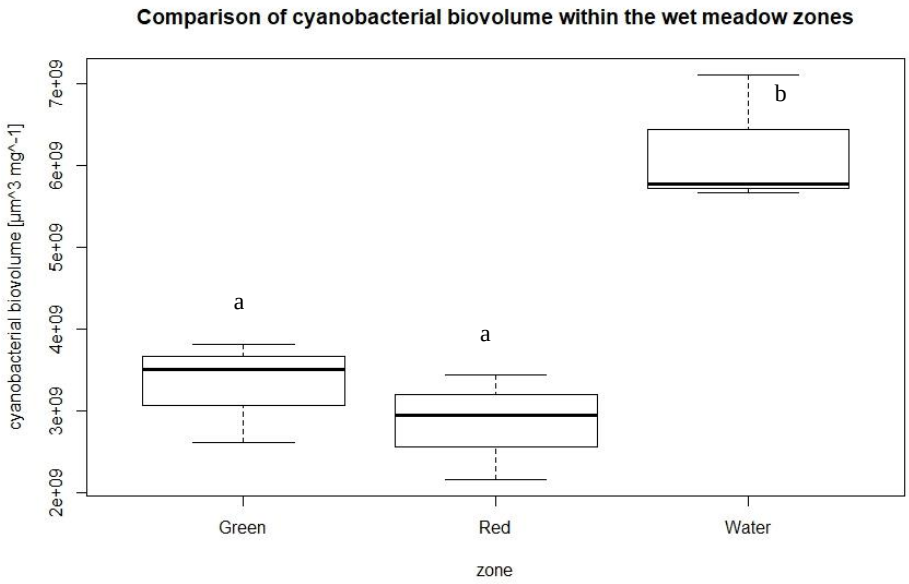

Figure 8: Boxplot comparing cyanobacterial biovolume between wet meadow zones. p-value of Anova test p=0.00211; n=3. Significant differences in Tuckey HSD test are indicated by different letters.

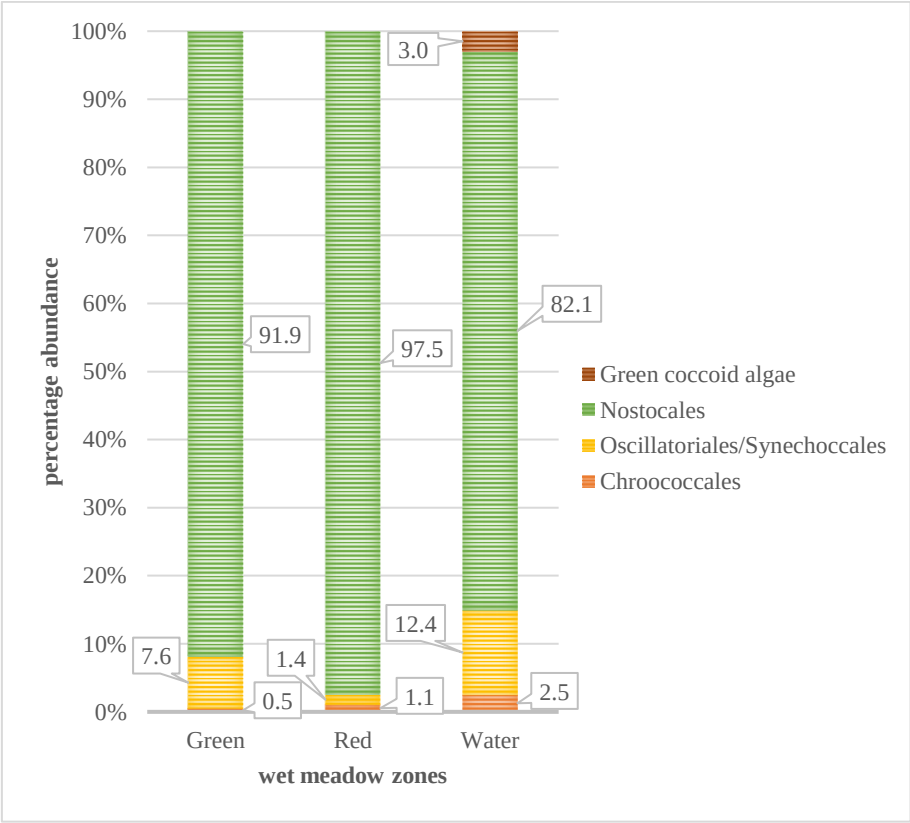

Figure 9: Percentage composition of cyanobacterial/microalgae orders in different parts of wet meadow.

Up to now, all reported moss-associated cyanobacteria belonged to the order Nostocales, which would be in accordance with our results, showing Nostocales as the most abundant. First report about such an association was made by (DeLuca et al., 2002), who observed *Nostoc* sp. association with feather moss from boreal forests of northern Scandinavia. Later

on, besides *Nostoc* sp., (Jean et al., 2012) also observed feather moss associated *Calothrix* sp. in North Carolina (USA), and (Gentili et al., 2005; Houle et al., 2006), additionally to *Nostoc* sp. and *Calothrix* sp. reported also *Stigonema* sp. from boreal forests of northern Scandinavia and Quebec, respectively. (Ininbergs et al., 2011) reported *Nostoc* sp., *Cylindrospermum* sp. and *Stigonema* sp. from northern Scandinavia. In contrast, (Lindo & Whiteley, 2011) didn't observe moss-associated *Nostoc* sp. in coastal rain forest of Vancouver Island (British Columbia, Canada), but instead reported only *Scytonema* sp. However contrarily to our results, cyanobacteria of order Chroococcales and Oscillatoriales/Synechoccales and green coccoid algae were till now, not reported as moss-associated. In contrast to our results and other studies of moss-tightly associated cyanobacteria and microalgae, (Lesniak, 2012), who studied moss-loosely-associated microalgae and cyanobacteria from water of wrung moss samples, reported 10 different diatom genera of significant abundance, and in general higher cyanobacterial species richness with *Nostoc* sp., *Leptolyngbya* sp. and *Microchaete* sp. representing the most abundant genera. Results of the post-freezing experiment (see Table 10), where primarily cyanobacteria of the order Nostocales were found after the post-freezing, together with above mentioned findings, suggests that only cyanobacteria of the order Nostocales form tight association with mosses. This would supports findings of (Bay et al., 2013), who suggested that the host-mosses are the key players in choosing their cyanobacterial partners and choose cyanobacteria that are able to fix atmospheric nitrogen (such as cyanobacteria of Nostocales order).

3.4 Sanger sequencing

3.4.1 First try

Even though specific cyanobacteria primer pair CYA359F/CYA781R (Nübel et al., 1997) was used for the amplification of the 16 S rRNA gene from two smashed moss samples for the first pilot test, mainly moss chloroplasts were amplified (see Table 12). In seven out of eight samples for the smashed moss samples ("W_M" and "R_M"), the moss chloroplasts were most likely detected according to BLAST search. The water sample however seemed more promising, since no moss chloroplasts were amplified. Instead, three cyanobacteria sequences with the fourth sequence being unfortunately of poor quality were detected.

Table 12
Blast results of the first pilot test using primer pair CYA359F/CYA781R

Sample	Cyanobacteria	Moss	Bad sequence
W_M	1	3	0
R_M	0	4	0
R_W	3	0	1

The first index in the sample name refers to the wet meadow zone where "W" stands for water zone and "R" for red zone. The second index refers to the sample type where "M" stands for the moss and "W" for water sample.

(Nübel et al., 1997) developed the cyanobacteria-specific primers based on published 16S rRNA sequences as a tool for cyanobacteria-biodiversity studies. They are still nowadays used in studies for specific cyanobacteria amplification (see e.g. Arróniz-Crespo et al., 2014; Pushkareva et al., 2015, 2018). However, (Burja et al., 2001) found out that they are not only specific for cyanobacterial, but target also a eukaryotic 16S rRNA gene fragment, which is located within the chloroplast of algae and land plants. This can be explained by the endosymbiotic theory according to which plant chloroplasts are originating from a common cyanobacterial ancestor (Kutschera & Niklas, 2005). According to results of (Burja et al., 2001), with the primer pair CYA359F/CYA781R, about 75 % of Streptophyla (land plants where mosses also belong to) can be targeted. For the primer pair CYA106F/CYA781R, it would be about 45 %. To increase the primer annealing temperature to try to target only cyanobacteria would therefore not help and the idea was rejected. Therefore, to try to mechanically dislodge cyanobacteria from the moss seemed like the best option to improve the primer specificity, since from the water samples the cyanobacteria were well amplified.

3.4.2 Second try

In general, the pre-treatment by vortex and sonication did improve the primer specificity. The primer pairs CYA106F/CYA781R and CYA359F/CYA781R revealed to be comparably efficient, amplifying two to three cyanobacteria out of four sequences from the pre-treated samples (see Table 13). From the remaining smashed moss sample, one cyanobacteria and three mosses were amplified. This confirms the hypothesis discussed in 3.2.1, that vortex and sonication of the samples don't separate all cyanobacteria from the mosses. The primer pairs CYA106F/CYA1444R and CYA359F/CYA1444R were shown to be rather inefficient, amplifying mainly bacteria for all types of samples, except of the remaining smashed sample, where again, mainly moss chloroplasts were amplified. CYA1444R primer was recommended by professor Ólafur Andrésson (personal correspondence, September 2016) as he is using it

for the cyanobacterial amplification from moss samples in Iceland. Perhaps it is due to the different biotope with different types of bacteria, which are not amplified by this primer, that it didn't work in our case. Generally, the amplification of bacteria can be explained by the universality of the 16S rRNA gene.

Table 13
Comparison of different primer results

Sample[a]	Forward primer	Reverse primer	Cyanobacteria	Plant[b]	Bacteria	Bad sequence
G_V	106	781Ra/b	3	0	1	0
G_S	106	781Ra/b	3	1	0	0
G_M	106	781Ra/b	1	3	0	0
R_V	106	781Ra/b	3	1	0	0
R_S	106	781Ra/b	3	0	1	0
G_V	359	781Ra/b	2	0	1	1
G_S	359	781Ra/b	3	1	0	0
R_V	359	781Ra/b	2	0	2	0
R_S	359	781Ra/b	2	0	2	0
G_S	106	1444	0	0	4	0
G_M	106	1444	0	3	1	0
G_V	359	1444	0	0	4	0
G_S	359	1444	0	1	3	0
G_M	359	1444	1	1	1	1

[a] The first index in the sample name refers to the wet meadow zone where "G" stands for green zone and "R" for red zone. The second index refers to the sample type preparation, where "V" stands for vortexed sample, "S" for sonicated sample and "M" for the remaining smashed moss sample.

[b] Mainly mosses.

The problem of losing information due to incomplete separation of cyanobacteria from the mosses and due to non-cyanobacterial amplification could be to some extent solved by using next generation sequencing. The ideal option would probably be to isolate DNA from two subsamples of every moss sample, first sonicated and secondly the remaining smashed moss. One can gain considerable amount of sequences from one sample using next generation sequencing. Therefore, even if the majority of amplified sequences from the remaining smashed sample would be of non-cyanobacterial origin, additional information, i.e. small number of cyanobacterial sequences, could be still gained. The price-performance ratio changed and nowadays the next generation sequencing would be favoured over the used Sanger sequencing technique.

Another possibility would be to try to amplify *nifH* gene instead of 16S rRNA, as for example in the studies of (Ininbergs et al., 2011; Warshan et al., 2016). However, since

the *nifH* gene sequencing is less routine than the one of 16S rRNA gene, public databases contain less data. This leads to another important issue concerning public databases. Incorrect taxonomic cyanobacterial sequence classification represents an important problem in public databases (Komárek, 2006; Willame et al., 2006). This can further lead to great misinterpretations and basically spreads as a chain reaction when researches, based on incorrectly classified sequences in public databases, upload new ones under incorrect classification.

3.4.3 Phylogenetic analysis

From resulting 21 sequences most likely belonging to the cyanobacterial phylum after BLAST check, 12 different OTUs were found and one sequence was classified as chimeric using UPARSE (Edgar, 2013). The representative sequence for each OTU can be found in Appendix C and Table 14 summarizes the corresponding best isolate hits from BLAST search against Nucleotide collection (nr/nt) of the NCBI database.

Table 14
List of the best isolate sequence hits for individual OTUs

OTU	# seq.[a]	Best sequence match isolate hit					
		Name	Order	Query cover	E value	Identity	Accession
OTU 1	4	*Nodosilinea* sp.	Synechococcales	100%	0	99%	LN849924.1
		Leptolyngbya sp.	Synechococcales	100%	0	99%	HM217072.1
		Plectonema sp.	Oscillatoriales	100%	0	99%	AY430152.1
		Phormidium angustissimum	Oscillatoriales	100%	0	99%	KX818204.1
OTU 2	4	*Leptolyngbya frigida*	Synechococcales	100%	0	99%	AY493612.1
		Leptolyngbya sp.	Synechococcales	100%	0	99%	AY790851.1
OTU 3	3	*Tolypothrix* sp.	Nostocales	100%	0	99%	LM992903.1
OTU 4	2	*Nostoc* sp.	Nostocales	100%	0	98%	GU062469.1
OTU 5	2	*Leptolyngbya nostocorum*	Synechococcales	100%	0	99%	JX413496.1
OTU 6	1	*Leptolyngbya foveolarum*	Synechococcales	100%	8.00E-157	93%	KC463180.1
OTU 7	1	*Aphanothece sacrum*	Chroococcales	100%	2.00E-168	95%	LC229081.1
		Aphanothece stagnina	Chroococcales	100%	2.00E-168	95%	FR848374.1
OTU 8	1	*Nostoc* sp.	Nostocales	100%	0	99%	KF704323.1
OTU 9	1	*Nostoc* sp.	Nostocales	100%	0	100%	EU022706.1
OTU 10	1	*Phormidesmis* sp.	Synechococcales	100%	1.00E-150	93%	KU219738.1
OTU 11	1	*Leptolyngbya antarctica*	Synechococcales	100%	1.00E-159	94%	AY493607.1
OTU 12	1	*Nostoc* sp.	Nostocales	83%	4.00E-135	95%	GU062469.1

[a] number of sequences per group

It must be noted that these 21 sequences represent only limited amount of information about the cyanobacterial community. It can be assumed that all the cyanobacteria belong to the orders of Nostocales, Synechococcales, Oscillatoriales and Chroococcales, which is in accordance to the results obtained from microscopy. The best BLAST hits for OTU 1 is not consistent, indicating both Synechococcales and Oscillatoriales.

The sequenced locus was unfortunately too short (380 bp) to allow a robust phylogenetic analysis. The phylogenetic signal was weak and bootstrap support was mostly low (<70%). The resulting phylogenetic tree is therefore not included in this thesis. The primer pair CYA359F/CYA781R was however used by other studies describing cyanobacterial communities. (Arróniz-Crespo et al., 2014) used the same primer pair to amplify cyanobacteria from vortexed moss samples. After reducing the sequences to OTUs, he performed a maximum-likelihood phylogenetic tree from the same locus length (380 bp) with better bootstrap support. However, he didn't find any clear and certain relationships in the phylogenetic tree either. (Pessi et al., 2016) used the same primer pair to classify cyanobacterial OTUs to genus level from aquatic mats. Similarly, (Pushkareva et al., 2015,2018) used this primer pair to describe cyanobacterial communities from soil crusts and classified resulting OTUs into order's level. Nevertheless, a solid cyanobacterial phylogenetic analysis requires sequences around at least 1000 bp long.

4 Conclusion

Moss-associated cyanobacteria are key players in the ecology of wet meadows in the High Arctic. They represent a dominant source of nitrogen and thus studying these associations can expand our knowledge about the wet meadow functioning.

In this thesis, three different methods to mechanically dislodge cyanobacteria from mosses were investigated. Unfortunately, all three methods were to some extent destructive and made the subsequent identification of cyanobacteria and biovolume estimation difficult. Nevertheless, the degree of destructiveness was the highest in the method using liquid nitrogen. With the method of sonication and vortex, it was unfortunately not possible to dislodge all cyanobacteria from the mosses. Despite that fact, the method of sonication (60% amplitude, 2 min) was favoured over the liquid nitrogen and was determined as the method of choice. Using this method, cyanobacterial/microalgae biovolume of three different micro-environments of the wet meadow were compared. Cyanobacteria of the order Nostocales, with the ability to fix nitrogen, were revealed to be the most abundant in all three micro-environments.

Furthermore, Sanger pilot sequencing was performed by amplifying the 16S rRNA gene with cyanobacterial specific primers. The problem of amplification of moss chloroplasts instead of cyanobacteria was partly solved by pre-treatment of the moss samples by vortex or sonication prior to DNA isolation. The primer pairs CYA106F/CYA781R and CYA359F/CYA781R were shown as the most efficient. In total, 26 cyanobacterial sequences were obtained which were clustered into 12 different OTUs. Nevertheless, the method of choice for further investigation would be new generation sequencing, as the price-performance ratio became more favourable over time.

For future studies of the wet meadow, a combination of estimation of cyanobacterial/microalgae biovolume from moss samples treated with liquid nitrogen and next generation sequencing would be probably the best possibility how to estimate biomass of cyanobacteria that are able to fix atmospheric nitrogen. With the liquid nitrogen treatment, it seems that all moss-associated cyanobacteria can be quantified, but the exact identification is nearly impossible. With the help of next generation sequencing, the identification could become more precise. Moreover, more moss replicates should be analysed to increase the robustness of the results.

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Appendix A: Photo documentation

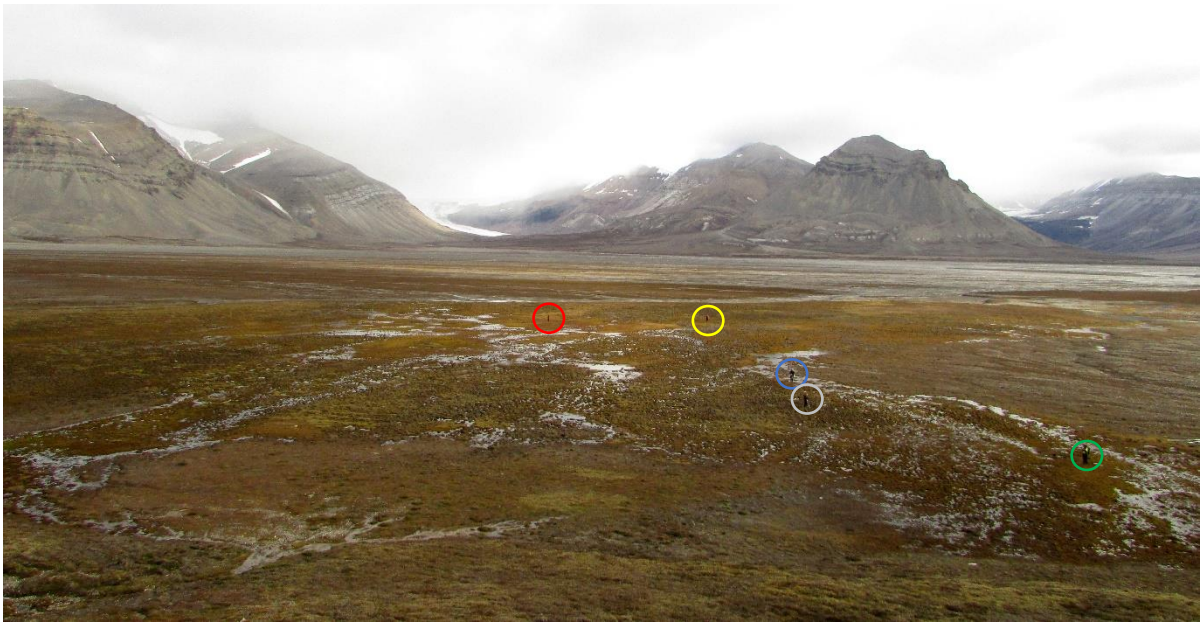

Figure 10: Studied wet meadow with 5 micro-environments as described by (Lesniak 2012): red, yellow, water, grey and green.

Figure 11: Wet meadow detail, typical hummock tundra.

Figure 12: Sampling detail n°1.

Figure 13: Sampling corer.

Figure 14: Sampling detail n°2.

Figure 15: Air-drying of samples.

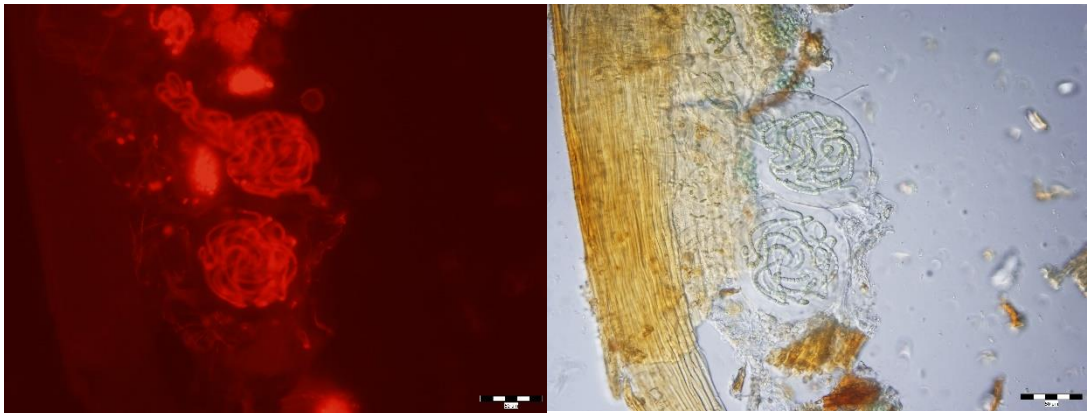

Figure 16: *Nostoc* sp. on moss fragment after sonication; scale bar = 50 µm.

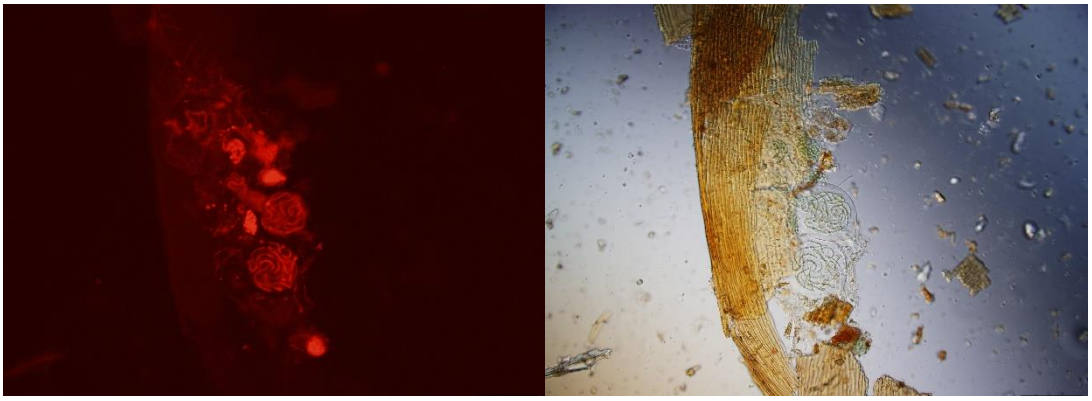

Figure 17: *Nostoc* sp. on moss fragment after sonication with smaller amplification; scale bar = 100 µm.

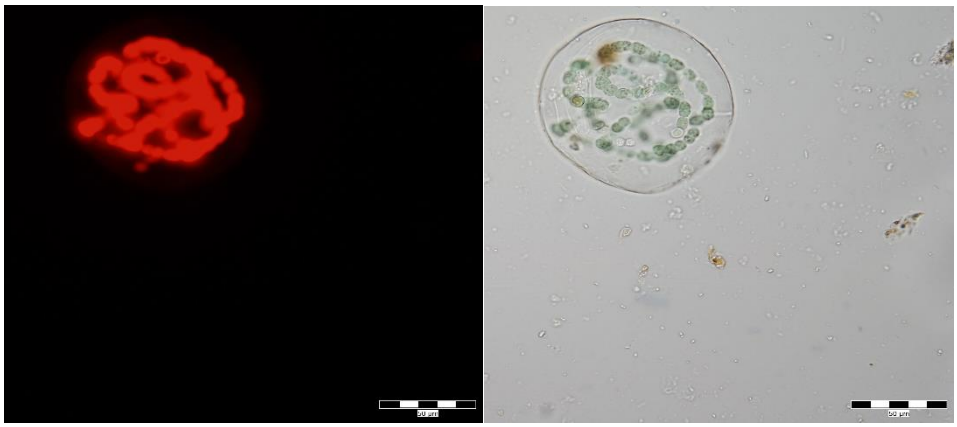

Figure 18: Cyanobacteria of Nostocales order (*Nostoc* sp.); scale bar = 50 µm.

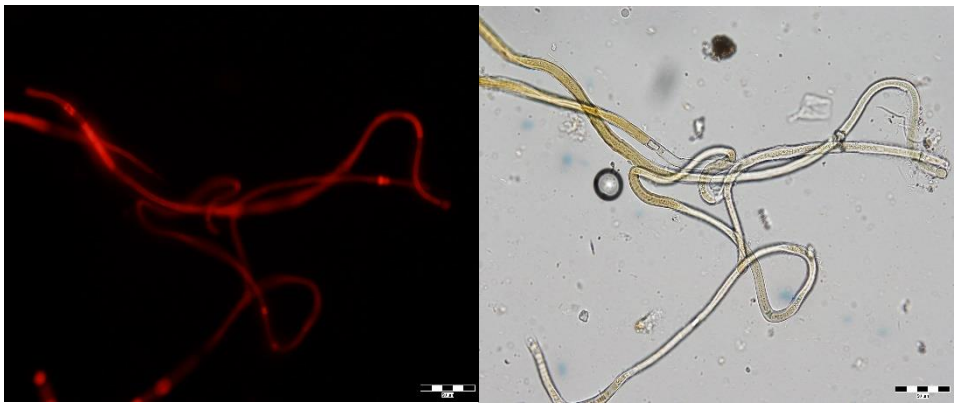

Figure 19: Cyanobacteria of Nostocales order (*Tolypothrix* sp.); scale bar = 50 µm.

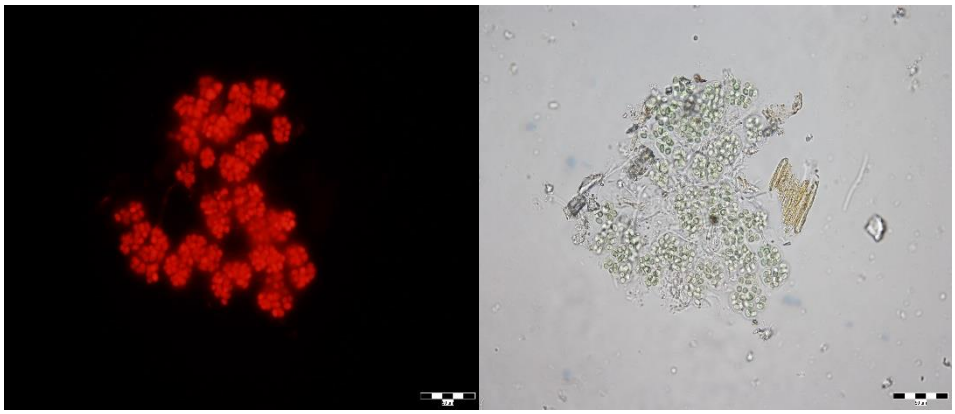

Figure 20: Cyanobacteria of Chroococcales order (*Aphanothece* sp.); scale bar = 50 µm.

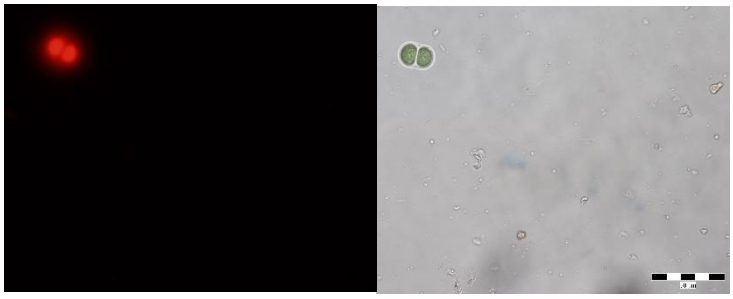

Figure 21: Cyanobacteria of Chroococcales order (*Chroococcus* sp.); scale bar = 50 µm.

Appendix B: Detailed results from the comparison of dislodging methods

Table 15
Summary of cyanobacteria/microalgae biovolume from the comparison of dislodging methods

		S1_20	S2_20	S1_60	S2_60	V	N2
Cyanobacterial biovolume [µm3 mg-1]	Mean	1.20E+09	1.54E+09	1.41E+09	2.55E+09	7.52E+08	3.27E+09
	SD	1.89E+08	2.95E+08	2.55E+08	2.50E+08	1.91E+08	3.53E+08
Chroococcales biovolume [µm3 mg-1]	Mean	4.82E+07	1.40E+08	1.40E+07	2.49E+06	4.51E+07	1.64E+07
	SD	4.73E+07	3.86E+07	3.97E+06	1.64E+06	3.09E+07	1.30E+07
Oscillatoriales/Synechoccales [µm3 mg-1]	Mean	3.53E+07	1.70E+08	6.94E+06	0	6.81E+07	5.00E+07
	SD	3.15E+07	6.74E+07	1.20E+07	0	4.97E+07	5.17E+07
Nostocales biovolume [µm3 mg-1]	Mean	1.10E+09	1.23E+09	1.39E+09	2.46E+09	6.31E+08	3.21E+09
	SD	1.84E+08	2.20E+08	2.67E+08	3.96E+08	1.37E+08	3.15E+08

Appendix C: Cluster representative sequences

>CL01|MOSTABUND|n=4/3
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CAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGCGGTTTATTAAGTCTGTTGTCAAAGCCCACAG
CTCAACTGTGGATCGGCAATGGAAACTGGTGAACTTGAGTGTGGTAGGGGTAGAGGGAATTCC-CGGTGTAG
C-GGTGAAATGCGTAGATATCGGGAAGAACACCAGTGGCGAAGGCGCTCTACTGGGCCACAACTGACGCTGA
-GGGACGAAAGCTAGGGGAGCG

>CL02|MOSTABUND|n=4/1
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AA-GATCTGACGGTACCTGATGAATCAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATG
CAAGCGTTATCCGGATTTATTGGGCGTAAAGCGTCCGCAGGTGGTTTACCAAGTCTGCTGTTAAAGCGCGAG
GCTTAACCTCGTACAGGCAGTGGAAACTGGGAGACTAGAGTGTGGTAGGGGTAGCGGGAATTCC-CAGTGTA
GC-GGTGAAATGCGTAGATATTGGGAAGAACACCAGCGGCGAAAGCGCGCTACTGGGCCATCACTGACACTC
A-GGGACGAAAGCTAGGGGAGCG

>CL03|MOSTABUND|n=3/1
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GCTCAACCTCATAAAGGCAGTGGAAACTACACGGCTAGAGTGCGTTCGGGGCAGAGGGAATTCC-TGGTGTA
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AGCTCAACTAGATAAGAGCAGTGGAAACTACATAGCTAGAGTACGTTCGGGGCAGAGGGAATTCC-TGGTGT
AGC-GGTGAAATGCGTAGAGATCAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTAGGCCGCAACTGACACT
GA-GGGACGAAAGCTAGGGGAGCG

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GGGACGAAAGCTAGGGGAGCG

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CAAGCGTTATCCGGAATCATTGGGCGTAAAGCGTCCGTAGGTGGTTTTTCAAGTCTGCTGTCAAAGCCTGGGG
CTTAACCCCAGATAGGCAGTGGAAACTGGGAGACTAGAGTTCGGTAGGGGTAGCGGGAATTCC-CAGTGTAG
C-GGTGAAATGCGTAGATATTGGGAAGAACATCGGTGGCGAAGGCGCGCTACTGGGCCGAAACTGACACTCA
CAGGACGAAAGCTAGGGGAGCG

>CL08|SINGLETON|n=1
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CAAGCGTTATCCGGAATGATTGGGCGTAAAGCGTCCGCAGGTGGCTATGTAAGTCTGCTGTTAAAGCGTCTA
GCTCAACTAGATAAAAGCAGTGGAAACTACAAGGCTAGAGTGCGTTCGGGGCAGAGGGAATTCC-TGGTGTA
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>CL09|SINGLETON|n=1
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>CL10|SINGLETON|n=1
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>CL11|SINGLETON|n=1
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>CL12|SINGLETON|n=1
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