School of Doctoral Studies in Biological Sciences
University of South Bohemia in České Budějovice
Faculty of Science

BIOLOGICAL SOIL CRUSTS OF COLD DESERTS OF W HIMALAYA

Ph.D. Thesis

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ANNOTATION

Proposed thesis is focused on the role of soil microbial crusts in the extreme environmental conditions of high-elevation cold desert of W Himalaya. Despite the importance of microbial soil crusts in arid soils, the biodiversity of their microbial communities, their role and function are still unclear. Our knowledge about functioning of these outlying ecosystems in this part of the world is still very insufficient in general. The area of Ladakh is perfect place for studying the microbial soil crust – arid climate and extreme elevation aroud 6000 m a.s.l. represents unique condition for well-developed soil crusts communities. The whole region is unaffected by human activities or plant invasions, so we can study soil crusts in pristine natural condition.

Our investigations is focused on soil microbial community of BSCc in Ladakh region. It combines range of aspects connected with BSCs such as taxonomical composition, changes of diversity and activity in relation to environmental condition. The thesis is the first compilation of studies concerned on microbial communities in area of Ladakh and one of the first work investigating the ecophysiology of BCSs in cold desert.

DECLARATION [IN CZECH]

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Kateřina Čapková ……..

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LIST OF PAPERS AND AUTHOR'S CONTRIBUTION

The thesis is based on the following papers:

I. **Janatková K.**, Řeháková K., Doležal J., Šimek M., Chlumská Z., Dvorský M., Kopecký M. (2013) Community structure of soil phototrophs along environmental gradients in arid Himalaya. Environmental Microbiology 15:2505-2516 (IF=6.2)
Kateřina Čapková (née Janatková) was responsible for sample collection in the field, data assembly, participated in experiment preparation and writing the manuscript.

II. Řeháková K., Doležal J., Chroňáková A., Krištůfek V., Kuchtová B., **Čapková K.**, Scharfen J., Čapek P. (2015) Bacterial community of cushion plant Thylacospermum ceaspitosum on elevational gradient in the Himalayan cold desert. Frontiers in Microbiology 6 (IF=3.79)
Kateřina Čapková was responsible for sample collection in the field, participated in data assembly and in writing of the manuscript.

III. **Čapková K**., Hauer T., Řeháková K., Doležal J. (2015): Some like it high! Phylogenic diversity of high-elevation cyanobacterial community from biological soil crusts of Western Himalaya.
Microbial Ecology 71:113-123 (IF=2.79)
Kateřina Čapková performed cultivation and isolation of the cultured strains, was responsible for molecular analyses and writing of the manuscript.

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CHAPTER I

General introduction

General introduction

Biological Soil Crusts (BSCs)

BSCs definition

Biological soil crusts are the community of organisms (cyanobacteria, green algae, bacterie, microfungi) living at the uppermost millimetres of soils. These microorganisms bound together soil particles and create compact soil crust and by doing so, they create specific microenvironment they live in.

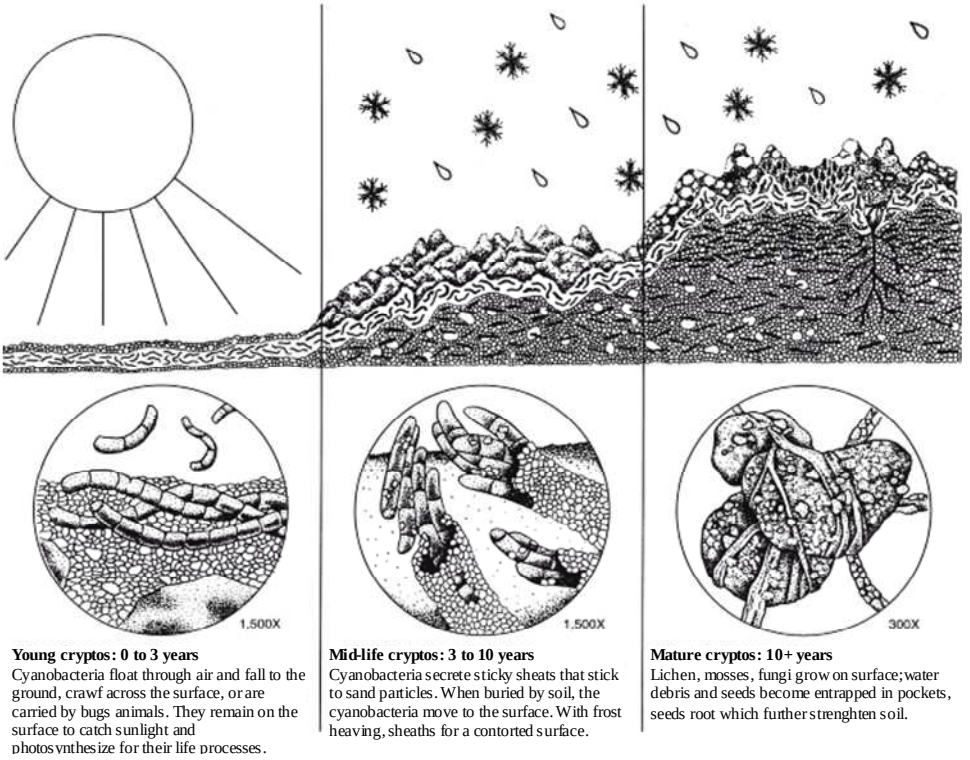


Fig. 1: Successional sequence for biological soil crusts. This example is for *Microcoleus vaginatus*-dominated crusts. Sequences in other ecoregions are similar but may involve different taxa. (picture from Belnap et al. 2001, Biological Soil Crusts: Ecology and Management)

Occurrence and importance of BSC

BSCs are an important component of the land surface of the arid and semi/arid regions around the world (West 1990). Physiological and structural adaptability of BSCs allows them succesfully colonize dry conditions of arid and semi/arid soils within wide range of temperatures (Belnap and Lange 2003). The most important adaptations for survival of BSCs in arid regions is their extraordinary ability to survive desiccation and structural variability which helps them to colonize wide ranges of temperatures. BSCs occure in hot deserts, where they form smooth and very thin moulds, as well as in cold climates where they tend to be much thicker and develop three-dimensional structures, up to several centimeters high, in response to freeze- thaw cycles, termed as rolling and pinnacled crusts (Belnap 2003).

Importance of BSCs in arid ecosystems lies in their ability to fix soil particles together and by doing so reduce soil erosion, and to decompose organic detritus, photosynthesize and fix atmospheric nitrogen. All these processes higly influenced multiple physico-chemical properties of the soil colonized by BSCs. The main function of BSC is prevention of soil erosion as mentioned, but similarily important is soil water retention and soil fertility improvement (Belnap and Lange 2003, Johansen and Schubert 2001). By their metabolic activity, cyanobacterial and microalgal communities (as first colonizers of high-mountain soils) affects nutrient availability for vascular plants and may improve water and nutrient status of plants growing inside BSCs moulds (Belnap and Harper 1995, Řeháková et al. 2011).

Recent understanding of BSC importance for ecosystems provoked deeper research of the biodiversity and functions of biological soil crusts, especially with respect to climate change, glacial retreat and vascular plant distributional shift (e.g. Schmidt et al. 2008; Lamb et al. 2011). Schmidt et al. (2009) research pointed out that the presence of microbial crusts can strongly promote the

occurrence and development of the vegetation after glacier retreat. Understanding the interactions between vegetation and BCS may help to alleviate the ongoing loss of vegetation and desertification in many desert regions around the World (Geist and Lambin 2004, Salvati et al. 2012).

Taxonomy and diversity of microbial communities of BSCs

Taxonomy and diversity of microbial communities of BSCs define BSCs features and functions. BSCs contain phototrophic and heterotrophic microorganisms.

(i) **Phototrophic microorganisms**. Cyanobacteria and eukaryotic microalgae – are important components of desert soil environments, especially in young successional stages (Nemergut et al. 2007; Řeháková et al. 2011). Such microbial communities play a key role in colonizing barren substrates, even above the actual vascular plant altitudinal limit. Here, together with lichens, mosses and microfungi, they persist in a complex structure of biological soil crusts (BSCs) (Paerl et al. 2000; Belnap and Lange 2001). The biomass of BSC is mainly represented by cyanobacterial taxa, which can be found all over the world. *Microcoleus vaginatus*, *Phormidium* sp. and *Nostoc* sp. have already been commonly reported from arid and semiarid environments (Garcia-Pichel et al. 2001; Yeager et al., 2004; Nagy et al., 2005; Freeman et al., 2009; Abed et al., 2010; Řeháková et al. 2011). Representatives of the order Chrooccocales (*Chroococcus* sp. and *Cyanothece* sp.) account for a minor part of biomass, similarly as in the biotopes from the Antarctic (Komárek et al. 2008). These two taxa are rarely reported from other desert soils in the world. *Chroococcus* creates lesser biomass of phototrophs mainly in the cold types of deserts such as Colorado Plateau or Great Basin desert (Rosentreter and Belnap 2001) or it is reported from temperate zone from alkaline soils in Ohio

(Rosentreter and Belnap 2001) or salt pasture in Hungary (Büdel 2001).

The prevalence of cyanobacteria in high altitude is caused by a combination of factors, particularly high soil pH, and high UV radiation. Numerous records for cyanobacteria in freshwater and soils indicate that their diversity and abundance are greatest at higher pH values, though the reasons for their success under these conditions are still unclear. *Microcoleus* and *Nostoc,* most abundant genera in BSCs from Ladakh, produce a thick mucilaginous sheath. Besides *Nostoc* has ability to produce scytonemin, which caused brown pigmentations of the sheath (Bowker et al. 2002; Matsui et al. 2012). Both these features protect them against the high UV radiation (Matsui et al. 2012) and desiccation risks. The genus *Phormidium*, other abundant cyanobacteria in microbial community, produces sheaths only occasionally, but it has another protective strategy. *Phormidium* is able to migrate from the surface to the deeper parts of soil when the light intensity or moisture level is not suitable for its growth; *Microcoleus* has also this ability (Garcia-Pichel and Castenholz, 2001).The BSCs are complex communities, where one member of ecosystem plays some role for the other member. The genus *Nostoc*, which grows in the surface of crusts, could protect other, less pigmented taxa such as *Microcoleus* and *Phormidium* (Bowker et al. 2002). All these adaptations facilitate their survival in soils of subnival zones and enable them to become dominant components of the phototrophic community.

(ii) **Heterotrophic microorganisms.** Although bacterial assemblages are important components of soil in arid ecosystems as well, the knowledge about composition, life strategies, and environmental drivers is still fragmentary, especially in remote high-elevation mountains. Bacteria are a common and fundamental part of microbial communities and constitute a major proportion of biodiversity in soil ecosystems (Gans et al. 2005; Griffiths et al. 2011). Vascular plants or phototrophic microorganisms and soil

microbial assemblages are indirectly connected through the soil substrate and interaction, which influence the soil fertility (Bardgett and Walker 2004; van der Heij den et al. 2008). Higher plants contribute to resources through litter fall and rhizodeposition and can also provide suitable microclimatic conditions to the belowground organisms. Microbial assemblages are able to convert the inaccessible minerals to accessible one for plants (Clarholm 1985; Carlson et al. 2010).

UV-protection of BSCs

The microenvironment in which the microbial communities of BSCs are living – the uppermost millimetres of the topsoil-is one of the most extreme habitats. They are exposed to high and low temperatures, extensive dessication and excessive sun radiation (Belnap and Lange 2001). Crust-forming cyanobacteria tolerate high levels of UV radiation and produce a wide range of UV protectants (e.g., scytonemin, carotenoids, or mycosporine-like amino acids). This is the most important benefit, which cuase dominant cyanobacterial abundance in BSCs. Cyanobacteria originate from the Precambrian era when the ozone shield was absent; they presumably faced high fluxes of UV radiation, which likely acted as an evolutionary pressure leading to the selection for efficient mechanisms for protection against UV radiation (Sinha et al 2002). Cyanobacterial tolerance of intense sunlight including UV radiation may have contributed to their success in colonizing high-altitude and high-latitude environments. Because extreme UV irradiance is a fundamental component of high altitude environment, BSCs pigment concentration is critical physiological trait that allow survival of microbial community. Even though occurence of UV protective pigments in cyanobacteria are known, it is less clear how these pigments can protect and affect activity of the whole microbial community of BSCs. This knowledge is, however, critical in order to evaluate *in-situ* functioning of BSCs.

Nitrogenase activity of BSCs

Limited productivity of vascular plants in arid regions generally results from low soil nutritient content. Nitrogen is the second limiting factor in deserts (after water availability; Evenari 1985), and biological soil crusts can be the dominant source of N for arid land ecosystems. Soil bacteria and cyanobacteria can contribute to soil fertility by fixing atmospheric nitrogen into a form that is available to higher plants (NH_4^+). Cyanobacteria can be heterocystic (i.e., they have special cells with anaerobic conditions inside where nitrogen fixation takes place), or non-heterocystic (i.e., they lack these specialized cells). For nitrogen fixation to occur in non-heterocystic cyanobacteria, the organisms need to be in an anaerobic environment, created by layering of cyanobacterial filaments just beneath the soil surface. (Belnap et al. 2001).

Nitrogen fixated by cyanobacteria is available for vascular plants in short term (Mayland et.al 1966 ; Mayland and MacIntosh 1996; Stewart 1967). The crust patches function mainly as a source (supplier) of nitrogen, and the vegetated patches function as sinks (consumers) of nitrogen. Five to 88% of nitrogen fixed by Nostoc has been shown to leak into the surrounding substrate (Magee and Burris 1954; Silvester et al. 1996; Belnap et al. 1997). The flow of nitrogen between the two patch types and the internal flows inside the vegetated patches, contribute to the increase of productivity and diversity in the vegetated patches and to their role as ‘‘Island of fertility’’ in desert landscapes (Smith et al. 1994; West and Skujins 1977). Vascular plants growing in biologically crusted soils show higher tissue concentrations of nitrogen than plants grown in uncrusted soils (Harper and Pendleton 1993; Belnap 1994, 1995; Belnap and Harper 1995).

Nitrogen fixation is highly dependent on past and present water and light regimes, as well as species composition (Rychert et al. 1978; Belnap 1994). In dry high altitude ecosystem, the similar functional relationship between soil crusts and plants was not

observed until now. It might be argued, that nitogenase activity of BSCs in high altitude ecosystems is drived by other environmental factors than in deserts. Above mentioned UV protection pigments might be one of those as high UV radiation levels deactivate cyanobacterial nitrogenase (Kumar et al., 2003).

Metabolic activity of Biological soil crusts

In arid regions, such as the Tibetan plateau, the activity of BSCs could be crucial for the sustainability and development of other organisms. BSCs make essential nutrients in the environment available by their activity. It is obvious, that BSCs can do it only when they are in active state, which is limited to brief periods when precipitation or dew hydrates microbial cells and allows metabolic activity of the otherwise dormant biota (Garcia-Pichel and Belnap 1996; Lange 2001). The same should be true also for BSCs in high altitude deserts. In addition to water availability, extreme temperature fluctuation are another environmental factor that would largely affects microbial metabolic activity. These two factors create a constrains of in-situ microbial community. In order to quantify functioning of BSCs at large scale and its ultimate connection to other organisms development and activity, it is neccesary to indentify combination of water availability and temperature, which allow BSCs activity. If for example, photosynthetic and heterotrophic activity of BSC microbial community is restricted to only few days in the year, when environmental conditions increase above the suboptimal level, functioning of BSCs as support for other organisms has to be revisited. In order to do so, laboratory experiments with high gradianets of temperature and water availability connected to modelling in-situ activity are needed.

Objectives of the thesis

Despite the importance of BSCs in arid and semiarid soils, which represent more than 1/3 of land's surface, our knowledge about their biodiversity and functions is still sparse as we identified in previous text and and therefore it requires further attention. Since our current knowledge of BSC function is mostly based on studies performed in Europe and North America, although the largest and highest mountainous areas are located in Asia in Himalaya region. Our knowledge about functioning of these outlying ecosystems in this part of the world is still very insufficient as BSC community has not been investigated in W Himalaya yet. On the Tibetan Plateau, which covers an area of almost 14 times as large as the area of the European Alps, extensive areas of subnival and alpine climatic zones host a unique and relatively species-rich flora (Miehe et al. 2011) and well develop BSCs with complex microbial communities (Řeháková et al. 2011). Proposed thesis should fill out this gap as it is the first attempt to identify the role of soil microbial crusts in the extreme conditions of high-elevation cold desert of W Himalaya in Ladakh (Jammu & Kasmir state, India).

The main objective of this thesis are: (i) to assess the taxonomic composition and biovolume of microbial communities of soil crusts , (ii) to find out whether diversity of phototrophs differs across an elevational gradient, (iii) to investigate how is the species compostion linked to soil physico-chemical properties, climatic conditions and vegetation cover, iv) make the first view to how can biological soil crusts contribute the soil evironmnet by nitrogen

Study area

The study area is situated in W Himalaya, Ladakh. Ladakh, for long centuries an independent Buddhist kingdom, has now been a part of the Republic of India, state Jammu & Kashmir.The Ladakh area is delimited by the Eastern Karakorum Range in the north and the Great Himalaya Range in the south, forming the southwestern extension of the Tibetan Plateau. Ladakh is characterized by an arid cold-desert environment because it lies in the rain-shadow of the Himalayas, which poses a barrier to seasonal monsoon precipitations. The climate is therefore generally arid with mean annual precipitations as low as 50–100 mm (Hartmann 1983; Wang 1988). At lower and middle elevations, evaporation exceeds precipitation. Elevation ranges from 3,550 m at the bottom of the Indus Valley to 7,672 m above sea level (m a.s.l.) with vascular plants occurring up to 6,060 m (near snowline). The mean monthly temperature rises above 0°C from June to August only and winter temperatures can drop below -30°C (Klimeš and Doležal 2010).

The fieldwork was conducted at two sites in two mountain areas of Ladakh: the Nubra Valley site (34°45′N, 77°35′E) is located in the Eastern Karakoram range; the Chamser Kangri site is in the southwestern extension of the Tibetan Plateau above Tso Moriri Lake (32°59‘N, 78°24’E) (see the map in Appendix). The Tibetan Plateau consists mainly of siliceous rocks (Precambrian granites, Tso Morari gneiss) (Epard 2008) the Nubra Valley consists mainly of granitoids (Philips 2008).

Soil samples were collected along altitudinal transects at both mountain sites. The transect at the Nubra Valley site was 9 km long, 4,600– 5,200 m a.s.l. The transect at the Chamser Kangri site was 12 km long, 5,300 –6,000 m a.s. l.. The altitudinal zonation of vegetation included steppes and semideserts at lower elevations (collectively referred to as steppes hereafter), and alpine meadows, screes, and the subnival zone close to glaciers. Screes represent a habitat that partly overlaps in altitudinal range with

alpine meadows and the subnival zone. The cover of vascular plant vegetation ranged from 0% to 50%.

Activities of Czech scientists in this part of Transhimalaya started in 1989 with an expedition of Czech Academy of Sciences. One of the participants, a botanist Leoš Klimeš, then decided for a systematic exploration of this little-known region and visited Ladakh regularly since 1997. His main goal was a thorough mapping of vascular flora and preparation of a modern and complete Flora of Ladakh. He was also interested in ecological issues such as clonality in plants and conducted several experiments. However, after his unexplained disappearence somewhere in the midst of the remote and wild part of Zanskar most of his work remained unfinished.

Since 2008, the research activities go on under the leadership of Jiří Doležal from the Institute of Botany in Třeboň. Main areas of research include monitoring of vegetation changes, nutrient economy in plants, relationship between vascular plants and soil microbial communities and other ecological topics concerning high altitudes.

Results and conclusions

The research of BSCs is a part of the multidisciplinary project which has been engaged in order to investigate vegetation changes, nutrient economy of plants, relationship between vascular plants and soil microbial communities and other relevant ecological features of extreme altitudes. Our research activities in Ladakh begun in 2008. Since this time, we have determined the microbial diversity of the BSCs along altitudinal gradient, started to investigate selected ecophysiological traits that allow cyanobacterial survival in subnival zone and finally we have started with determintion of the metabolic activity of microbial communities composing biological soil crusts.

The first aim of our investigation was to determine the biodiversity of microorganisms which forms the crusts, because no information about BSCs from this area were available. This part of the research was succesfully completed and represents the main part of proposed thesis (Chapter II). Our results revealed predominance of well-developed cyanobacterial community in BSCs, dominated by heterocytous *Nosto*c and diazotrophic *Microcoleus*. We also found that the species diversity of cyanobacteria and eukaryotic algae is relatively low. However, despite low biodiversity, the results of phylogenic analyses identified new genera.

In general, we found out that the biomass of cyanobacteria increase with altitude – so that the cyanobacterial abundance is highest at the uppermost elevations around 5900 m a.s.l. in the most extreme conditions (Chapter I, III). The observed trend may be caused by several factors and one of them could be the ability of cyanobacteria to product UV-protective pigments, which can facilitate their survival in soils of subnival zones and enable them to become dominant components of the microbial community. This led us to analyse the pigmet content of microbial community along the altitudinal gradient and test if the pigment content is one of the factors truly helping to cyanobacteria survive in high altitudes in such a high biomass. We used quantitative and qualitative HPLC analysis and extracted eight pigments from the composite soil samples including scytonemin, myxoxanthophyll, oscillofukosid, zeaxanthin, canthaxanthin, echinenon, b-karoten and surprisingly bacteriochlorophyll-a as well (it is very likely one of the first evidence of bacteriochlorophyll from desert soils). Currently, we are going to explore whether there is any trends in pigment production along to elevation gradient or changes of microbial communities biomass from BSCs.

Cyanobacteria are known of capability to sequester atmospheric nitrogen, therefore we further determined BSCs contribution to nitrogen input into this subnival ecosystem. Results of the

investigation of potential BSCs nitrogenase activity are presented as a second part of the thesis (Chapter I). We performed the pilot study about the potencial nitrogenaze activity of BSCs in controled laboratory conditions. The results showed relatively low activity compared to the temperate ecosystems. Nevertheless, it indicated that the BSCs contributed to the nitrogen cycle in subnival and alpine environment, mostly in the altitude 5700 m a.s.l.

Currently, we are focusing on estimation of potential photosyntetic and heterotrophic activity of BSCs from high-altitude cold deserts of Tibetian Plateau under laboratory conditions as well as in-situ conditions. Based on literature, temperature and moisture are the most important factors influencing the metabolic activity of microbial communities of BSCs. Therefore, we measured production and consumption of CO2 and O2 by BSCs in short term incubation experiment with manipulated temperature (4 – 30°C), moisture level (15 – 100% WHC) and light intensity (light, dark). Incubation setup allowed us to separate activity of heterotrophs and autotrophs from each other, substracting heterotrophic activity from autotrophic activity under light. We assumed, that the heterotrophic activity is similar under dark and under light. CO2/O2 consumption/production served as a proxy of phototrophic or heterotrophic metabolic activity. Heterotrophic activity is connected to CO2 release in respiratory cycle while O2 is consumed. Thus, CO2 production and O2 consumption is proxy of heterotrophic activity. In opposite phototrophs immobilize atmospheric CO2 in calvin cycle, while producing O2. Therefore, CO2 consumption and O2 production (after substracting heterotrophic activity) is proxy of phototrophic activity. Phototrophic activity can be according to commonly used definition also defined as a net primary production.

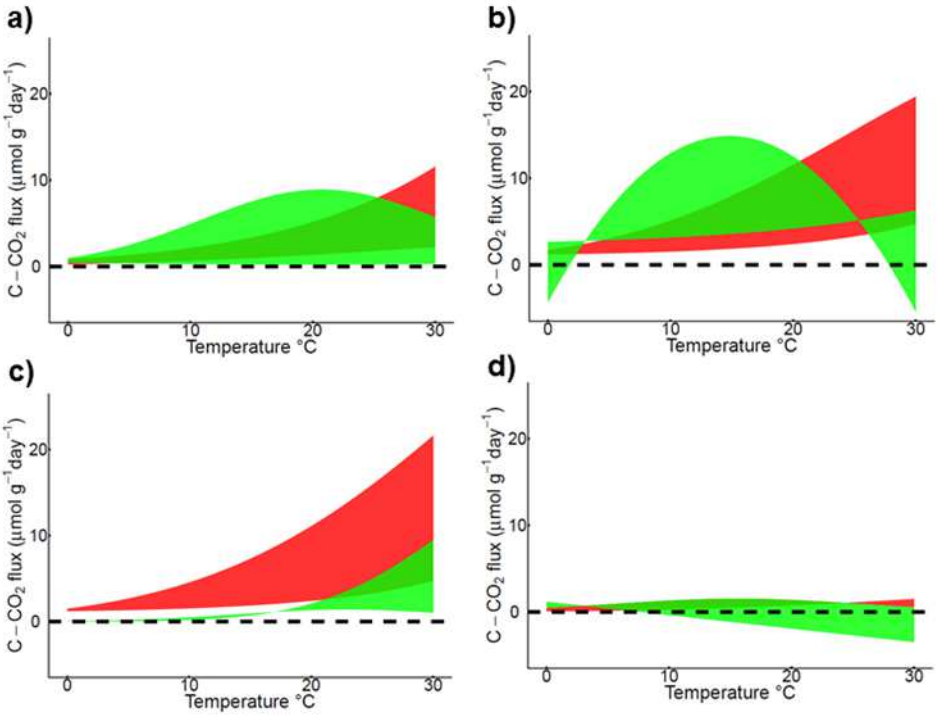
The results are evaluated in relation to collected data about stoichiometry of soil nutrients, phylogenetic structure of the communities and the information about biomass of microbial communities (given in Chapter I and III). All these data will be used for development of mechanistic mathematical model, which

will predict overall metabolic activity of BSCs in response to two major climatic drivers – temperature and moisture. The next step will be model upscaling. Model upscaling allow us to estimate metabolic activity of BSCs in diurnal due course and subsequently calculate annual carbon (C) exchange within the ecosystem using in situ data of temperature and moisture fluctuation.

In order to distangle the complex interactions driving BSCs activity, mathematical modelling is necessary to employ. It is because response of both heteroprophic and phototrophic activity folows non trivial pattern and both have different temperature and moisture optima for their activity. For example, the highest phototrophic activity was measured at 12°C in 5,750 m a.s.l. (Fig. 2a), whereas the greatest heterotrophic activity was measured at 30°C in 5,900 m a.s.l. (Fig 2b). Figure 2 also shows, that espetially phototrophic avtivity has different temperature optima at different elevation. At 5900 m a.s.l. it exponentially increase until 30, but it becomes negative at same temperature in 5750 m a.s.l.. We hypothesize, that this pattern is driven by interaction between heterotrophic and phototrophic part of the community and environmental and edaphic conditions. Therefore, among gas (CO2 and O2) exchange we also measured many chemical and microbial parameters including biomass, available soil C, N, P, activity of major hydrolytic enzymes etc., which help us to construct and parametrize mechanical model based on already existing ones (Allison & Gessner 2012; Manzoni & Porporato 2009; Allen et al. 2005; Manzoni & Porporato 2007). This should help us to identify the reasons for such complex patterns. If model parameters will differ along altitudinal gradient, than microbial community adaptations and its structure are solely responsible for that pattern.

photosynthetic

Fig.2. Range of possible carbon flux from heterotrophs (red colour) and phototrophs (green colour) the soil at 4 elevetions – a) 5,600, b) 5,750, c) 5,900 and d) 6,000 m a.s.l. under different temperatures and moistures. Carbon flux out of the soil is a proxy of heterotrophic activity. C flux into the soil represents gross C flux and thus phothosynthetic activity. It was calculated as difference between net C flux into the soil and C flux out of the soil as result of respiratory activity of heterotrophic part of the community.



Future prospectives

The thesis summarize first infromations about BSCs communities from outlying ecosystem of high-altitude cold desert of W Himalaya . These new findings will help for improve understanding of BSC communities and their role in ecosystems. Such infromation might be extremly important for future prediction of global climate change impact on the mountains ecosystems in the Indian Mts.. As such it could helpful for forecast future changes of these pristine and remote areas which represent large and unique ecosystems on the Earth.

However, there are still some uknowns, which needs to be resolved. First of all, it is important to evaluate pigment concentration data. The first reults suggested that not only cyanobacteria, but also heterotrophic bacteria produce pigments, which confirms the critical role of UV radiation for survival of microbes in high altitude deserts. The second, more complex, issue connected to microbial survival is confouning effect of water availability and temperature. The future work should be focused on to build and calibrate mathematical model, which would predict in-situ activity of BSCs in year time course.

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CHAPTER II

Community structure of soil phototrophs along environmental gradients in arid Himalaya

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Community structure of soil phototrophs along environmental gradients in arid Himalaya

Abstract

The well-developed biological soil crusts cover up to 40% of the soil surface in the alpine and subnival zones of the Tibetan Plateau, accounting for a vast area of Asia. We investigated the diversity and biomass of the phototrophic part (Cyanobacteria) of the microbial community inhabiting biological soil crusts and uncrusted soils in their surroundings on the elevation gradient of 5200-5900m a.s.l. The influence of soil physico-chemical properties on phototrophs was studied. The ability of high-altitude phototrophs to fix molecular nitrogen was also determined under laboratory conditions. The biological soil crust phototroph community did not differ from that living in uncrusted soil in terms of the species composition, but the biomass is three-to-five times higher. An increasing trend in the cyanobacterial biomass from the biological soil crusts with elevation was observed, with the genera Nostoc spp., Microcoleus vaginatus and Phormidium spp. contributing to this increase. Based on the laboratory experiments, the highest nitrogenase activity was recorded in the middle elevations, and the rate of nitrogen fixation was not correlated with the cyanobacterial biomass.

Key Words: desert crusts; bacterial diversity; nitrogen-fixation; western himalayas; colorado plateau; high-elevation; climate-change; north-america; cyanobacterial; succession

Introduction

Soil environments in high-altitude and high-latitude ecosystems provide habitats for numerous microorganisms, despite being subject to extremes of environmental stress, principally freezing and desiccation (e.g. Kaštovská et al., 2005; 2007; Gangwar et al., 2009; Blanco et al., 2012). Phototrophic microorganisms - cyanobacteria and eukaryotic microalgae - are important components of these soil environments, especially in young successional stages (Nemergut et al., 2007; Řeháková et al., 2011). Such microbial communities play a key role in colonizing barren substrates, even above the actual vascular plant altitudinal limit. Here, together with lichens, mosses and microfungi, they persist in a complex structure of biological soil crusts (BSCs) (Paerl et al., 2000; Belnap and Lange, 2001). BSCs carry out key processes in the development of soil (Kubečková et al., 2003; Tirkey and Adhikary, 2005), biogeochemical cycling (Johansen, 1993; Heckman et al., 2006) and plant colonization (Belnap and Harper, 1995). The diversity and abundance of soil cyanobacterial and microalgal communities as first colonizers of high-mountain soils may profoundly affect nutrient availability for pioneer vascular plants by enhancing plant seedling establishment (Belnap and Lange, 2001).

The biodiversity and functions of microbes in the mountain ecosystems have received increased attention, especially with respect to climate change, glacial retreat and vascular plant distributional shift (e.g. Schmidt etal., 2008; Lamb etal., 2011). Climate in most places on the Earth has become increasingly warm over the past two hundred years, with some regions such as high-mountain biomes experiencing an increase of as much as 2-3 K (Alley, 2007). As broad-scale distributions of organisms are shaped by climatic conditions (Walther etal., 2006), changes in climate necessarily result in shifts of range limits. Evidence of such shifts has already been found in a variety of mountain regions and

organisms (Parmesen and Yohe, 2003; Root et al., 2003; Walther et al., 2006; Erschbamer et al., 2011).

Most of the available studies, however, have been based on data from European mountains, while the largest and highest mountainous areas are located in Asia. On the Tibetan Plateau, which covers an area almost 14 times as large as the area of the European Alps, extensive areas of subnival and alpine climatic zones host a unique and relatively species-rich flora (Miehe etal., 2011) and well develop BSCs with complex microbial communities (Řeháková et al., 2011). Alpine zones of the Tibetan Plateau serve as grazing land for yaks, wild sheep and other mammals, while many plant species are utilized by local people for medicinal purposes. However, 67% of all glaciers, representing the only reliable water reservoir for local people and their livelihoods, are presently retreating (Rai and Gurung, 2005; Xu et al., 2009). This indicates that considerable environmental change in this high altitude region is underway.

Despite the importance of phototrophs in alpine soils, their biodiversity and functions are still unclear and require further attention, especially in remote mountain regions such as the Tibetan Plateau. The dry mountains in SW Tibet are situated in the rain-shadow north of the Great Himalaya, the so-called arid Trans-Himalaya. These provide unique opportunities for investigating the response of various organisms to the changing environment at one of the highest (c. 6000 m) elevations in the world inhabited by angiosperms (Klimeš and Doležal, 2010; Dvorský et al., 2011). The harsh environment of the area imposes great constraints on plants, which must cope with aridity and salinity at the lower altitudes, while extreme diurnal temperature fluctuations, strong winds and solifluction constrain plants at the higher altitudes. Under such conditions, the productivity is generally very low and the vegetation cover is sparse. These circumstances open the space for the extensive development of phototrophic communities in the BSCs and increase the importance of cyanobacteria and microalgae.

The aim of this study was to assess whether the taxonomic composition and biovolume of phototrophic microbial communities differ between soil crusts and barren soil and whether these differences are constant across an elevational gradient from 5300 to 5900 m a.s.l. covering various habitats, from dry alpine steppes, to wet alpine screes and the subnival zone. We also assessed whether the taxonomic diversity and biovolume of phototrophic microbial communities is linked to soil physico-chemical properties and nitrogenase activity (NA), and vegetation cover. The present investigation represents, to the best of our knowledge, the first study focused on BSCs in the remote region of the Tibetan Plateau.

Results

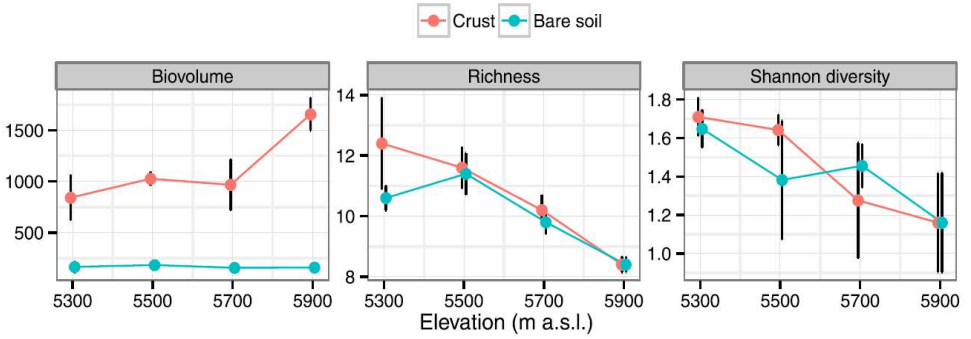
The composition of phototrophic communities

Phototrophic microorganisms were found in all examined samples, with Cyanobacteria being the dominant component of the communities. The phototrophic communities contained 16 morphotypes and the biovolume of a single morphotype ranged from 0 to 490 166 μm^3 mg^{-1} dry weight (DW) (Table 1). Richness and Shannon diversity of soil phototrophic communities significantly decreased with increasing elevation, both in crusts and bare soil (Fig. 1). The total biovolume of soil phototrophs was, however, three-to-five times higher in crusts than in bare soil, and increased significantly with elevation in the soil crusts only (Fig. 1).

The combined effect of elevation and soil type on the composition of soil phototrophic communities explained 40.7% of the total data variation and was highly significant (non-standardized canonical redundancy analysis (RDA): F= 3.006, P= 0.001, Supplement 1). The non-standardized RDA revealed that all morphotypes were much more abundant in soil crusts when compared with bare soils. Variance partitioning revealed that

23.8% was explained solely by soil type differences (F= 13.6, P = 0.001), and 15.2% by elevation (F= 2.89, P= 0.001). Less variation was explained by soil type and elevation in the standardized RDA. A non-significant result of RDA standardized by sample norm shows that, when abundance differences among soil habitats are removed, species proportions remain more or less constant between crust and bare soil in each elevation, while there are significant shifts in species composition along the elevation gradient.

Fig. 1. Comparison of soil phototrophic biovolume (µm^3 x mg dry soil x 10^3), cyanobacterial and algal morphotypes richness and diversity between crusts and bare soil in four elevation sites on the western slope of Chamser Kangri Massif, Tso Moriri Lake area, SW Tibet.



In the RDA ordination diagram from the non-standardized analysis, the main compositional changes along the first ordination axis are associated with soil type, clearly separating the soil crusts from bare soil (Fig. 2), while the second axis corresponds to the position of the four elevation sites. The RDA diagrams show that cyanobacteria from the orders Oscillatoriales and Nostocales predominated at the higher elevation sites (5700 and 5900 m), while Chroococcales were more abundant in the communities at the lower altitude sites (5300 and 5500 m) (Fig. 2). Hence, Oscillatoriales and Nostocales prevailed in subnival soils, particularly in soil crusts, while Chroococcales were dominant in the soil crusts of alpine steppes and screes at lower elevation (Fig.

2). Eukaryotic algae (Chlorophyceae and Tribophyceae) accounted for a small proportion (0-5.6%) of the total phototrophic biovolume (Table 1) and were more important components of the phototrophic communities of the bare soil at lower elevation (Fig. 2). In the two upper elevation sites, which had the highest biovolume of Oscillatoriales, *Microcoleus vaginatus* and *Phormidium* spp. accounted for most of the biomass (Fig. 3). The biovolume of cyanobacteria *Phormidium* and *Microcoleus vaginatus* increased with increasing elevation, but only in soil crusts, while the biovolume decreased with elevation for *Nodularia* and taxa from the order Chroococcales (Fig.3).

Table 1. **I. The biovolume of phototrophs** in μm^3 mg^{-1} dry soil x 10^3 (means) and **II. The nitrogenase activity** in nmol C_2H_4 g^{-1} (dry soil) day^{-1} (means) at soil crust and bare soil.

	Bare soil	Crust			
	Mean/SDEV	Mean/SDEV	Soil	Elevation	Interaction
I. Phototrophs					
Nostoc sp.	18/8	137/58	↑ 0.015	↑ 0.000	↑in 0.000
Phormidium spp.	44/24	298/144	↑ 0.071	↑ 0.001	↑in 0.015
Microcoleus vaginatus	72/42	490/273	↑ 0.045	↑ 0.002	↑in 0.013
Microcoleus sp.	17/38	110/243	ns	ns	ns
Nodularia sp.	7/7	40/32	↑ 0.000	↓ 0.003	↓in 0.000
Leptolyngbya sp.	4/4	29/17	↑ 0.001	ns	ns
Cyanothece (5 × 10)	$7 \times 10^{-1}/1$	6/8	↑ 0.017	ns	ns
Cyanothece (10 × 15)	0/0	1/2	ns	↓ 0.038	↓in 0.027
Chroococcus 1 (2.5 × 5)	$2 \times 10^{-1}/5 \times 10^{-1}$	1/1	ns	ns	ns
Chroococcus 2 (5 × 5)	$6 \times 10^{-3}/9 \times 10^{-3}$	$4 \times 10^{-2}/5 \times 10^{-2}$	ns	ns	ns
Chroococcus 3 (5 × 7.5)	$5 \times 10^{-3}/0.01$	$3 \times 10^{-2}/5 \times 10^{-2}$	↑ 0.000	ns	ns
Chrococcales (2.5 × 2.5) G1	$1 \times 10^{-1}/8 \times 10^{-2}$	$6 \times 10^{-1}/5 \times 10^{-1}$	↑ 0.001	ns	ns
Chrococcales (5 × 5) G2	1/1	6/6	↑ 0.006	ns	ns
Chrococcales (10 × 10) G3	$2 \times 10^{-1}/4 \times 10^{-1}$	$8 \times 10^{-1}/2$	ns	ns	ns
coccal microalgae (15 × 15) B1	$6 \times 10^{-1}/1$	$3/1 \times 10^{-1}$	ns	ns	ns
Chroococcales	2/2	16/10	↑ 0.000	↓ 0.037	↓in 0.042
Oscillatoriales	137/73	926/414	↑ 0.001	ns	↑in 0.034
Nostocales	26/13	178/77	↑ 0.000	↑ 0.037	↑in 0.028
eukaryotic algae	$6 \times 10^{-1}/1$	3/0	ns	ns	ns
Total biovolume	166/72	1111/392	↑ 0.000	ns	↑in 0.019
II. Nitrogenase activity					
1–2 days	0.11	0.71	↑ 0.080	ns	ns
4–5 days	0.87	2.29	ns	ns	ns
7–8 days	0.04	0.19	↑ 0.038	ns	ns

Also shown are *P*-values from GLMM analyses (for Type I error estimate) comparing two soil types, elevation differences and interaction between these predictors. An upward or downward pointing arrow indicates a positive or negative relationship between the dependent variable and altitude or soil (↑ = increase in crust), and ns indicate that the relationship is not significant. ↑in = increase only in soil crust.

Soil physico-chemical parameters

The collected soils were variable in their physico-chemical properties (Fig. 4). High pH values indicated alkaline soils in the investigated arid area, which received less than 100 mm of rain per year. This is reflected by low soil water content, ranging from 6% to 20% (expressed as volumetric water content), and high sodium concentration (data not shown). Salt-build-up is common, with saline ($NaCl^-$ affected) soils found particularly at lower elevations. Soil from lower altitude (alpine steppe and scree) sites had higher nitrate, phosphorous, magnesium and potassium concentrations; with the exception of nitrate, this was particularly true of bare soil. Soil crusts from higher altitude sites had a higher concentration of organic carbon, amonium and calcium in bare soil (Figs 2 and 4). The coarsest soil was found in semi-arid alpine steppes and screes, where the fraction > 0.5 mm represented up to 73% of the soil particles; the soil with highest proportion of fine particles was from middle elevations (Fig. 2).

Fig. 2. Redundancy analysis biplots (RDA, not standardized by sample norm) of soil phototrophs (response variables) in relation to soil types (crust, bare soil) and elevation (5300, 5500, 5700, 5900) on the western slope of Chamser Kangri, SW Tibet. Response variables are represented by vectors (arrows) and are related to physico-chemical characteristics of soil (dotted arrows) and environmental variables represented by centroides. The angles between arrows indicate correlations between variables.

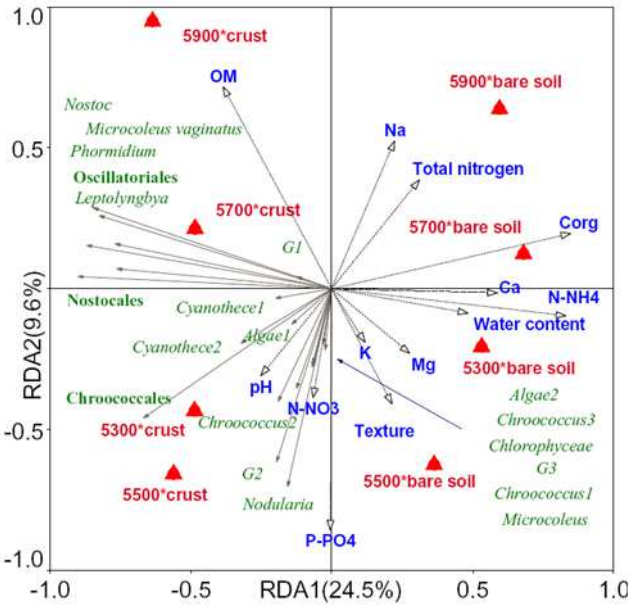


Fig. 3. Comparison of soil cyanobacterial biovolume (μm^3 x mg dry soil x 10^3) between crusts and bare soil in four elevations on the western slope of Chamser Kangri, SW Tibet.

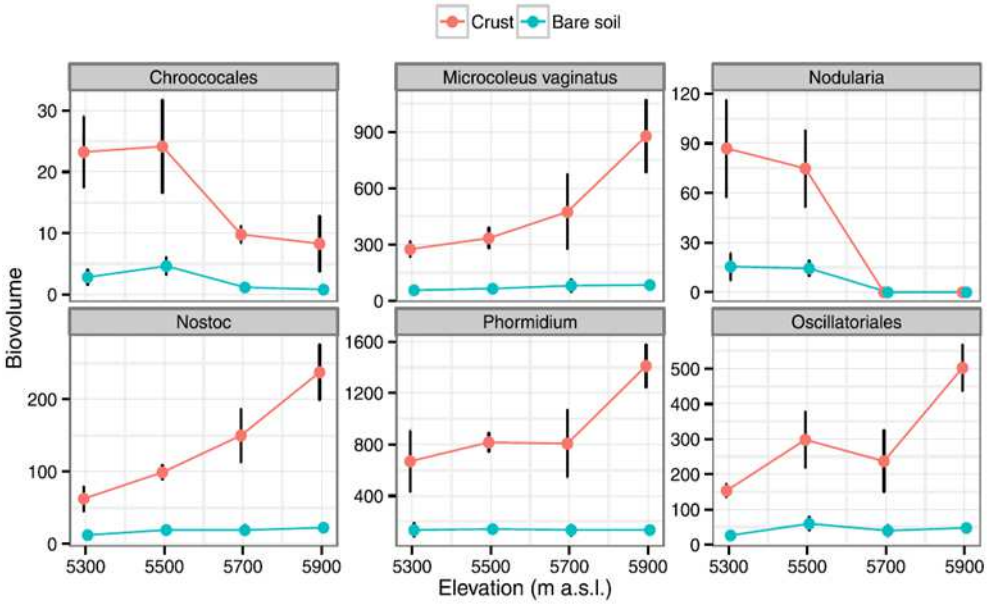
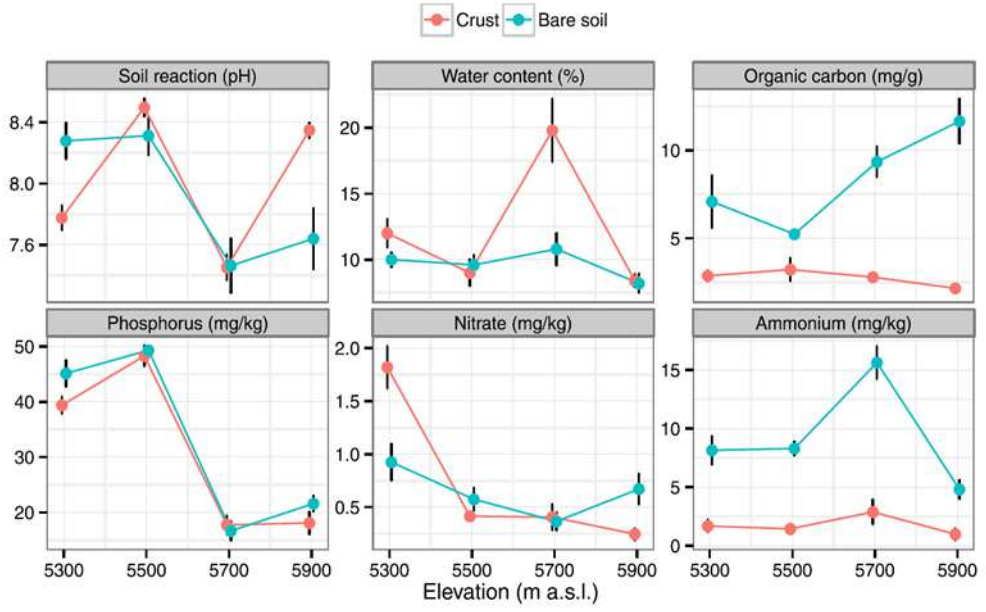


Fig. 4. Comparison of concentration of selected physico-chemical parameters between crusts and bare soil in four elevations on the western slope of Chamser Kangri, SW Tibet.



Potential nitrogenase activity in biological soil crusts and in bare soils

This is first time, when the NA was investigated in the nival and subnival zone of Himalaya Mts. N_2-fixing activity (NA) was recorded for most collected soil samples and the ability of Himalaya's soil to fix nitrogen at least in the laboratory condition was observed. Rates of potential NA (indication of N_2 fixation) are presented in Table 1 and Fig. 5. The results showed that NA depended on elevation, and was, in general, much higher in soil crusts than in bare soil. The highest rates of NA were repeatedly found in soil crusts from an elevation of 5700 m, that is in soils which are the least water limited (Fig. 5). The lowest NA values occurred in both the dry and cold ends of the environmental gradient. The range of NA is wide as NA oscillated from 0 to 5 nmol C_2H_4 g^{-1} day^{-1} (Fig. 5). The changes in course of NA in the desiccation experiment were observed only for the altitude 5700 m. NA increased during the first 4-5 days of desiccation, when it reached a rate of 5 nmol C_2H_4 g^{-1} day^{-1} and then decreased to 0.3 nmol C_2H_4 g^{-1} day^{-1} towards 7-8 days (Fig. 5). During this time the mass of water per gram of dry soil was initially 5.5%, falling to less than 1%.

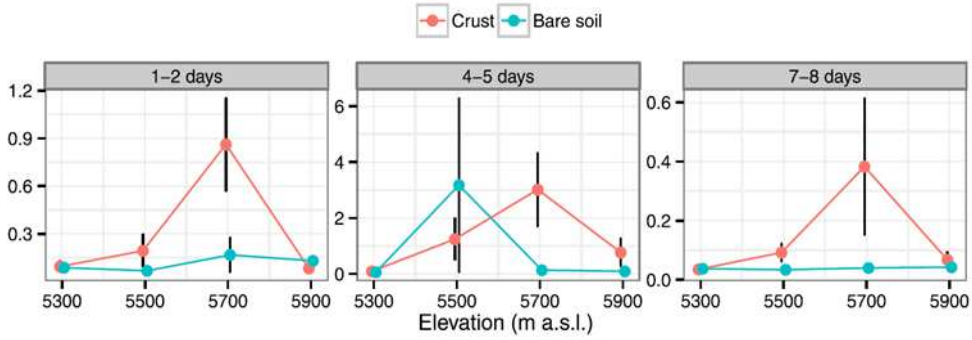


Fig. 5. Comparison of nitrogenase activity [nmol C_2H_4 g^{-1} (DW) day^{-1}] between crusts and bare soil in four elevations on the western slope of Chamser Kangri, SW Tibet. Measurements were done repeatedly at the days 1–2, 4–5 and 7–8 of incubation. Heading 1–2 means: start of incubation in day 1 and measurement of NA in day 2. Other two headings had same meaning. The scale in single graphs is different.

Microclimate

The vegetation season in 2010 (defined here as the period with mean daily air temperatures above zero) lasted nearly 5 months (155 days) at the lowest elevation of 5300 m, 3.5 months (107 days) at 5600 m and 3 months (90 days) at 5700 m. At the highest elevation (5900 m) the vegetation season was restricted to less than 2 months (56 days) (Fig. 6). Even during August, when temperatures generally reached their highest value, the temperature regularly dropped below zero at night, and repeatedly to about -5°C at the highest sites. The duration of sub-zero temperatures during a 24 h period was the main difference between the sites. While the temperature never dropped below zero at the lowest elevation, it usually fell below zero for about 2-3 h at the middle elevations; at the highest elevation freezing lasted between 8 and 16 h every day, particularly in the second half of August when many plants still flowered and fruited. Daily temperatures rose to 8-17°C at all four elevations but for a much shorter time per day at higher elevations.

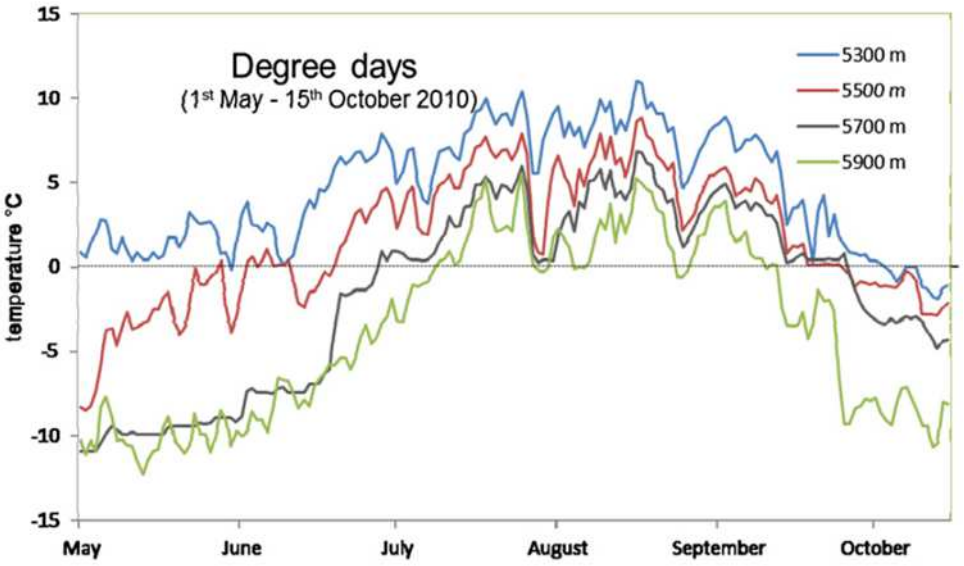


Fig. 6. Mean daily air temperatures measured at four elevations on the western slope of the Chamser Kangri, SW Tibet, Ladakh.

Discussion

Despite an increased interest in the nature of BSCs, there are still many aspects, which need to be studied. One of them is the diversity and role of primary producers (phototrophs) - cyanobacteria and algae - which are the important and dominant components of BSC (Nemergut et al., 2007; Řeháková et al., 2011). The subnival zone of Ladakh is suitable places for development of BSC as almost 40% of soil in this zone is covered by BSC dominated by cyanobacteria. Despite their importance, little attention has thus far been paid to them in the mountain ecosystems of Ladakh.

Phototrophic communities - biomass and diversity

Most of the phototroph's biomass was found in the BSC (Fig. 1) at levels three-to-five times higher than in bare soil, even when the measured nutrients had higher concentrations in the bare soil (Figs 2 and 4). Other factors likely contributed to the development of biomass in the biological crusts, or the turnover of nutrients in the crusts may be faster with nutrients being bound only in the microbial loop and not released into the substrata (Schmidt et al., 2007). An interesting finding of our research was a significant increase in cyanobacterial biomass in BSC with increasing altitude, while the biomass in the bare soil did not change significantly through the whole elevational gradient. This increase of cyanobacterial biomass within soil crust with increasing altitude is in sharp contrast to the commonly observed trend of a decreasing biomass of organisms with more severe environmental conditions (Ma et al., 2004; Baiser et al., 2010). The observed trend may be caused by several factors. First, there is less competition with vascular plants for resources such as nutrients, water and light. At the highest elevation studied a relativejy rich vascular plant community developed, being composed of dwarf rosette species,

but with negligible cover of less than 10% (Dvorský et al., 2011). Second, the temperature profile during the vegetation season provides better growing conditions for the microorganisms than for higher plants. The vegetation season lasts about one and half months at the highest elevation (5900 m a.s.l.) and during these days the temperature often drops below freezing for more than 8 h (Fig. 6). The short growing season and shortage of water are conditions, which microorganisms are resistant to (Zelikova *et al.,* 2012). Phototrophic microorganisms have a much quicker metabolism and a shorter generation time than vascular plants. In addition to this, the low amount of accessible water primarily activates the crust rather than the bare soil (Cable and Huxman, 2004) where the evaporation is much quicker due to lower retention of water, which is much better in BSC (Aguilar et al*.,* 2009). This also supports the growth of microorganisms in BSC.

The biomass of BSC was mainly created by cyanobacterial taxa, which are important primary producers within soil crusts all around the World. *Microcoleus vaginatus, Phormidium* sp. and *Nostoc* sp. have already been commonly reported from arid and semiarid environments (Garcia-Pichel *et al.,* 2001; Yeager *et al.,* 2004; Nagy *et al.,* 2005; Freeman *et al.,* 2009; Abed *et al.,* 2010; Řeháková *et al.,* 2011). Representatives of the order Chrooccocales (*Chroococcus* sp. and *Cyanothece* sp.) account for a minor part of biomass, similarly as in the biotopes from the Antarctic (Komárek *et al.,* 2008). These two taxa are rarely reported from other desert soils in the world. *Chroococcus* creates lesser biomass of phototrophs mainly in the cold types of deserts such as Colorado Plateau or Great Basin desert (Rosentreter and Belnap, 2001) or it is reported from temperate zone from alkaline soils in Ohio (Rosentreter and Belnap, 2001) or salt pasture in Hungary (Biidel, 2001). The species mentioned in the literature above where originally described from water ecosystems or wet dripping walls (*Chroococcus turgidus, C. tenarf*). Their occurrence in the desert soil is highly improbable. To solve the proper

taxonomic position of Himalayas *Chroococcus* the phylogenetic analyses is needed. Also morphotype *Chroococcus-Wke* in the deserts of Oman was observed by Abed and colleagues (2010). Cyanobacterium *Cyanothece* was observed in the desert soil previously but also very rarely. We were able to find only two references. Firts *Cyanothece aeruginosa* from Sand Barrens in Ohio (Rosentreter and Belnap, 2001). *Cyanothece aeruginosa* (Nägel; Komárek 1976) is originally described from clear moorland waters; therefore the correct identification is highly improbable. Second report is cyanobacterium from Solar Lake identified as morphospecies *Cyanothece*-like, called in the article also *'Halothece*' (Abed and Garcia-Pichel, 2001).

Although the species composition of phototrophs is comparable across the investigated altitudinal gradient, their biomass significantly increased (*Microcoleus, Nostoc, Phormidium)* or decreased (*Cyanothece, Nodularia)* with altitude. *Microcoleus* and *Nostoc* are genera, which produce a thick mucilaginous sheath. Besides *Nostoc* has ability to produce scytonemin, which caused brown pigmentations of the sheath (Bowker *et al.,* 2002; Matsui *et al.,* 2012). Both these features protect them against the high UV radiation (Matsui *et al.,* 2012) and desiccation risks (Kubečková *et al.,* 2003) present in the arid environment of the Ladakh Mountains. The genus *Phormidium* produces sheaths only occasionally, but has another protective strategy. *Phormidium* is able to migrate from the surface to the bulk of the soil when the light intensity or moisture level is not suitable for its growth; *Microcoleus* also has this ability (Garcia-Pichel and Castenholz, 2001). The BCS are complex community, where one member of ecosystem plays some role for the other member. The genus *Nostoc,* which grows in the surface of crusts, could protect other, less- pigmented taxa such as *Microcoleus* and *Phormidium* (Bowker *etal.,* 2002). All these adaptations facilitate their survival in soils of subnival zones and enable them to become dominant components of the phototrophic community.

Nitrogenase activity in subnival soils

Crust ecophysiology has not been previously characterized in the region of Ladakh. Because of the welldeveloped cyanobacterial community in the studied soil, where almost 16% of total phototrophic biomass is created by order Nostocales, mainly genus *Nostoc,* we surveyed their contribution to nitrogen input into the subnival ecosystem. Microbial activity in desert communities is restricted to brief periods when precipitation or dew hydrates microbial cells, thus allowing metabolic activity of the otherwise dormant biota (Garcia-Pichel and Belnap, 1996; Lange, 2001). Temperature is another environmental factor, which influences nitrogen fixation (Gallon *et al.,* 1993) and high UV radiation levels deactivate cyanobacterial nitrogenase (Kumar *et al.,* 2003). To avoid the restriction caused by the unfavourable environmental conditions for NA, we set up controlled experimental conditions (temperature 21 °C, full-water saturation of soil, low UV radiation) for NA measurement. All investigated crust samples had detectable NA while almost no activity was detected in bare soils irrespective of elevation (Fig. 5). In spite of the high variability in measured values during the experiment, some significant results were apparent. The NA was independent on the total biomass of cyanobacteria in BSC. The total biomass increased in the BSC with elevation, but NA did not follow this trend. Even if the taxonomic composition of primary producers was comparable across elevation, the intensity of NA differed.

The NA activity was repeatedly highest in crusts from the elevation 5700 m a.s.l., despite the same ‘propitious’ conditions for all measured samples during experiment. The nitrogen-fixing organisms have probably ‘memory’ about *in situ* conditions in natural ecosystems and keep it even in the laboratory. The reason for the highest NA in middle elevations could be that climatic and edaphic factors are more favourable here for NA. BSC are less water-limited than in the lowest (driest) and highest (coldest)

elevations studied (Fig. 6). At the same time, the temperature does not drop below freezing so often during the vegetation season as in the 5900 m a.s.l. region (Fig. 6) and the UV irradiance is lower than in the highest elevation.

Crusts from the elevation 5700 m a.s.l. had very similar course of the NA reaction to watering and consequent desiccation as the natural populations of *Nostoc* from Svalbard. Colonies of *Nostoc* were capable of photosynthesis and nitrogen fixation until they lost 40% of their wet weight (Kvíderová *et al.,* 2011). The stability of the NA even with relatively low water content in the cyanobacterial tissue or BSC can be explained by the complex response of microorganisms to desiccation. The major water loss occurs in the mucilaginous envelopes of the colonies or in the intercalary space of the soil during the first days of desiccation but the intracellular water content is not affected (Kvíderová *et al.,* 2011).

The majority of organisms are not able tolerate 10% water content treshold in their body, because there is no longer sufficient water to form monolayer around proteins and membrane. Consequently metabolic and enzymatic activities are stopped (Alpert, 2006; Tashyreva and Elster, 2012)**.**

Although the NA determined in laboratory conditions was in general relatively low, not exceeding approximately 6 nmol C_2H_4 g^{-1} day^{-1}, it can be a significant source of N *in situ* in subnival soils. Hrčková *et al.,* (2010) found NA in cyanobacterial species isolated from deglaciated soils in Polar Regions, which were similar to NA in cyanobacteria isolated in temperate and tropical regions. This indicates the potential of cyanobacteria to nitrogen fixate regardless of the environmental conditions they are exposed to.

The conducted investigation in Himalayas Mts. revealed relatively small taxonomical diversity of phototrophs in the studied BSCs and barren soil nevertless phototrophs contributed significantly to the biomass of soil microorganisms in subnival and alpine zone. With increasing elevation their biomass and relevance in BSC is growing. Because diazothrofic taxa created up to 16% of

total cyanobacterial biomass of BSC, their contributed to the nitrogen cycle in subnival and alpine environment, mostly in the altitude 5700 m a.s.l.

Experimental procedures

Study area

Fieldwork was conducted in August 2010 on the western slope of Chamser Kangri Peak above Tso Moriri Lake (32°59‘N, 78°24’E). This is located in the southwestern extension of the Tibetan Plateau in Ladakh, Jammu and Kashmir States, India (Supplement 2). An arid environment only occasionally affected by monsoon, which seldom cross the main Himalayan range, characterized the whole area. The parent rock consists mainly of siliceous rocks (Tso Morari gneiss) (Epard and Steck, 2008). The altitudinal zonation of vegetation included steppes at lower elevations (5000-5300 m), alpine screes and meadows (5300-5600), and the subnival zone close to glaciers (5600-6000 m). The cover of vascular plant vegetation ranged from 0 to 50%. The general vegetation description of the study area is provided by Klimeš and Doležal (2010), Klimešová and colleagues (2010) and Dvorský and colleagues (2011).

Soil sampling

The sampling was done at four locations along an elevational transect from 5300 to 5900 m a.s.l., so as to cover the physiognomically different major vegetation types of steppes, alpine screes, alpine meadows and the subnival zone. Places devoid of soil and vascular plants such as very steep unstable slopes, glaciers, big boulder moraines and elevations > 6000 m a.s.l. were avoided. At each site on a gradient, the five pairs of composite samples were taken randomly, one from the bare

surface layer of soil and the other from soil crust nearby. Soil was taken from an area of 10 cm^2 and 1-3 cm deep, with a sterile spatula. In total, 40 soil samples were collected (4 elevations x 2 soil types x 5 replicates). The soil air-dried on aluminum plates for 10 h immediately after collection, because the field conditions do not allowed other storage of collected material. The BSC and arid land soil are exposed to the drying and freezing commonly in the Himalayas Mts. This method of preservation is recomended for arid land soils and BSC samples by Campbell and colleagues (2010), because it prevents microbial activity in a naturally occurring manner, without the cell damage that may be associated with freezing and, particularly, thawing cycles. The samples were placed in sterile 100 ml polypropylene bags (Nasco Whirl-Pak®), and transported to the laboratory for analysis.

Physico-chemical characteristics of soil

Subsamples of soil were used for the determination of pH, organic matter content, and texture as described by Kaštovská and colleagues (2005). For the determination of total nitrogen, NH_4^+ and NO_3^-, methods by Zbiral and colleagues (1997), Kopáček and Hejzlar (1993), and Wolf (1982) were used. The technique described in Mehlich (1978) was used for the extraction of phosphorus (P) from samples and the concentration of P was measured by using ascorbic acid-molybdate and a SHIMADZU UV-1650PC spectrophotometer. The macro-elements (Ca, Mg, K, and Na) were extracted from the soil according to US EPA method 200.2 (HCl-HNO_3) http://www.epa.gov/epaoswer/hazwaste/test/3050b.pdf) and determined spectrochemically using US EPA method 3050 (Kimborough and Wakakuwa, 1991). Soil organic carbon was determined by wet oxidation with acidified dichromate (Rowell, 1994). Mid-season volumetric water content was measured at each soil sampling point immediately during the

collection by a Hydrosense II Soil Moisture Measurement System (Campbell Scientific, Australia).

Algal and cyanobacterial abundance and diversity

The samples for abundance and diversity investigation were prepared as follows. One gram of mixed soil was diluted in 5 ml of distilled water. The slurry was disintegrated at the beginning manually with pestle and consequently with sonifi- cator (Bandelin Sonorex) for 1 min. Twenty microlitre of slurry was put under cover glass cover area 22 x 22 mm. Ten stripes (area of one stripe 11 mm^2) were counted. Samples were observed under microscopy Olympus BX 60, magnification 400x.

The number and biovolume of microalgal and cyanobacterial cells as well as the taxonomic composition of communities were determined using epifluorescence microscopy (Olympus BX 60). Green and blue excitation (MWB filter cube blue excitation 450-480, emission 515+for eukaryotic algae; MWG filter cube green excitation 510-550, emission 590+ for cyanobacteria) was used (Kaštovská *et al.,* 2005). The term 'eukaryotic' alga is used in this paper for the taxa from classes Chlorophyceae and Tribophyceae. The green coccoid algae included species from the classes Chlorophyceae and Tribophyceae, because it is impossible to distinguish them under the epifluorescence microscope. In our soil samples, it was possible to recognize two morphotypes according different cell dimensions.

Cyanobacteria were classified into three orders according to their morphology: Chroococcales (single-celled organisms), Oscillatorialles (filamentous cyanobacteria without heterocytes and akinetes) and Nostocales (filamentous or colonial cyanobacteria with heterocytes and akinetes). In Oscillatoriales, the taxa *Phormidium* spp., *Leptolyngbya* spp., *Microcoleus vaginatus* and *Microcoleus* sp. were determined according to the width of the filaments, shape of the vegetative cells and presence/absence of

mucilaginous sheaths. In Nostocales, two morphotypes were distinguished according to their life form (colonies or filaments) and the shape of the vegetative cells and heterocytes: *Nostoc* sp. and *Nodularia* sp. For the order Chrooccocales, it was possible to recognize taxa *Chroococcus* spp., *Cyanothece* spp. and 'unidentified balls' according to the vegetative cells' shape and dimension, and division of cells. For the dimension of single morphotypes (see Table 1).

Nitrogenase activity

Nitrogenase activity was estimated as acetylene-ethylene reducing activity (Hardy *et al.,* 1973) in all collected soil samples. Stored, dry soils were re-wetted using distilled water; the amount of water necessary to moisten soils to *c.* 24% w/w (23.8 ± 2.3, mean ± SD; moisture is expressed as mass of water per gram of dry soil in percent) was set in a preliminary experiment, details not shown. Thirty gram portions of moist soils were placed in 120 ml serum bottles and pre-incubated under laboratory conditions (22°C, daylight 16 h; day 0). After 24 h, 10 ml of acetylene was added and immediately a 0.5 ml sample of the internal atmosphere was taken with a gas-tight syringe (day 1). Sampling was repeated in the same way after 24 h of incubation (that is on day 2). The bottles were then opened and let to dry for 48 h (days 2-4). Then the determination of NA (days 4-5) and the air-drying (days 5-7) were repeated and finally the NA was determined (days 7-8). The soils were air dried on day 8. Therefore, altogether three subsequent measurements of NA were done using the same bottles with the soils under study. The amount of ethylene in the headspace samples was determined Hewlet Packard 5890 gas chromatograph equipped with a flame ionization detector (FID). A standard mixture of 99.9 p.p.m. ethylene in N_2 was used for calibration purposes. The NA was expressed in nmol C_2H_4 per gram of dry soil per day.

For details on ethylene determination and calculations see Šimek and colleagues (1987).

Microclimatic measurements

At each elevation site, we recorded air temperature and relative air humidity using a HOBO U23 Pro v2 datalogger placed on the soil surface and shielded against direct sunlight. The measurements were recorded every 2 h for the whole year.

Statistical analysis

Differences in cyanobacterial and algal composition between four elevation sites and two soil types (crust and bare soil) were analysed by RDA, which is a constrained ordination method, in the CANOCO 4.5 program (ter Braak and Šmilauer, 1998). RDA was used because environmental variables were in the form of categorical predictors (dummy variables). Standardization by species (dependent variables) was used because the data analysed were of various types and units.
The variance partitioning procedure was performed using several RDAs with explanatory variables and covariables to remove their effects and to obtain the net effect of an individual factor. Using this approach, we constructed tests analogous to the testing of particular terms in anova models but for multivariate data; for details, see Lepš and Šmilauer (2003). Four analyses were carried out; (i) Soil type x Elevation (categorial variable, coded as a set of indicator variables) as the sole explanatory variable - the analysis accounts for all, the main effect of soil type, elevation and their interactions, (ii) and (iii) Soil type being an environmental variable, and Elevation a covariable and vice versa - this accounts just for additive (main) effects of the environmental variables. Note that because Soil type and Elevation are orthogonal, the amount of explained variability (i.e. sum of canonical axes) is not affected by

the use of a covariable, (iv) The interaction Elevation x Soil is the environmental variable and Elevation and Plot identifiers are covariables. This accounts for the interaction between elevation and site (i.e. for non-additivity of their effects). This corresponds to the interaction in Repeated Measures anova. The significance of these relationships was tested using the Monte Carlo permutation test (999 permutations) constructed for a split-plot design (see Lepš and Šmilauer, 2003, p. 219). The results of multivariate analyses were visualized in the form of a biplot ordination diagram. The relation of soil physicochemical characteristics to cyanobacterial and algal composition was visualized by their passive projection to the RDA ordination plane.

Both RDA with and without standardization by sample norm were used. RDA without standardization reflects both the differences in the species abundances and also the relative proportions of species, while standardized RDA takes into account the proportions of species only. There is an important implication of significant effect in the standardized-by-samples and the non-standardized analyses. The former analysis tests the null hypothesis that there is no effect of the environment on species composition. To reject this hypothesis, it is enough if the absolute values of species abundances differ between treatments, even if the proportion of individual species remains constant. The latter analysis tests the null hypothesis that the relative proportions of species do not differ between sites. The test of the first hypothesis is usually more powerful, but the rejection of the second hypothesis is more ecologically interesting.

The univariate data were further analysed by mixed-effect models. The effect of soil type (crust, bare ground), elevation (5300, 5500, 5700, 5900 m) and their interactions on individual cyanobacterial and algal species/morphotypes was analysed using a linear mixed-effect models or generalized linear mixed-effect models, depending on the nature of a particular response variable (assuming Gaussian, quasi- Poisson, or quasi-binomial

distributions). Each pair of samples was considered a 'main-plot' and represented a factor with a random effect nested in the elevation site. Soil type and elevation were the fixed effect factors. All the tests were based on the restricted maximum likelihood approach. The statistical significance of the main effects and interactions were assessed by computing Bayesian highest probability (HPD) intervals using Markov chain Monte Carlo simulations (1000 permutations), as this is as this is more appropriate than normal confidence limits for generalized linear mixed models (GLMMs). To control for familywise error rate, the false discovery rate procedure was performed (Benjamíni and Hochberg, 1995). Analyses were done using the lme4 and language packages in the R program (R Development Core Team, 2010).

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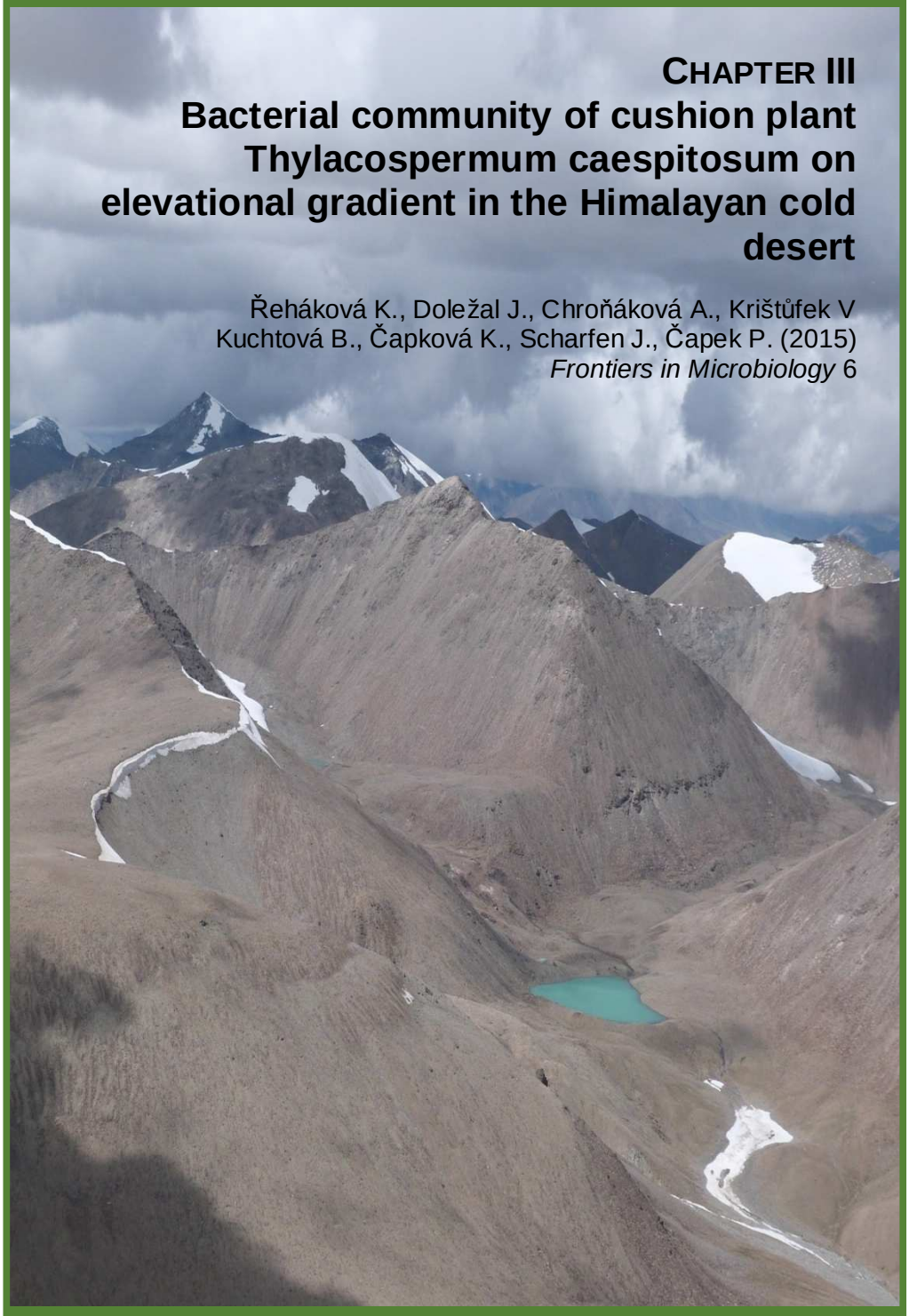
Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplement 1. Summary of results of redundancy analyses (RDA) for phototrophic soil composition, standardized (S) or not (N) by sample norm.

Supplement 2. Location of four studied elevation sites near Tso Moriri Lake, SW Tibetan Plateau in NW India (top left picture). Studied soil crusts on the western slope of Chamser Kangri Masif at elevation 5700 and 5900 m a.s.l. (top and middle right). Southeastward view of Chamser Kangri (6666 m a.sl.) with glacier tongue descending to 5700 m.



CHAPTER III
Bacterial community of cushion plant Thylacospermum caespitosum on elevational gradient in the Himalayan cold desert

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Bacterial community of cushion plant Thylacospermum ceaspitosum on elevational gradient in the Himalayan cold desert

Abstract

Although bacterial assemblages are important components of soils in arid ecosystems, the knowledge about composition, life-strategies, and environmental drivers is still fragmentary, especially in remote high-elevation mountains. We compared the quality and quantity of heterotrophic bacterial assemblages between the rhizosphere of the dominant cushion-forming plant *Thylacospermum ceaspitosum* and its surrounding bulk soil in two mountain ranges (East Karakoram: 4850-5250m and Little Tibet: 5350-5850m), in communities from cold steppes to the subnival zone in Ladakh, arid Trans-Himalaya, northwest India. Bacterial communities were characterized by molecular fingerprinting in combination with culture-dependent methods. The effects of environmental factors (elevation, mountain range, and soil physico-chemical parameters) on the bacterial community composition and structure were tested by multivariate redundancy analysis and conditional inference trees. Actinobacteria dominate the cultivable part of community and represent a major bacterial lineage of cold desert soils. The most abundant genera were Streptomyces, Arthrobacter, and Paenibacillus, representing both r- and K-strategists. The soil texture is the most important factor for the community structure and the total bacteria counts. Less abundant and diverse assemblages are found in East Karakoram with coarser soils derived from leucogranite bedrock, while more diverse assemblages in Little Tibet are associated with finer soils derived from easily weathering gneisses. Cushion rhizosphere is in general less diverse than bulk soil, and contains more r-strategists. K-strategists are more associated with the extremes of the

gradient, with drought at lowest elevations (4850-5000 m) and frost at the highest elevations (5750-5850 m). The present study illuminates the composition of soil bacterial assemblages in relation to the cushion plant T ceaspitosum in a xeric environment and brings important information about heterotrophic bacteria in Himalayan soil.

Keywords: heterotrophic microbial community, subnival soil, life strategy, Ladakh, mountains, Himalayas

Introduction

Bacteria are a common and fundamental part of microbial communities and constitute a major proportion of biodiversity in soil ecosystems (Gans et al., 2005; Roesch et al., 2007; Fulthorpe et al., 2008; Griffiths et al., 2011). There are two scientific views on their distribution and both opinions have support. The first is that microbial populations can exhibit a geographic distribution (e.g., Fierer and Jackson, 2006; Adler et al., 2007; Griffiths et al., 2011). The second suggests ubiquitous distribution, i.e., everything is everywhere, but the environment selects (e.g., Fenchel and Finlay, 2003; Hubert et al., 2009). The relative importance of contemporary and historical factors in determining spatial patterns in microbial communities can only be evaluated through further studies that systematically sample and record data from various distances, habitats and environmental conditions (Chu et al., 2010).

Bacterial diversity and function are influenced by abiotic parameters, especially pH and nitrogen concentration (Fierer et al., 2009, 2012; Shen et al., 2013), and by biotic interaction with other members of the biotope (Eskelinen el al., 2009; Stark et al., 2012; Ciccazzo et al., 2014). Soil bacteria living in the mountain ecosystems have to withstand harsh environmental conditions such as large temperature and moisture fluctuation and a high intensity of UV radiation (Ley et al., 2004; Zumsteg et al., 2011). Elevational gradients in mountains serve as powerful study systems for answering questions on how the functional diversity and biomass of bacteria can be affected by different microclimatic conditions provided by these gradients. The altitudinal gradient also predicts the composition of the plant community, which will form individual vegetation zones within mountains. The vascular plant species composition directly or indirectly control and/or mediate all multitrophic interactions in the soil ecosystem (van der Heijden et al., 2008; Chakraborty et al., 2012). To eliminate the

influence of plant composition, bacteria in the bulk soil in close vicinity and from rhizosphere of *Thylacospermum caespitosum* were studied. This is the dominant plant of nival and subnival zones of Trans-Himalaya Mountains.

Our hypothesis was that this cushion plant would ameliorate the environmental conditions for microorganisms and would support their survival in subnival zone and consequently influence the species composition of bacteria. This investigation focused on the interaction between this cushion plant and microbial assemblage, and the amelioration effect of *Thylacospermum* on soil bacterial assemblages with emphasis on heterotrophic bacteria.

In this study, two elevational gradients covering the whole distributional range of *T. ceaspitosum* in the Himalaya Mts. were investigated to assess the interactions between bacterial assemblage, soil and microclimatic conditions in different elevations. Composition of bacterial communities was characterized by the method of Capillary Electrophoresis Single Strand Conformation Polymorphism (CE-SSCP) based on 16S rRNA gene (Stach et al., 2001; Zinger et al., 2007, 2008). For more detailed identification of heterotrophic bacterial communities, the culture-depending methods were used.

Our aims were: (1) to evaluate quantitative and qualitative changes in soil bacterial community and physico-chemical properties along an elevational gradient; and (2) to assess the influence of the cushion vascular plant Thylacospermum ceaspitosum on the quantity and quality of heterotrophic bacteria and soil biochemical characteristics in the nival and subnival zones of Indian Trans-Himalayas.

Study Area

The research was performed in Ladakh, Jammu and Kashmir State, India. The Eastern Karakoram Range delimited study area from the north and the Great Himalaya Range from the south. The region is only rarely affected by monsoonal rains and generally receives very little annual precipitation (<100 mm, Hartmann, 1983; Wang, 1988). Precipitation at lower and middle elevations (3500-5300 m) exceeds evaporation, while above 5300 m the water availability tends to increase due to melting snow from glaciers and occasional spells of rain and snow during summer monsoons (Miehe et al., 2001).

The investigation was done in July and August 2009 at two distinct localities (Tso Moriri, Nubra) where *T. ceaspitosum* is the dominant plant in subnival and nival zones. *Thylacospermum caespitosum* (Caryophyllaceae JUSS., subfamily Alsinoideae (DC.) FENZL, tribus Alsineae LAMARCK and DC.) is one of the most prominent vascular plants in alpine and subnival zones of Ladakh. It is perennial and forms very dense and solid cushions (Klimešová et al., 2011). Its distributional range includes high mountains in Kazakhstan, Kyrgyzstan, NW India, Nepal and China. In the study region, *T. caespitosum* occurs from 4500 to 5900 m elevation (Klimeš and Doležal, 2010).

Tso Moriri is situated in the valley of Lunglung stream on the western side of Chamser Kangri near Tso Moriri Lake (32°59'N, 78°24'E). Tso Moriri geomorphologically belongs to the Tibetan Plateau. Bedrock is formed by siliceous rocks (Precambrian granites, Tso Moriri gneiss) and by calcareous and saline sediments (Phillips, 2008). The cold steppes, alpine grassland and subnival vegetation were characteristic of the studied elevational gradient (Dvorský et al., 2011, 2013). The second locality was a side valley near the village of Tiggur in Nubra Valley, in the northern part of Ladakh (34°45'N, 77°35'E), belonging geomorphologically to the Eastern Karakoram Range. The bedrock mostly consisted of

Nubra-Siachen leucogranites (Phillips, 2008). Zonation of the vegetation was similar to the Tso Moriri, but the vegetation zones were shifted downwards by 300-400 m meters because of the higher elevation of surrounding mountains and their large-scale glaciation.

Materials and Methods

Soil Sampling

Soil was collected along two elevational gradients in 2009. In each transect, four sites were selected to cover the entire elevation range of *T. caespitosum:* in Nubra Valley at 4850, 5000, 5100, and 5250 m and in Tso Moriri at 5350, 5600, 5750, and 5850 m. Soil samples were taken from six randomly selected cushions of *T. caespitosum* within each elevation. One soil sample (150 g) was collected from the rhizosphere below the cushion and one sample from the bulk soil outside the cushion. The bulk soil sample was a composite of two samples taken from the eastern and western sides (75 + 75 g). The samples were air dried for 24 h on an aluminum plate, and then placed into sterile 540 ml polypropylene bags (Nasco Whirl-Pak®) and transported to the laboratory for analysis. Drying of samples is the only possible soil preservation technique under field conditions in the investigated localities. The closest power supply is accessible after 2 days of traveling and then only irregularly. The soil of Ladakh Mts. is exposed to the drying (average annual precipitation <100 mm) and freezing conditions commonly found in the Himalayas Mts (Bhutiyani et al., 2007). This method of preservation is recommended for arid land soils and BSC samples by Campbell el al. (2010) because it prevents microbial activity in a naturally occurring manner, without the cell damage that maybe associated with freezing and, particularly, thawing cycles.

Measurement of Microclimatic Conditions

At each elevation site, we recorded air temperature and relative air humidity using a HOBO U23 Pro v2 datalogger placed 10 cm above the soil surface and shielded against direct sunlight. The measurements were recorded every 2 h from August 2008 to August 2011. Additionally, at three sites (Nubra 5000 m, Tso Moriri 5600 m, and 5850 m), we chose one cushion of average size and placed a temperature logger (iButton® DS1923, Maxim Integrated Products) in the soil below it and another one in the soil of the adjacent open area 50 cm from the cushion. Inside the cushions, the loggers were placed 2 cm deep in the substrate under the cushion tissue, where the colonizing species were thought to be rooting. In the open areas, the loggers were buried 2 cm under the soil surface. The measurements were recorded every 2 h from September 2009 to August 2010.

Physico-chemical Characteristics of Soil

After transport to the laboratory, the subsamples were oven- dried at 100°C, ground in a mortar and sieved to 2 mm fraction after the removal of roots. The following concentrations of nutrients were determined: total N (TN), $P\text{-}PO_4$, Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}. Other physico-chemical data were also measured - pH, water content, organic matter content (OM), and texture (percentage content of particles bigger than 0.5 mm in diameter). Soil chemical analyses were conducted in accordance with standardized methods of the Association of German Agricultural Analytical and Research Institutes (VDLUFA 1991). Soil pH was potentiometrically measured in a suspension with 0.01 M $CaCl_2$.

Analyses of Bacterial Community Structure

The pattern of bacterial community structure was obtained by Capillary-Electrophoresis Single Strand Conformation Polymorphism (CE-SSCP), a method which is based on sorting DNA amplicons by electrophoresis under native conditions, according to their length and their nucleotide composition. The acquired SSCP profile is used as a picture of the entire bacterial community (Zinger el al., 2007, 2008).

Soil DNA extractions were carried out in triplicate from 0.25 g of dry soil from each soil sample with the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Ozyme, St. Quentin en Yve- lines, France) according to the manufacturers instructions. DNA extracts from three spatial repetitions of each sample were pooled to make a compounded sample.

The V3 region of bacterial 16S RNA genes was amplified with the primers W49 (5'-ACGGTCCAGACTCCTACGGG-3') and W104-FAM labeled (5'-TTACCGC GGCTGCTGGCAC-3') (Delbes et al., 2000). PCR reactions (25 p.1) were set up as follows: 2.5 mM of MgCb, 1 U of AmpliTaq GoldTM buffer, 0.4 mg of bovine serum albumin, 0.1 mM of each dNTP, 0.26 mM of each primer, 2 U of AmpliTaqGold DNA polymerase (Applied Biosystems, Courtaboeuf, France), and 10 ng of DNA template. The PCR reaction was carried out as follows: an initial phase at 95°C for 10 min; followed by 30 cycles of denaturation (95°C/30s), annealing (56°C/15s), and extension ($72^{<5}$C/15s) and followed by final step at 72°C for 7 min. The amplicons of each sample were then submitted to CE-SSCP, which were performed on an ABI Prism 3130 XL genetic analyzer (Applied Biosystems, Courtaboeuf, France) (Zinger et al., 2008). The obtained CE-SSCP profiles were normalized and used for statistical analysis.

Quantification of Soil Bacteria

Mix-samples were collected from the top 3 cm of the soil at 0.5 m distances from the cushion-bulk soil. Soil below *T. caespitosum* cushions was collected from the boundary line between plant and substrate-rhizosphere soil.

Five grams of field-soil were suspended in 45 ml of 0.2% solution of calgon (sodium hexametaphosphate) and homogenized in an ultrasonic bath (50 kHz, 4 min). Samples were serially diluted (to 10^4-10^6) and plated (0.2 ml) in quadruplicate onto R2A agar Difco (pH 7.2) for estimation of cultivable cell population density (colony forming units-CFU) after 8 days of incubation in the dark at 20°C. A colony forming curve was generated for each soil sample by counting newly visible colonies every 24 h for a 192-h long incubation period and plotting the cumulative number of colonies at each time point (Sigler et al., 2001). Plates that contained between 30 and 300 colonies were selected for enumeration only. “Fast growers” (r-strategists) were defined as bacteria that produce visible colonies within 72 h, “slow growers” (K-strategists) within 73-192 h, respectively. The total number of bacteria (*T)* in each sample was estimated with DAPI (4', 6-diamidino-2-phenylindole) staining and microscopic counting (Bloem et al., 1995). Cultivable-to-total cell ratio (C/T) (expressed as percentage of CFU from total bacterial counts) was calculated to determine the index of succession state of microbial communities.

Identification of Soil Isolates

The isolates for identification using sequencing of 16S rRNA gene were chosen according the growth strategy and character of colony (pigmentation, shape, and consistency). Each strain represented a unique combination of shape, pigmentation, and consistency and speed of growth. Twelve strains of r- and 12 strains of K-strategists from bulk soil were selected. Nine strains of r- and 12 strains of K-

strategists from the rhizosphere were chosen. The 16S rRNA gene amplification was performed using universal bacterial primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5' - A AGGAGGT GAT CC AGCCGC A- 3') (Edwards et al., 1989), and sequencing. The total volume of PCR reactions was 50 µL. The final reaction mixtures contained (final concentrations) FastStart PCR Master (Roche; 1 x) and primers (Metabion; 500 nM each). Cell lysate, prepared by one cycle of freezing and boiling of bacterial culture in water, or genomic DNA (1 µL) served as a template. Thermal cycling was performed as follows: initial denaturation at 94°C for 2 min; followed by 35 cycles of denaturation (94°C/15s), annealing (61°C/30s) and extension (72°C/45s); followed by final extension at 72°C for 5 min. Amplified 16S rRNA genes were purified with the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) and sequenced using the primers pA and/or pH.

The obtained 16S rRNA gene sequences were edited by Bioedit 7.0.4.1 software (Hall, 1999). The edited sequences were compared against the database of type strains *EzTaxon-e Database* (Kim et al., 2012; http://eztaxon-e.ezbiocloud.net) to retrieve the most relative species. The 16S rRNA gene sequences are available in GenBank under accession numbers KC354443-KC354487.

Data Analyses

In statistical analyses, we distinguish two soil environments: (1) rhizosphere inside cushions and (2) bulk soil from open areas outside cushions.

Bacterial Patterns Derived from CE-SSCP Profiles

To test the respective effects of cushion habitat, elevation and soil physico-chemical characteristics and their interactions on the microbial assemblage variation derived from microbial SSCP profiles, we performed a distance-based RDA on a Bray-Curtis dissimilarity matrix. This was calculated from square-root transformed percentage data standardized by sample totals, separately for the two mountain ranges Nubra and Tso Moriri. The variance partitioning procedure in distance-based RDA was performed with explanatory variables and co-variables to remove their effects and to obtain a net effect of an individual predictor. Using this approach, we constructed tests analogous to the testing of particular terms in ANOVA models but for multivariate data; for details, see Lepš and Smilauer (2003). Differences in bacterial assemblages were tested by 999 permutations. The results of multivariate analyses were visualized in the form of a bi-plot ordination.

To explore further the source of variation in bacterial assemblages in the whole dataset combining Nubra and Tso Moriri, we used multivariate regression trees (MRT) (Death, 2002). The MRT hierarchically splits the dissimilarity matrix into the more homogenous subsets according to the selected gradient. We used the same Bray-Curtis dissimilarity matrix as for ordination analyses and pruned the tree according to 1-SE rule (Breiman et al., 1984).

In order to reveal differences in the soil bacteria and physicochemical parameters between the rhizosphere and bulk soil and their dependence on elevation, we used generalized linear

mixed-effect models. The pair-sample identity was a random effect factor, and elevation and soil origin (rhizosphere and bulk soil) were fixed effect factors. The tests were based on the likelihood-ratio approach, approximating the difference in model deviances with a χ^2 distribution. To control for familywise error rate, the false discovery rate procedure was performed (Benjamíni and Hochberg, 1995). Analyses were run using the *lme4* package (R Development Core Team, 2013) in R software.

We further modeled the effect of cushion, elevation, and soil physico-chemical parameters on the soil bacterial variables (total bacteria, C/T, CFU, r-strategy) using conditional inference trees [a type of classification and regression tree (CART)]. This method belongs to non-parametric regressions, making a dichotomous tree which can be used as a predictive model to get some insight into which environmental factors contribute to high/low values of soil bacterial variables. This type of classification and regression tree has several crucial advantages over other approaches (e.g., traditional CART algorithm), including (1) the statistical testing of each split through permutation, (2) no need for problematic pruning of over-fitted trees, and (3) no selection bias toward variables with many possible splits or missing values (Hothorn et al., 2006).

The statistical methods were applied using *Canoco* 5 (ler Braak and Smilauer, 2012), *mvpart* (Death, 2002), *party* (Hothorn et al., 2006), *lme4* packages within R 3.03 (R Development Core Team, 2013).

Results

The Response of the Bacterial Assemblages to Environmental Factors

The combined effect of elevation, cushion habitat, and soil physico-chemical parameters on composition of bacterial assemblages derived from CE-SSCP profiles explained 55.2% of the total data variation in Nubra, which was highly significant (db-RDA, $P < 0.001$). Concerning marginal effects of each explanatory variable (analyses with no covariables), db-RDA in Nubra showed that the contribution to data variation was 38.9% for soil physico-chemical parameters ($F = 2.1$, $P = 0.008$), 30.7% for altitude ($F = 6.5$, $P = 0.002$), and 2.6% for cushion ($F = 1.636$, $P = 0.088$). Variance partitioning revealed that 8.9% was explained solely by elevation, 17.8% by soil physico-chemical parameters, and 0.7% by cushion. Because these three variables were intercorrelated, only the net effect of elevation proved to be significant, while that of soil physico-chemical parameters and cushion became insignificant, being explained by elevation during variance partitioning. This was indicated by the first ordination axis in the ordination diagram that separated less diverse microbial assemblages of the semi-deserts at 4850 m—characterized by coarsest soil, especially outside cushions— from more diverse assemblages at 5000 and 5100 m with higher soil organic matter and nutrient concentrations (Figure 1). The second axis separated the bacterial assemblages of the highest Nubra site at 5250 m from lower elevation sites. Differences in bacterial assemblages between rhizosphere soil and bulk soil outside the cushion were highest at the lowest elevation of 4850 m, and negligible at higher sites in Nubra. Diversity in bacterial assemblages, expressed by the Shannon-Wienner index, increased with elevation and in bulk soil in Nubra, with the lowest values in the rhizosphere soils at 4850 and 5000 m, and highest at the bulk soil at 5250 m.

In Tso Moriri, db-RDA showed that the contribution to data variation was non-significant in soil physico-chemical parameters (F = 1.1, *P* = 0.46), but was significant for the interaction of elevation and cushion that together explained 24.8% variability in bacterial assemblages (F = 6.6, *P* = 0.018). Variance partitioning confirmed the overwhelming effect of elevation, explaining solely 14.8% variation {F = 2.5, *P* = 0.048), but also significant was the net effect of the cushion (4.2%, F = 1.9, *P* = 0.042), and marginally significant the elevation x cushion interaction (9%, F = 1.3, *P* = 0.096). This was indicated by the first axis in the db-RDA ordination diagram that separated less diverse microbial assemblages of the bulk soils at 5600 and 5750 m—characterized by high pH—from more diverse assemblages of bulk soil at 5350 m with higher phosphate nutrient concentrations (Figure 2). The second axis separated the rhizo- sphere bacterial assemblages at lower elevations—characterized by higher potassium and magnesium—from rhizosphere assemblages at 5850 m with higher OM and concentration of calcium and sodium.

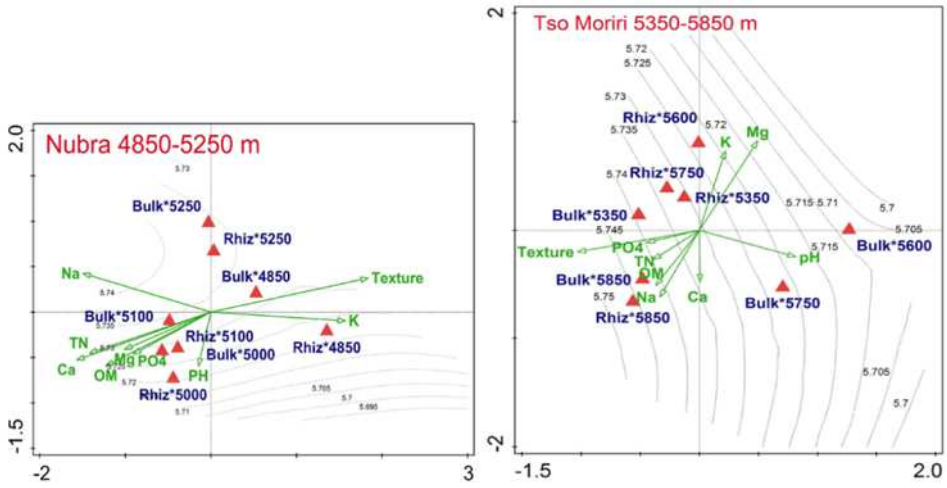

Fig.1 and 2 db-RDA analyses of the bacterial dissimilarity matrices (Bray-Curtis) and vector-fitting of the environmental variables calculated separately for Nubra and Tsomoriri. Communities were grouped to the centroid by elevation and cushion interaction.

The multiple regression tree (MRT) analysis (Figure 3) shows that composition of bacterial assemblage variation in the whole dataset (Nubra and Tso Moriri together) was primarily predicted by soil texture (coarseness). Less diverse assemblages from coarser soil on leucogranite bedrock in Nubra were separated from more diverse bacterial assemblages from finer soil at Tso Moriri. The finer soil at Tso Moriri is derived from more easily weathering gneisses. Within coarser soil Nubra assemblages, lower calcium and phosphate concentration separated less diverse rhizosphere communities of lower elevations (< 5000 m) from more diverse assemblages of higher elevation sites. Bacterial assemblages in finer texture soils (less than 58.5% of soil particles>0.5mm) were split in next node by elevation, clearly separating the remaining Nubra sites from all Tso Moriri communities, where texture, calcium and pH play a role in further splitting.

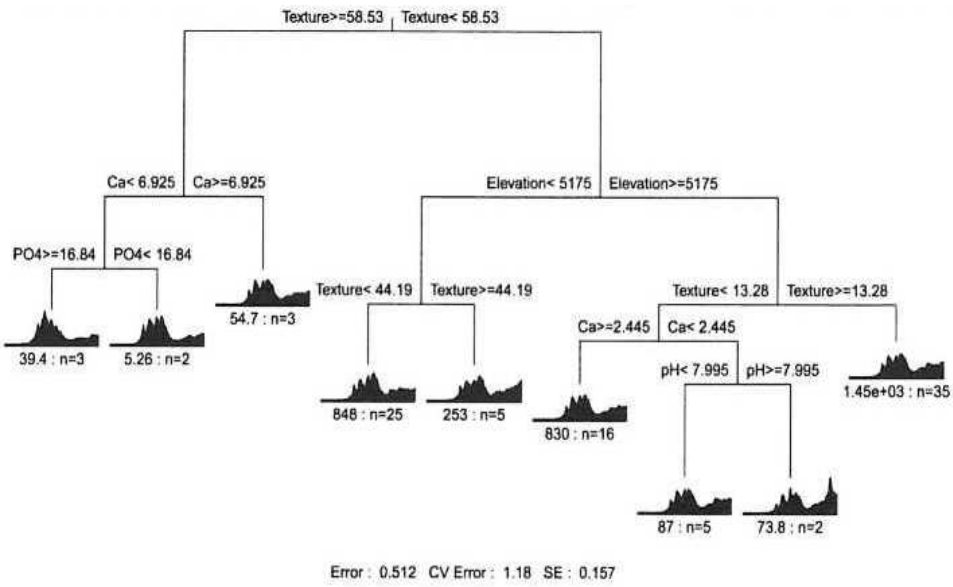

Fig. 3: Environmental factors predicting variation in bacterial assemblages in Nubra and Tso Moriri. We identified the differences with multivariate regression tree which hierarchically splits bacterial dissimilarity matrix into more homogenous subsets according to the elevation, cushion and soil physico—hemical parametres. The dissimilarity matrix was calculated from SSCP profiles data with Bray-Curtis index and the tree was pruned according the 1-SE rule.

Growth Strategies of Cultivable Bacteria

An abundant bacterial community was recorded at both local¬ities in all studied elevations (108 cells/g OM). The total bac¬terial counts were higher in Tso Moriri than Nubra. At both localities, the total bacterial counts were higher in the rhizo¬sphere at the lowest sites (Nubra 4850 m, Tso Moriri 5350 m), whereas more bacteria were recorded in bulk soils at higher sites at Nubra (significant cushion x elevation interaction, Table 1, Figure 4).

The cultivable bacteria isolated from the soils allowed determination of growth strategies. The amount of cultivable bacteria (CFU) was two orders lower (106 cells/g OM) than the total bac¬terial count (Figure 4). The CFU of aerobic heterotrophic bacte¬rial population represented 1-56% of the total bacterial counts. At both localities, CFU values decreased with increasing eleva¬tion and a larger proportion of CFU (2-56%) was found in bulk soil (Figure 4). The lower elevation sites (4850 and 5000 m) in Nubra had significantly higher proportions of CFU in bulk soil (30-56%) outside cushions than inside them, while the higher-elevation sites (5100 and 5250 m) did not differ significantly (Table 1). All elevation sites in Tso Moriri had a higher propor¬tion of CFU outside than inside cushions.

At both localities, the percentage of cultivable bacteria, expressed as culturable-to-total cell ratio (C/T), was higher in bulk soils, with the one exception at 5100 m elevation in Nubra. C/T ratio values tend to decrease with increasing elevation both in the bulk soil and the rhizosphere in Tso Moriri, while in Nubra it tends to increase in the rhizosphere with elevation (significant cushion x elevation interaction, Table 1, Figure 4).

The analysis of life strategy (r/K) of bacteria, based on the time interval needed to form a colony on an agar plate, showed significantly more r-strategists in the rhizosphere than in the bulk soil outside the cushion (Figure 4, Table 1). The significant interaction between cushion and elevation was found in Nubra; the

percentage of r-strategists increased in the rhizosphere with increasing elevation, while the opposite was found for the bulk soil outside the cushions. A decreasing trend was observed for both soil environments in Tso Moriri (Figure 4, Table 1).

To reveal the diversity of the cultivable part of bacterial communities, sequencing of 16S rRNA gene was provided on the isolated strains, both for r/K-strategists. The taxonomic composition of r- and K- strategists was different in the bulk soil and the rhizosphere (Table 2). The r-strategists were less diverse than the K-strategists on species, genus, and family and even phylum level. Only four families of r-strategists were determined in both types of studied soils, while seven families from bulk soil and 10 families from rhizosphere were recorded for K-strategists (Table 2). The families of r-strategists found in bulk soil and rhi¬zosphere were the same. Surprisingly, both r- and K- strategists were covered by the phylum Actinobacteria and specialized K-strategists were mostly represented by Firmicutes, Proteobacteria and Cytophaga-Bacteriodes-Flavobacterium groups. The fami¬lies of K-strategists were more diversified between soil types; only three of them were identical (Micrococcaceae, Streptomycetaceae, and Microbacteriaceae). The other bacteria representing the K-strategists were isolated rarely and also represented the unique bacterial isolates for both types of soils.

Species composition of cultivable bacteria showed small similarity between r/K-strategists and also between the soil types (Table 2). There were 35 species (19 genera) identified at both soil types, with 13 (seven genera) unique in bulk soil, 21 (10 genera) unique in the rhizosphere, and only the Atrhrobacter, Streptomyces, and *Paettibacillus genera* were common to both soils. On the species level, only *Streptomyces cirratus* and *Paenibacilus amyloliticus* were identified in both soils, otherwise the species composition did not overlap (Table 2). Two unidentifiable isolates were found in studied soil, and they are

potentially species of bacteria new to science (Table 2).

TABLE 1 | Microbiological and soil physico-chemical characteristics at two localities Nubra and Tso Moriri in all investigated elevations.

		Nubra					Tso Moriri					
		4850	5000	5100	5250	Cushion	Elevation	5350	5600	5750	5850	Cushion	Elevation
Total bacteria 10^8/g DW	R	**41.7**	36.8	**6.9**	28.2		** ↓in↑out	56.7	57.5	66.0	47.1		
	B	**27.1**	30.8	**35.3**	29.9			49.5	51.3	65.3	54.3		
CFU 10^6/g DW	R	**4.8**	**2.8**	2.3	3.3	***out>in	*** ↓	**6.4**	**2.0**	**2.9**	**1.5**	***out>in	*** ↓
	B	**12.0**	**7.3**	4.2	5.0			**10.6**	**5.3**	**5.9**	**3.8**		
C/T %	R	**12.5**	**14.1**	**35.6**	12.7	*out>in	** ↑in↓out	**12.7**	**3.5**	4.3	3.4	***out>in	*** ↓
	B	**39.5**	**34.7**	**16.8**	12.3			**27.0**	**10.3**	10.0	7.3		
r-Strateg %	R	**50.7**	**67**	63.3	**72.8**	***in>out	*** ↑in↓out	72.7	68.3	58.0	57.5	**in>out	** ↓
	B	**41.7**	**45.2**	57.3	**32.2**			64.2	54.3	62.3	46.8		
TN mg/kg	R	487	1686	1163	985			833	697	1252	1415		** ↑
	B	613	1385	778	1024			738	957	1148	1554		
P-PO_4^{3-} mg/kg	R	17.6	19.1	12.6	12.3	*out>in	*** ↓	28.2	14.1	24.8	12.7	*out>in	* ↓
	B	**23.0**	**25.7**	14.4	13.0			**39.5**	**20.8**	23.1	17.2		
Ca mg/g	R	5.2	32.6	12.7	17.7			2.9	2.5	2.4	2.5	*out>in	** ↓in↑out
	B	7.3	33.3	17.3	17.6			2.5	3.0	**3.0**	**3.6**		
Mg mg/g	R	8.1	8.5	7.6	7.5	*out>in		3.5	2.8	2.5	2.2		*** ↓
	B	7.6	**9.9**	**8.6**	8.3			3.1	3.1	2.6	2.5		
K mg/g	R	6.5	3.7	3.9	3.7		*** ↓	3.0	1.8	2.2	1.9		* ↓> in
	B	5.9	4.4	4.0	4.1		*** ↑	2.4	2.0	2.2	2.2		
Na mg/g	R	0.6	0.6	0.9	0.9			0.3	0.2	0.5	0.5		*** ↑> out
	B	0.4	0.7	0.9	0.9			0.1	0.2	0.6	0.6		
OM %	R	2.4	**7.2**	**4.4**	2.8	**in>out		2.3	2.0	2.6	2.8		
	B	1.8	3.0	2.7	2.9			2.5	2.2	2.3	2.8		
Soil particles>0.5 mm %	R	54.0	19.9	14.4	42.5		* ↓	11.9	10.6	15.1	20.1		** ↑
	B	55.4	25.9	18.0	35.9			13.3	10.2	13.4	18.6		
pH	R	8.4	8.5	8.6	8.4	**out>in		7.7	7.7	7.1	6.9	***out>in	** ↓
	B	8.5	8.8	**9.0**	**8.8**			**8.1**	**8.3**	**8.0**	**7.8**		

*Availability of soil nutrients and composition of microbial assemblages in the rhizosphere (R) T. caespitosum or in the bulk soil (B). An upward or downward pointing arrow indicates a positive or negative relationship between the dependent variable and elevation, based on the likelihood-ratio test in generalized linear mixed-effect models (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$). Also shown are post-hoc Tukey tests on paired differences (significantly higher values inside/outside cushion are in bold). The presented values are mean from six replications at one elevation.*

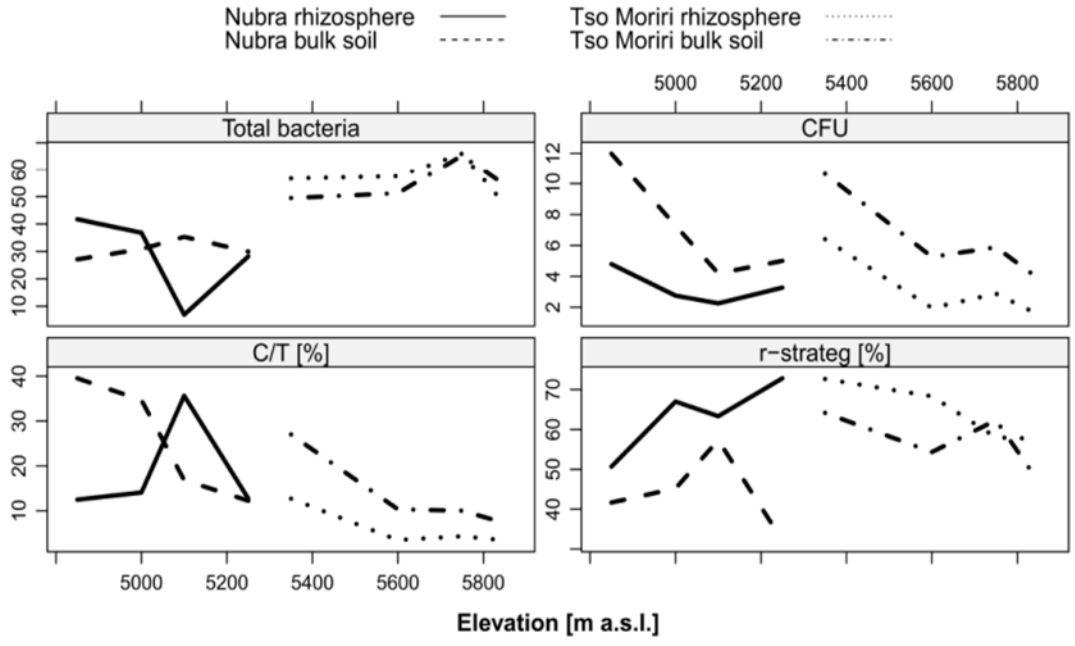

FIGURE 4 | Characteristics of heterotrophic bacterial communities at two localities Nubra and Tso Moriri in all studied elevations. Total count of bacteria*10^8/g DW of soil; CFU, culturable cell population density after 8 days of cultivation*10^6/g DW of soil; C/T, percentage of CFU from total bacterial counts; r-strateg—percentage of bacteria that produce visible colonies within 72 h.

TABLE 2 | Species composition and growth strategy of bacteria in bulk soil and rhizosphere of *Thylacospermum*.

	Bulk soil		Rhizosphere		Total	
	r-strateg	K-strateg	r-strateg	K-strateg	r-strateg	K-strateg
ACTINOBACTERIA						
Brevibacteriaceae						
*Brevibacterium luteolum** (94.9%)		+				+
Intersporangiaceae						
Knoellia locipacati (98.2%)				+		+
Microbacteriaceae						
Agrococcus citreus (99.6%)		+				+
Clavibacter michiganensis (99.4–99.5%)	+	+			+	+
Curtobacterium flaccumfaciens (100%)			+		+	
Microbacterium phyllosphaerae (98.7%)			+		+	
Mycetocola manganoxydans (99.6%)				+		+
Micrococcaceae						
Arthrobacter agilis (99.6%)		+				+
Arthrobacter humicola (99.6%)	+				+	
Arthrobacter nitroguajacolicus (99.7%)	+				+	
Arthrobacter oryzae (99.6%)		+				+
Arthrobacter pascens (99.7–99.9%)	+		+		+	
Arthrobacter polychromogenes (99.7%)	+				+	
Arthrobacter tumbae (99.7%)		+		+		+
Kocuria rosea (99.5%)	+				+	
Micromonosporaceae						
Micromonospora saelicesensis (98.7%)				+		+
Nocardioidaceae						
Kribbella catacumbae (99.5%)		+				+
Promicromonosporaceae						
*Isoptericola dokdonensis** (95.2%)				+		+
Streptomycetaceae						
Streptomyces badius (99.3%)			+		+	
Streptomyces citratus (99.7–99.9)	+	+	+		+	+
Streptomyces cyaneofuscatus (100%)	+				+	
Streptomyces glomeroaurantiacus (99.6%)	+				+	
Streptomyces humidus (99.7%)			+		+	
Streptomyces lilmocidini (99.3–99.6%)			+		+	
Streptomyces niveus (99.4–99.7%)		+				+
*Streptomyces scabiei*sporus (99.6%)				+		+
FIRMICUTES						
Paenibacillaceae						
Paenibacillus alginolyticus (99.7%)				+		+
Paenibacillus amylolilicus (99.7%)	+		+		+	
Paenibacillus borealis (99.2%)				+		+
Staphylococcaceae						
Staphylococcus caprae (100%)				+		+
Staphylococcus warneri (100%)				+		+
PROTEOBACTERIA						
Alpha-proteobacteria						
Acetobacteraceae						
Roseomonas aestuarii (99.6%)				+		+
Rhodospirillaceae						
Skermanella aerolata (99.6%)		+				+

TABLE 2 | Continued

	Bulk soil		Rhizosphere		Total	
	r-strateg	K-strateg	r-strateg	K-strateg	r-strateg	K-strateg
Beta-proteobacteria						
Alcaligenaceae						
Pigmentiphaga litoralis (96.6%)				+		+
BACTEROIDETES-CYTOPHAGA-FIRMUCUTES group						
Cytophagaceae						
Dyadobacter psychrophilus (96.6%)		+				+
Total no. species	9	11	8	12	15	22
Total no. genus	5	6	5	10	7	16
Total no. families	4	7	4	10	4	14

Numbers in parentheses are nucleotide similarity of single strains.
**means low nucleotide similarity on genus level.*

Cushion, Elevation, and Soil Parameters Impact on Cultivable Bacteria

The conditional inference tree analysis of total bacteria counts showed primarily a locality effect, separating high-elevation sites of Tso Moriri (5350-5850 m) with more bacteria from Nubra sites (4850-5250 m). Nubra sites were separated at the next node by Na, with significantly more bacteria found in soils with lower concentration of sodium (<0.856 mg/g). At Tso Moriri, more total bacteria was found in soil with lower organic matter (<3.18%) and Ca (<2.78 mg/g) contents (Figure 5).

The analysis for CFU showed a primary habitat effect, separating bulk soils with higher CFU values from rhizosphere soils. Rhi- zosphere bacterial communities were separated at the next node by the concentration of phosphate. The higher amount of CFU was presented in less phosphate-limited soils (>17.28 mg/kg). The least CFU in rhizosphere were found in samples with lower concentrations of PO4-P, Ca, and total N. The amount of CFU in bulk soils reached a higher number at elevations sites lower than 5000 m, and in soils containing less sodium (Figure 6).

The conditional inference tree analysis of C/T ratio showed primarily an elevation effect, separating lower-elevation sites below

5350 m with higher C/T ratio from the higher-elevation sites (above 5350 m). The next divisions were in both cases based on cushion habitat, with the highest C/T ratio values found in bulk soils from lower elevations below 5000 m (Figure 7).

The analysis for proportion of r-strategists showed a primary cushion habitat effect, separating bulk soils from the rhizosphere. The r-strategists from the rhizosphere were later separated by calcium content, where the critical concentration was 6.16 mg/g. Tso Moriri lower elevation sites (5350-5600 m) had more r-strategists than sites above 5600 m. The abundance of r-strategists in bulk soil was determined firstly by locality. pH influenced the abundance of r-strategists at Nubra sites, where the more abundant communities were found in soil with pH higher than 8. At Tso Moriri the abundance of r-strategists was influenced by TN concentration (Figure 8).

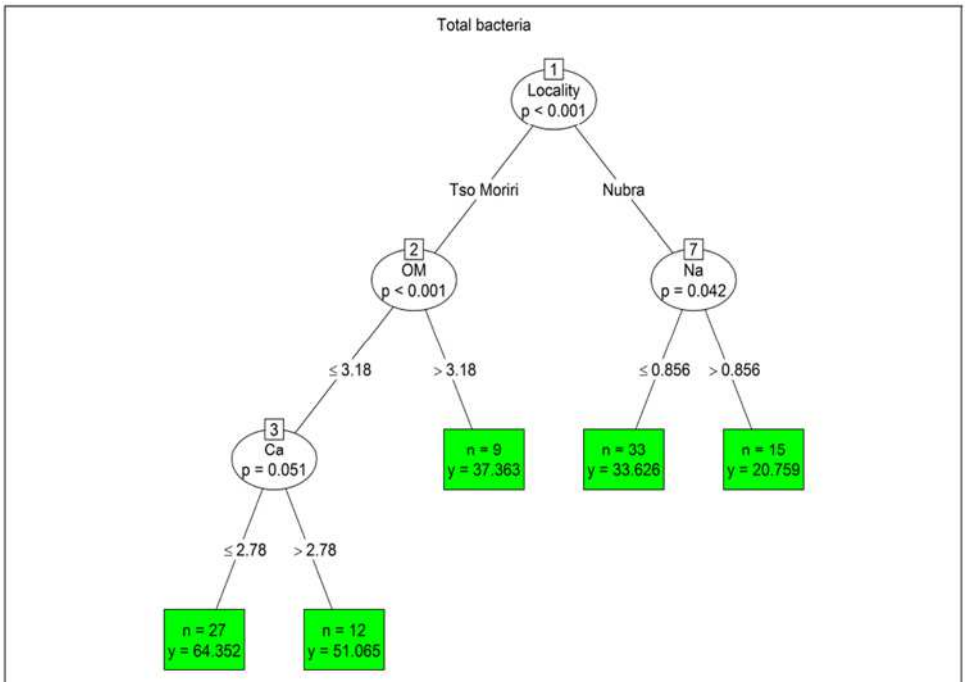

FIGURE 5 | Conditional inference trees testing the effect of cushion, elevation, and soil physico-chemical parameters on the total bacterial counts. Hierarchical splitting of the data was based on selecting predictors that best distinguish a variable's responses and dividing samples into two groups according to the predictor's splitting value. Significant predictors are shown in oval windows, while the number of releves in each group (*n*) and their mean species richness (*y*) in green boxes.

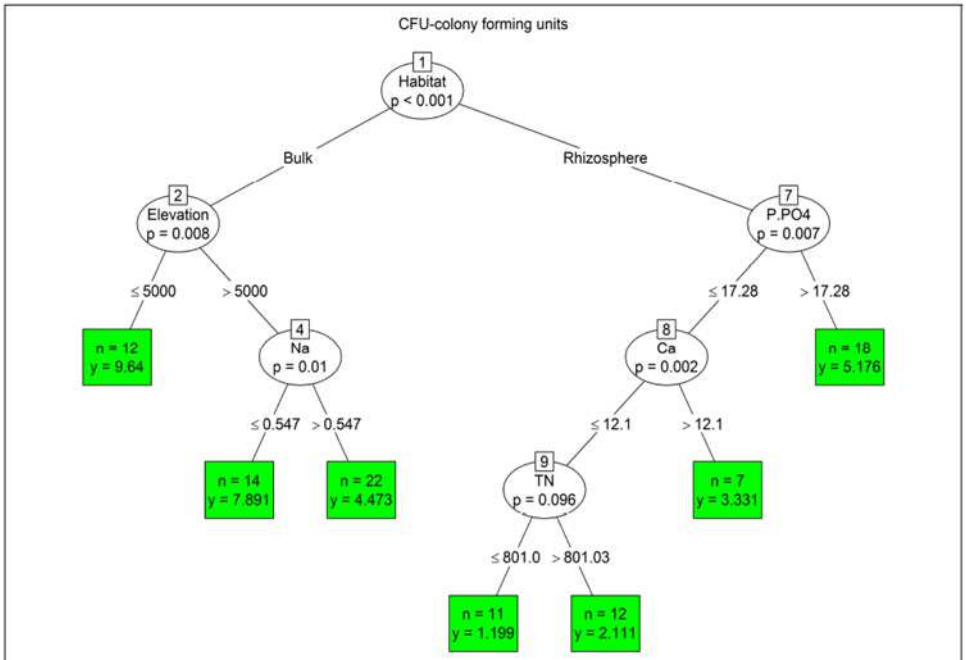

FIGURE 6 | The conditional inference tree analysis of CFU (for details see Figure 4).

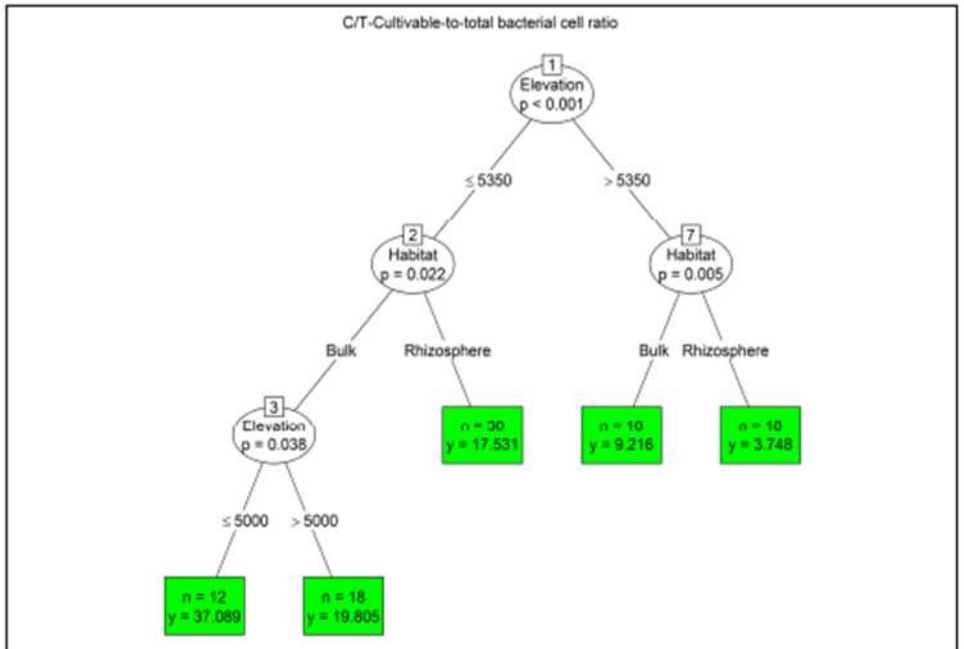

FIGURE 7 | The conditional inference tree analysis of proportion of cultivable bacteria to total bacterial count (for details see Figure 4).

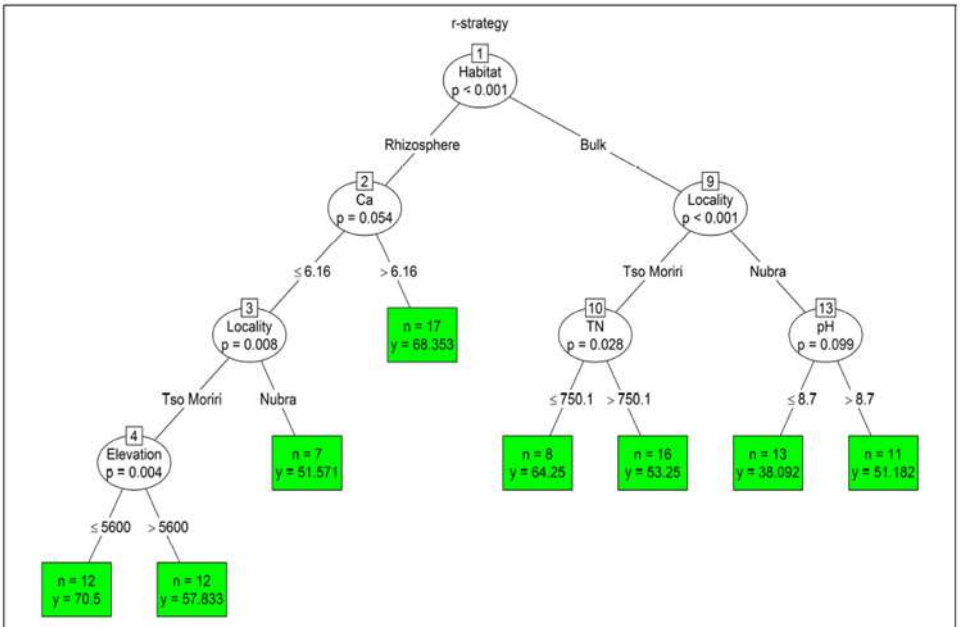

FIGURE 8 | The conditional inference tree analysis of r-strategist (for details see Figure 4).

Microclimatic Conditions

At Tso Moriri, the vegetation season, defined as the period with mean daily soil temperatures above freezing lasted nearly 5 months at 5350 m, 3.5 months at 5600 m, and 3 months at 5750 m (data not shown). At the highest elevation (5850 m), the vegetation season was restricted to less than 2 months (56 days, Figure 9). The mean air annual/summer temperatures decreased from — 4.4/7.3 to — 10.4/4.4°C between 5350 and 5850 m, while relative air humidity increased from 61/50 to 84/53%. The sites differed mainly in the duration of the sub-zero temperature spells over the course of a 24-h period. While air temperature 10 cm above ground never dropped below zero at the lowest elevation during August, it usually fell below zero for about 2-3 h at the middle elevations; at the highest elevation at 5850 m freezing lasted between 6 and 10 h every day, particularly in the second half of

August (Figure 9) when many plants still flowered and fruited. Daily air temperatures rose to 8-17°C at all four elevations but for a much shorter time per day at higher elevations. In Nubra, the vegetation season lasted between 3 months (mid-May to beginning of September) at 5250 m and 5 months (the begin¬ning of May to mid-October) at 4850 m (Figure 9). The mean air annual/summer temperatures decreased from — 1.6/7.7°C to —3.6/7.1 and the relative air humidity increased from 39/38 to 87/69% with increasing elevation. At all sites, air temperatures in the warmest month of August remained above zero both day and night.

Cushions from a low site in Nubra (5000 m) provided warmer microsites, as measured 2 cm below ground, compared with open areas (annual mean T = —0.3 vs. —3.4JC). They also had twice as many degree-days (1227 vs. 601 at TbaSe = 0 C) and a frost-free period lasting a month longer (data not shown). On the other hand, the differences at the highest site in Tso Moriri were only minor, the open areas being even slightly warmer than cushions.

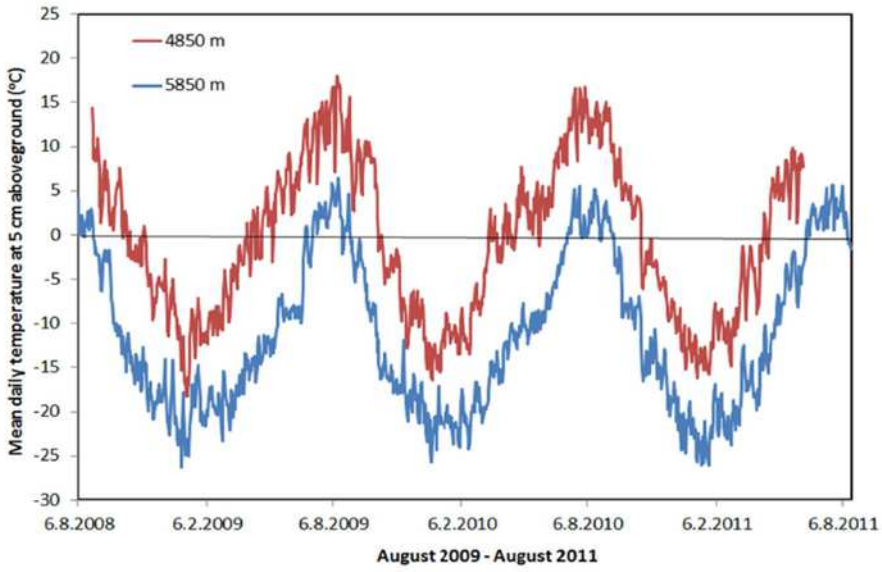

FIGURE 9 | The course of the mean daily temperature at 5 cm aboveground at the lowest studied elevation (Nubra 4850 m) and the highest (Tso Moriri 5850 m) during years 2008–11.

Soil Chemical Properties

At both localities, the contents of PO^-P were higher in the bulk soil (Table 1, Figure 10). PO4-P and K content significantly decreased with elevation (Figure 10, Table 1). Sodium content increased with elevation in Tso Moriri (Table 1). Organic mat¬ter content was higher in the rhizosphere in Nubra, while in Tso Moriri the values between rhizosphere and bulk soil did not differ (Figure 10, Table 1). More Mg was found in the bulk soil at Nubra and this was significant at 5000 and 5100 m elevations. Similar magnesium concentrations were recorded in Tso Moriri with a significant decrease with elevation (Figure 10, Table 1). Calcium concentration had similar values in the rhizosphere and the bulk soil at both localities and decreased with elevation (Figure 10, Table 1). The pH had higher values in bulk soils than in the rhizosphere and the decreasing trend was observed with increasing elevation, significant in Tso Moriri (Figure 10, Table 1).

Discussion

Vascular plants and soil microbial assemblages are indirectly connected through the soil substrate and interaction, which influence the soil fertility (Bardgett and Walker, 2004; van der Heij den et al., 2008). Higher plants contribute to resources through litter fall and rhizodeposition and can also provide suitable microclimatic conditions to belowground organisms. Microbial assemblages are able to convert the inaccessible minerals to accessible one for plants (Clarholm, 1985; Bonkowski, 2004; Carlson el al., 2010). We expected the dominant cushion plants of the high-elevation Himalayas cold deserts to ameliorate the environmental conditions for soil microorganisms. This way, the bacterial assemblages become more diverse in the rhizosphere of *T. caespitosum* than in the bulk soil of the subnival zone. The present investigation is, to the best of our knowledge, the first study focused on the interaction

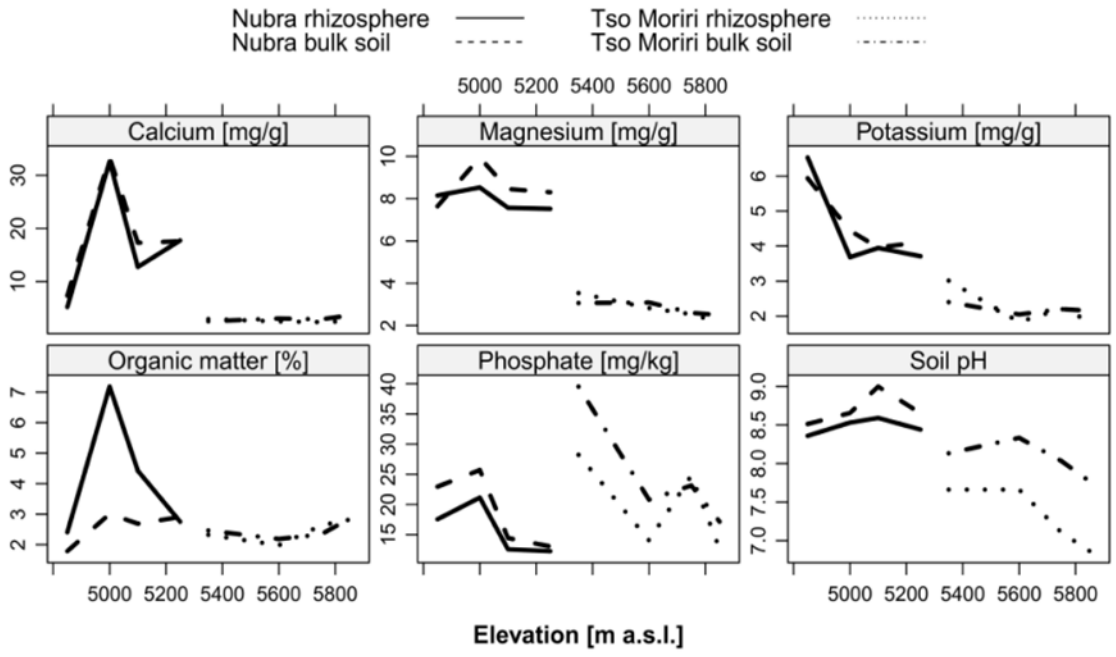

FIGURE 10 | Physico-chemical properties (Ca^{2+}, Mg^{+}, K+, organic matter$^{-}_{3}$, PO^{3-}_{4}, pH) of bulk and rhizosphere soil at two localities Nubra and Tso Moriri in all studied elevations.

between this cushion plant and its microbial assemblages in the dry Himalaya.

We found that *T. caespitosum* cushions do not have a significant impact on the total number of bacteria at both localities, and on the structure of the bacterial community in Nubra (Table 1, Figure 1). The cushions did, however, influence the bacterial community structure in Tso Moriri (Figure 2) and the cultivable part of bacterial community at both localities (Figure 4, Table 1). Hence, the cushion plants do not structure the microbial assemblages evenly along the investigated elevational gradient. The structuring effect is evident with increasing stress such as drought, nutrient and thermal limitation. The similar pattern of structuring bacterial diversity along the elevational gradient was reported from the water streams in China, where the temperature
was the most important driver (Wang et al., 2012). Roy et al. (2013) found that the assemblages from the rhizosphere of Silene acaulis diifered from the bulk soil assemblages in the French Alps. Our study indicates that such differences exist in the dry Himalayas, but they are elevation-dependent, increasing with higher abiotic stress at higher elevations. Thus, plant-bacteria association became more common as environmental conditions became less harsh and plants became more abundant (King et al, 2012).

We found a significant influence of the cushion on the cultivable part of the bacteria. The proportion of r-strategists is larger in the rhizosphere, where there is more organic matter and a lower pH, making phosphorus more accessible for organisms. The higher moisture and stable temperature also help create the conditions demanded by zymogenous (r-strategist) microorganisms (Langer et al., 2004). The rhizosphere also releases exudates (such as pentose) which are preferentially utilized by r-strategists (De Ley et al., 1993; Koch, 2001; Elliott et al., 2008; Eíhottová et al., 2009). The higher proportion of K-strategists was recorded at the localities with persistent, severe conditions such as drought at lower

elevations (4850-5000 m) (Cruz-Martinez et al., 2009) or regular freezing/thawing during the vegetation season at higher elevations (5750-5850 m) (Figure 9). These unfavorable conditions evoke competition about resources, which can support the growth of organisms with lower biomass turnover and high metabolic efficiency (Dworkin et al., 2006). At these localities, stable assemblages are created, capable of utilizing and exploiting the recalcitrant material. The “climax stadium” of bacterial assemblage is probably developed here.

The spatial differences in the proportion of cultivable and non-cultivable types were found along the elevational gradient and between localities **(**Figure 4). The relatively high degree of cultivability (1-54%) was surprising, but similar results were reported for microbial populations from other harsh environments such as sea ice (Junge et al., 2002), subglacial sediments (Foght et al., 2004) or recently from dry soil in the Andean Puna (Fcrrero el al, 2010). This discovery contrasts with widely cited value (<1%) of the total count being cultivable (Amann et al., 1995). This could be caused by the relative simplicity of cold environments and their associated biota (Foght et al., 2004). The percentage of cultivable bacteria is much lower in higher elevations, especially in Tso Moriri. An increasing proportion of non- cultivable types may be associated with later successional stages since these types direct less energy into growth and/or have very specific growth requirements that may not be readily provided in artificial media (Garland et al., 2001). We suggest that generally arid soil in the southwestern part of the Tibetan Plateau, where Tso Moriri is situated, is in climax stage, not being affected by large disturbances associated with glaciation fluctuation for the millennium. Glaciological studies have indicated that contrasting patterns of glaciations exist across adjacent regions of the Himalaya, which are likely due to a combination of orographic and climatic influences (Owen et al., 2008; Dortch et al., 2010). Ice sheet glaciations did not evolve during the Last Glacial Maximum

on the majority of the Tibetan Plateau (Kirchne et al., 2011). There is some evidence for the glacial advance in Korzog Range c.a. 4.7-2.7ka BP (Leipe et al., 2014) on the other side of Tso Moriri Lake, which is way more glaciated than our study site because of northeast-facing slopes. In contrast, Nubra is a rel¬atively young glacier forefield (up to 100 years old). The early successional states usually contain a higher proportion of cul¬tivable types (Garland et al., 2001; Sigler et al., 2002; Krištůfek el al., 2005).

We used conditional inference trees to identify important environmental parameters structuring the bacterial assemblages in the diy Himalayan soils. A combination of locality, habitat and elevation together with soil chemical factors showed to be important predictors of variation in the bacterial assemblages. However, each parameter had different drivers. The phylogenetic structure of the bacterial community was primarily explained by the mountain range type (Tso Moriri vs. Nubra). The r-/K- strategists and numbers of cultivable bacteria were determined by the microhabitat type (inside vs. outside the cushion), while the C/T ratio was mostly influenced by elevation. Another impor¬tant parameter affecting biomass and composition of bacteria was the concentration of cations, mainly sodium and calcium. Hasse el al., 20Ü1 showed that sodium played a significant role in the energy metabolism and pathogenicity of bacteria. Also the sporu- lation and germination are influenced by the concentration of divalent cations, including calcium. The fast uptake of calcium was observed in stage IV of sporulation (Young and Filz-James, 1962) and may ultimately comprise up to 3% of the dry weight of the spores (Murrell and Warth, 1965). The concentration of cations remarkably influenced the physiological properties of spores, in particular a resistance to heat, radiation, enzymes, disinfectants and other deleterious agents (Warth, 1979). We propose that the bacteria living in high-elevation soils have higher uptake of cations, which could help them to withstand severe conditions in the nival and subnival zones. This question needs to be studied more.

It is important to know which bacterial species or groups of species are present in subnival ecosystems and in the rhizosphere of the dominant vascular plant of this zone. We need to know the present species diversity, because ongoing climate warming in the Himalayas will likely influence the relative importance, frequency and composition of functional groups, their trophic interactions, and processes controlling these interactions (Chakraborty et al., 2012).

The taxonomic composition of isolated bacteria was very variable and was dominated by Actinobacteria (Table 2). Soil Actinobacteria, with a diverse machinery of enzymes, are important players in the decomposition of soil recalcitrant matter and weathering of mineral elements. Even more, mostly Streptomycetes produce phytohormones and soil enzymes, solubilize phosphate, and thus play a role as plant growth promoters (Vyas et al., 2009; Gulati et al., 2010). By contrast, Proteobacte- ria were a less abundant member of the studied soil assemblages. This is different from the typical soil bacterial assemblages with regular distribution of Proteobacteria, represented mainly by e.g., *Pseudomonas, Stenotrophomonas*, *Xanthomonas, Acinetobacter, Variovorax,* and other genera (Krištůfek et al., 2005; Liu et al., 2012). The increased abundance of gram-negative soil bacteria, represented mainly by a-, ß-, and y-Proteobacteria, is usually associated with rhizosphere of higher plants (Fierer et al., 2007). The *Arthrobacter* genus comprised a significant portion of the bacterial isolates from the investigated bulk soil, representing both growing strategies-fast (r-) and slow (K-), where five out of eight species were identified as r- strategists. *Strep- tomyces* represented relatively diversified taxa identified in both soil types (Table 2). Interestingly, six out of eight Streptomycete species *{S. badius, S. cirratus*, *S. cyaneofuscatus, S. glomeroauran- tiacus, S. humidus,* and *S. litmocidini*) produced visible colonies early (up to day 3 of cultivation) and fitted the r-selection strategy. Other fast growing Streptomycetes, *S. rochei,* and 5. *cremeus,* were described previously by Yang and Lou (2011) from spring in Carst

area in China. This indicates that the *Arthrobacter* and *Streptomyces* genera cannot be assigned to the r- or K- selection category, as different species inside a particular genus differed in their ecological demands. This situation is analogous to the superior taxon Actinobacteria (Fierer et al., 2007). The community composition might be influenced by the fact that soil sample desiccation preceded the cultivation of bacteria, but the shortage of water is common for the Himalayan soils. However, there is an assumption that Actinobacteria evolved as a terrestrial clade of bacteria dominating the arid soils by an average of 64% (Battistuzzi and Hedges, 2009) and that together with Cyanobacteria they comprise the major components of Terrabacteria harboring the most developed adaptation mechanisms on terrestrial life strategy. These adaptations include desiccation resistance of the peptidoglycan layer of Gram-positive bacteria (Actinobacteria and Firmicutes), as well as spore production (Gram-positive taxa and Cyanobacteria), which confer resistance to multiple stresses (desiccation, UV radiance, or high salt concentration) typical for terrestrial habitats, and even more pronounced in mountain areas. Among the isolates, one psychrophilic strain (*Dyadobacterpsychrophilus* strain Lad-5K) was detected and there are some assumptions that other strains can grow under low temperatures, being adapted to cold soils in high mountains.

This is the first study surveying the quality, quantity and life strategy of bacterial assemblages in Ladakh Mts., India along elevation gradient in association with dominant alpine plants T. *ceaspitosum.* Species composition of whole bacterial assemblages, species composition of cultivable, aerobic bacterial taxa in bulk soil and *Thylacospermum* rhizosphere were also investigated. It is apparent that physico-chemical parameters of soil are tightly coupled with the bacterial diversity in the extreme environment of mountain cold desert. The present study illuminates the complex plant-soil microbial relationship in a xeric subglacial

environment and brings the primary data about diversity of heterotrophic bacteria for the Himalayan soils.

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CHAPTER IV

Some like it high! Phylogenetic diversity of high-elevation cyanobacterial community from biological soil crusts of Western Himalaya.

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Some like it high! Phylogenetic diversity of high-elevation cyanobacterial community from biological soil crusts of Western Himalaya.

Abstract

The environment of high-altitudinal cold deserts of Western Himalaya is characterized by extensive development of biological soil crusts, with cyanobacteria as dominant component. The knowledge of their taxonomic composition and dependency on soil chemistry and elevation is still fragmentary. We studied the abundance and the phylogenetic diversity of the culturable cyanobacteria and eukaryotic microalgae in soil crusts along altitudinal gradients (4600-5900 m) at two sites in the dry mountains of Ladakh (SW Tibetan Plateau and Eastern Karakoram), using both microscopic and molecular approaches. The effects of environmental factors (altitude, mountain range, and soil physico-chemical parameters) on the composition and biovolume of phototrophs were tested by multivariate redundancy analysis and variance partitioning. Both phylogenetic diversity and composition of morphotypes were similar between Karakorum and Tibetan Plateau. Phylogenetic analysis of 16S rRNA gene revealed strains belonging to at least five genera. Besides clusters of common soil genera, e.g., *Microcoleus, Nodosilinea,* or *Nostoc*, two distinct clades of simple trichal taxa were newly discovered. The most abundant cyanobacterial orders were Oscillatoriales and Nostacales, whose biovolume increased with increasing elevation, while that of Chroococales decreased. Cyanobacterial species richness was low in that only 15 morphotypes were detected. The environmental factors accounted for 52 % of the total variability in microbial data, 38.7 % of which was explained solely by soil chemical properties, 14.5 % by altitude, and 8.4 % by mountain

range. The elevation, soil phosphate, and magnesium were the most important predictors of soil phototrophic communities in both mountain ranges despite their different bedrocks and origin. The present investigation represents a first record on phylogenetic diversity of the cyanobacterial community of biological soil crusts from Western Himalayas and first record from altitudes over 5000 m.

Keywords: Soil crusts, Cyanobacterial diversity, Western Himalayas, High-elevation, Desert,Phosphorus

Introduction

The phototrophic microbial communities are important components of soils in arid and semi-arid ecosystems around the world. The knowledge of their taxonomic composition and dependency on soil chemistry and vegetation is, however, still unclear and requires further attention. Especially the remote mountain regions such as Western Himalaya are insufficiently explored in this regard. The dry mountains in the Western Himalaya are situated in the rain-shadow north of the Great Himalaya range. The harsh environment of this arid area is characterized by extreme diurnal temperature fluctuations, strong winds, and high UV radiation with sparse vegetation cover. These circumstances open the space for the extensive development of biological soil crusts (BSCs) and increase the importance of cyanobacterial communities, which are the dominant component in this area. BSCs carry out key processes in the development of soil [1, 2], biogeochemical cycling [3, 4], and plant colonization [5] in extreme environmental areas.

The diversity and abundance of soil cyanobacterial and microalgal communities as first colonizers of high- mountain soils may profoundly affect nutrient availability for pioneer vascular plants [6]. The biodiversity and functions of microbes in the mountain ecosystems have received increased attention, especially with respect to climate change, glacial retreat, and vascular plant distributional shift e.g., [7, 8]. These changes in biodiversity can alter ecosystem processes and the resilience and the resistance of ecosystems to environmental change [9, 10]. Without baseline data on soil diversity, however, we cannot track the effects of climate change, and without an understanding of the drivers of community composition, we cannot predict how climate change may affect these soil communities.

In our previous study, we investigated the abundance and the diversity of cyanobacteria in the BSCs along an altitudinal gradient (5300-5900 m a s 1.) in a SW extension of Tibetan Plateau, Ladakh [11]. The diversity was determined only according to the

morphology of cyanobacteria, bringing the basic picture about the cyanobacterial diversity of BSCs from this region.

The aim of this study is detailed assessment of cyanobacterial diversity based on molecular phylogenetic taxonomy, using the 16S rRNA gene sequencing of unial- gal strains. DNA sequence data are therefore well placed to provide more insight into the cyanobacterial diversity of the BSCs from the high altitudes of the Western Himalayas. By using the molecular advance, we are able to compare the taxonomical composition with other regions to assess whether cyanobacteria of Ladakh BSCs are mainly cosmopolitan or they rather form isolated populations. We also incorporate the morphological and molecular data in an effort to provide accurate identifications and description of gained strains.

The study is extended by comparing the taxonomic composition of phototrophs, based on molecular phylog- eny, in two mountain ranges in the Western Himalayas— southwestern spur of Tibetan Plateau with Eastern Karakoram. These mountain ranges have different bedrocks and origin, allowing us to compare the cyanobacterial diversity in the alpine and subnival soils in relation to local environmental conditions. The present investigation represents, to the best of our knowledge, a first record on phylogenetic diversity of the cyanobacterial community of BSCs from Western Himalayas and first record on phylogenetic diversity in altitudes over 5000 m elevation.

Methods

Sampling Sites and Sample Collection

The study area is in Ladakh, Jammu, and Kashmir States, India, and is characterized by an arid cold-desert environment because the precipitation seldom crosses the high crest of the main Himalayan range [12]. The fieldwork was conducted in August

2010 in two mountain areas of Ladakh: in the Eastern Karakoram Range in the Nubra Valley site (34°45'N, 77°35'E) and in the southwestern extension of the Tibetan Plateau on the western slope of Chamser Kangri peak above Tso Moriri lake (32°59′N, 78°24'E). The Chamser Kangri Plateau consists mainly of gneisses [13], while the Nubra Valley consists mainly of leucogranits [14]. The samples of BSCs were collected along two elevational transects at both mountain sites. The transect in E Karakoram was 9 km long, the sampling sites were in four altitudes 4600,4800,5000, and 5200 m. The transect at the Tibetan Plateau was 12 km long, with sampling sites in four altitudes between 5300,5500, 5700, and 5900 m. The sampling points on each transect cover the different major vegetation types. The altitudinal zonation of vegetation included steppes and semi-deserts at lower elevations, and alpine meadows, screes, and the subnival zone close to glaciers. The cover of vascular plant vegetation ranged from 0 to 50 %. The data on vegetation type and cover at the same sampling points were documented by [15-18].

At each sampling point on each transect, five composite samples of BSCs were collected. In total, 20 samples of BSCs were collected from each mountain range (four elevations, five replicates). Soil was taken from an area of 10 m^2 and 2- 4 cm deep (the thickness of BSC) with a sterile spatula. The soil was air-dried on aluminum plates for 10 h immediately after collection, because the field conditions do not allow other storage of collected material. The BSC and arid land soil are commonly exposed to the drying and freezing in the Himalayas. This method of preservation is recommended for arid land soils and BSC samples by Campbell et al. [19], because it prevents microbial activity in a naturally occurring manner, without the cell damage that may be associated with freezing and, particularly, thawing cycles. The samples were placed in sterile 100 ml polypropylene bags (Nasco Whirl- Pak®) and transported to the laboratory for analysis.

Physico-Chemical Characteristics of Soil

Subsamples of soil were used for the determination of pH, organic matter content, and texture as described by Kaštovská and colleagues [20]. For the determination of total nitrogen, methods by Zbiral and colleagues [21] were used. The technique described in Mehlich [22] was used for the extraction of phosphoms (P) from samples, and the concentration of P was measured by using ascorbic acid-molybdate and a SHIMADZU UV-1650PC spectrophotometer. The macroelements (Ca, Mg, K, and Na) were extracted from the soil according to US EPA method 200.2 (HC1-HN03) http:// www.epa.gov/epaoswer/hazwaste/test/3050b.pdf) and determined spectrochemically using US EPA method 3050 [23]. Soil organic carbon was determined by wet oxidation with acidified dichromate [24]. Mid-season volumetric water content was measured at each soil sampling point immediately during the collection by a Hydrosense II Soil Moisture Measurement System (Campbell Scientific, Australia).

Abundance and Diversity of the Phototrophic Microbial Communities

The samples for abundance and diversity investigation were prepared as follows. One gram of mixed soil was diluted in 5 ml of distilled water. The slurry was disintegrated at the beginning manually with pestle and consequently with sonificator (Bandelin Sonorex) for 1 min. Twenty microliter of slurry was put under cover glass cover area 22 x 22 mm. Ten stripes (area of one stripe is 11 mm^2) were counted. Samples were observed under microscopy Olympus BX 60, magnification 400x.

Biovolume and the number of microalgal and cyanobacterial cells as well as the taxonomic composition of communities were determined using light and epifluorescence microscopy (Olympus BX 60). Green and blue excitations (MWB filter cube blue

excitation 450-480, emission 515+ for eukaryotic algae; MWG filter cube green excitation 510— 550, emission 590+ for cyanobacteria) were used [20]. The term “eukaryotic” alga is used in this paper for the taxa from classes Chlorophyceae and Tribophyceae. Cyanobacteria were classified into three orders according to their morphology: Chroococcales (single-celled organisms), Oscillatorialles (filamentous cyanobacteria without heterocytes and akinetes), and Nostocales (filamentous or colonial cyanobacteria with heterocytes and akinetes). In Oscillatoriales, the taxa were determined according to the width of the filaments, shape of the vegetative cells, and presence/absence of mucilaginous sheaths. In Nostocales, morphotypes were distinguished according to their life form (colonies or filaments) and the shape of the vegetative cells and heterocytes. For the order Chrooccocales, it was possible to recognize taxa according to the vegetative cells’ shape and dimension and division of cells. For the dimension of single morphotypes, see Table 1.

Cultivation, Isolation, and Sequencing of Strains

The soil subsamples were placed on plates with solid BBM medium (Bold-Basal/Bristol Medium, the basic medium for cultivation of terrestrial algae), prepared as described in Bischoff and Bold 1963 [25]. Cyanobacterial strains were picked up from colonies that grew on plates and transferred onto new plates in order to obtain unialgal colonies. Cultures were maintained in ambient light at 15 °C. Strains were morphologically characterized in high-resolution Olympus photomicroscope BX 60. The DNA of isolates was extracted using the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) following manufacturer’s protocols. The 16S rRNA gene and the internal transcribed spacer (ITS) region were amplified with primers 16S27F (5'- AGA GTT TGA TCC TGG CTC AG -3') and 23S30R 23S30R (5'- CTT CGC CTC TGT GTG CCT AGG T -3') [26].

Amplification was carried out as follows: one cycle of 5 min at 94 °C; 10 cycles of45 s at 94 °C, 45 s at 57 °C, and 2 min at 72 °C; 25 cycles of 45 s at 94 °C, 45 s at 54 °C, and 2 min at 72 °C; and a final elongation step of 7 min at 72 °C. PCR product was used as a template for sequencing (Applied Biosystems 3130x1 Genetic Analyzer) with primers 16S27F, 23S30R [26], primer CYA781F(a) (5' AAT GGG ATT AGA TAC CCC AGT AGT A - 3') [27], and the reverse complement of Primer 14(5'-TGT ACA CAC CGC CCG TC-30 [28]. The length of the sequences was 1000 bps.

Phylogenetic Analyses

Obtained sequences were aligned using MAFFT v. 7 [29] with default settings together with sequences of other 66 operational taxonomic units (OTUs) representing main groups of simple trichal cyanobacteria and with sequences of other 67 OTUs representing main groups of heterocystous cyanobacteria. The alignment lengths are 1385 bp. Phylogenetic calculations involved Bayesian inferences performed in MrBayes 3.2.2 [30], maximum likelihood (ML) analysis in PhyML 3.0 [31], and maximum parsimony analysis in Sea View 4.5.1 [32].). For Bayesian inference, two runs of eight Markov chains were executed for 1 million generations with default parameters, sampling every 100 generations (the final average standard deviation of split frequencies was lower than 0.01). The first 25 % of sampled trees were discarded as bum-in. The ML tree was constructed applying the GTR+I+ T model chosen according to Akaike information criterion provided by jModelTest 2 [33]. A total of 1000 bootstrap replicate searches were conducted to evaluate the relative support of branches. A maximum parsimony analysis involved 1000 replicate searches using the tree bisection-reconnection (TBR) branch swapping algorithm. Sequences obtained as part of this work were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession numbers LN849921-LN849937 and LN877213.

Statistical Analysis

Differences in cyanobacterial and algal composition between elevation sites, two mountain areas (East Karakoram versus Tibetan Plateau), and due to variation in soil chemical properties were analyzed by canonical redundancy analysis (RDA), which is a constrained ordination method, in the Canoco 5 [34]. RDA was used because environmental variables were in the form of both categorical (dummy variables) and continuous predictors. Standardization by species (dependent variables) was used because the data analyzed were of various types and units. The variance partitioning procedure was performed using several RDAs with explanatory variables and covariables to remove their effects and to obtain the net effect of an individual factor. Using this approach, we constructed tests analogous to the testing of particular terms in ANOVA models but for multivariate data; for details, see Lepš and Šmilauer [35]. Three sets of analyses were carried out: (1) elevation x mountain (categorical variable, coded as a set of indicator variables) x Soil chemistry as the explanatory variables— the analysis accounts for all, the main effect of mountain range, elevation, soil chemistry, and their interactions, (2) mountain range being an environmental variable, and elevation and soil chemistry a covariable and vice versa—this accounts just for additive (net) effect of each environmental variable, (3) separate analyses for each mountain range, repeating the variance partitioning procedure of tests 1 and 2 with elevation and soil chemical properties to account for their net effects. The significance of these relationships was tested using the Monte Carlo permutation test (999 permutations, see Lepš and Šmilauer, [35]). The results of multivariate analyses were visualized in the form of a biplot ordination diagram.

We further used a generalized linear model with altitude as the explanatory variable to test for changes (increase, decrease, hump shape, and valley shape) in algal and cyanobacterial

abundance. The significance of the linear model was tested first; if the form of linear dependence was not accepted, a second-order polynomial was fitted using the glm function in the R program [36]. To control for familywise error rate, the false discovery rate procedure was performed [37].

Results

Phylogenetic Diversity of Cyanobacterial Community

The molecular analyses showed that the phylogenetic diversity of cyanobacterial community from BSCs is highly similar between E Karakorum and Tibetan Plateau. Phylogenetic analysis of 16S rRNA gene revealed strains belonging to at least five genera. Besides clusters of common soil genera, e.g., *Microcoleus* or *Nodosilinea* two distinct clades of simple trichal taxa occurred (Fig. 1). Both represent probably new taxa and their life cycle, morphology, and ultrastructure need to be studied more in detail. First is marked as cluster A in Fig. 1. It is formed by unidentified members of family Leptolyngbyaceae.

The two original sequences are from strains isolated from two different localities, third sequence in this cluster is from microbial mat in calcareous river in Spain. The second cluster marked as B in Fig. 1 contains sequences of strains similar to genus *Geitierinema.* As notable from Fig. 1, these types belong to a different, yet undescribed genus, because sequences of type species of *Geitierinema, G. splendidum* are in very distant cluster with members of *Crinalium.*

The number of heterocytous types is lower than simple trichal types, but there occur two facts notable in Fig. 2: 1) several strains of soil *Nodularia* types cluster with planktic *Nodularia,(2*) *Calolhrix*-like strains isolated from Ladakh form a cluster outside other Rivulariaceae with p-distance 0.051 or higher, which means, that they represent a new genus. The phylogenetic analysis confirmed

the previous results about the cyanobacterial composition based only on morphological identification and considerably increased our knowledge about the cyanobacterial diversity of BSCs from W Himalayas.

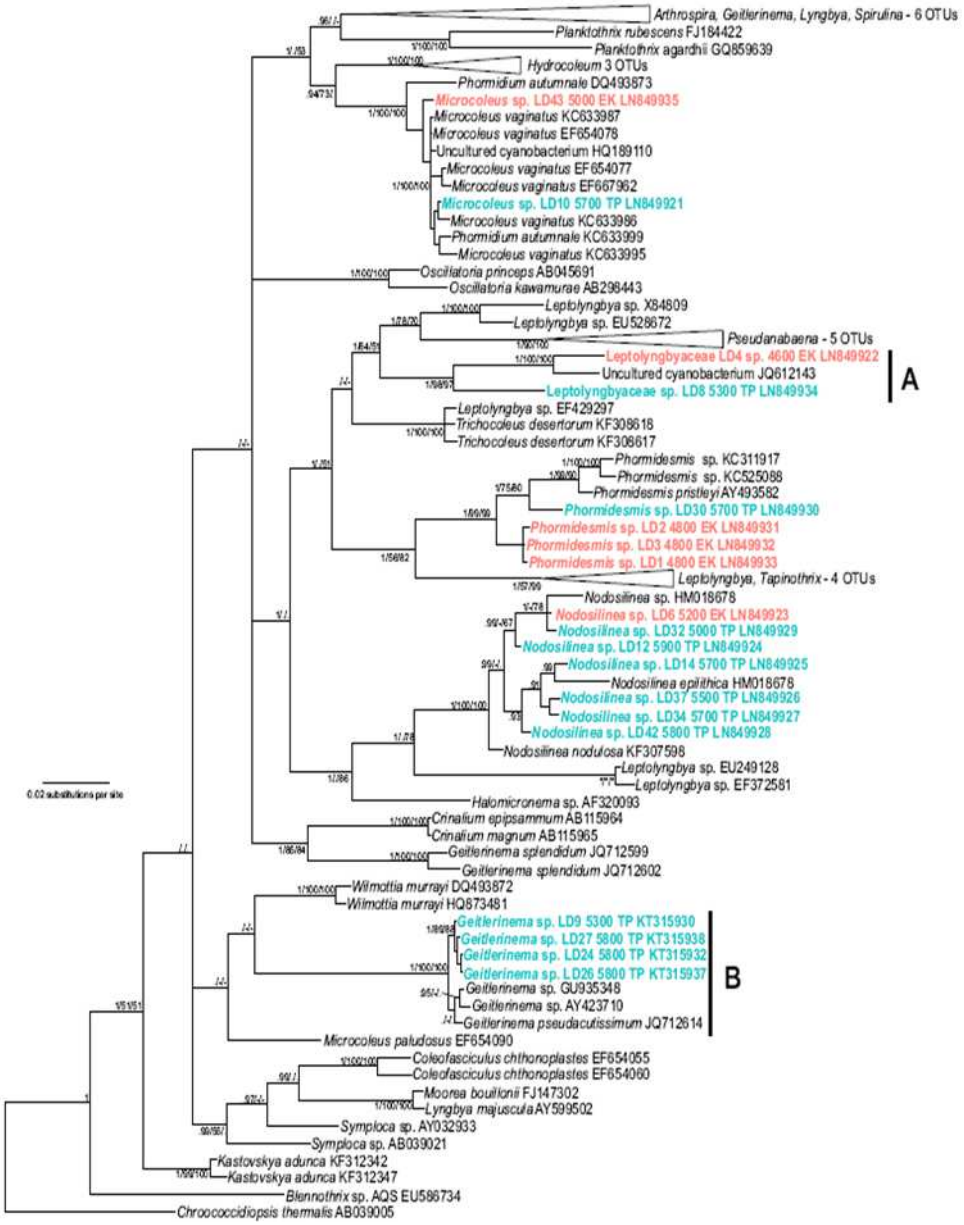

Fig. 1 Phylogenetic relationships of simple trichal cyanobacteria dominant in soil crusts in Ladakh using topology given by Bayesian analysis. OTUs typed in orange are from Eastern Karakoram, OTUs typed in blue are from Tibetan Plateau. Clusters A and B represent probably genera new to science. The given support values are given for Bayesian posterior probabilities >0.9 and for Maximum likelihood and Maximum parsimony ≥50 %

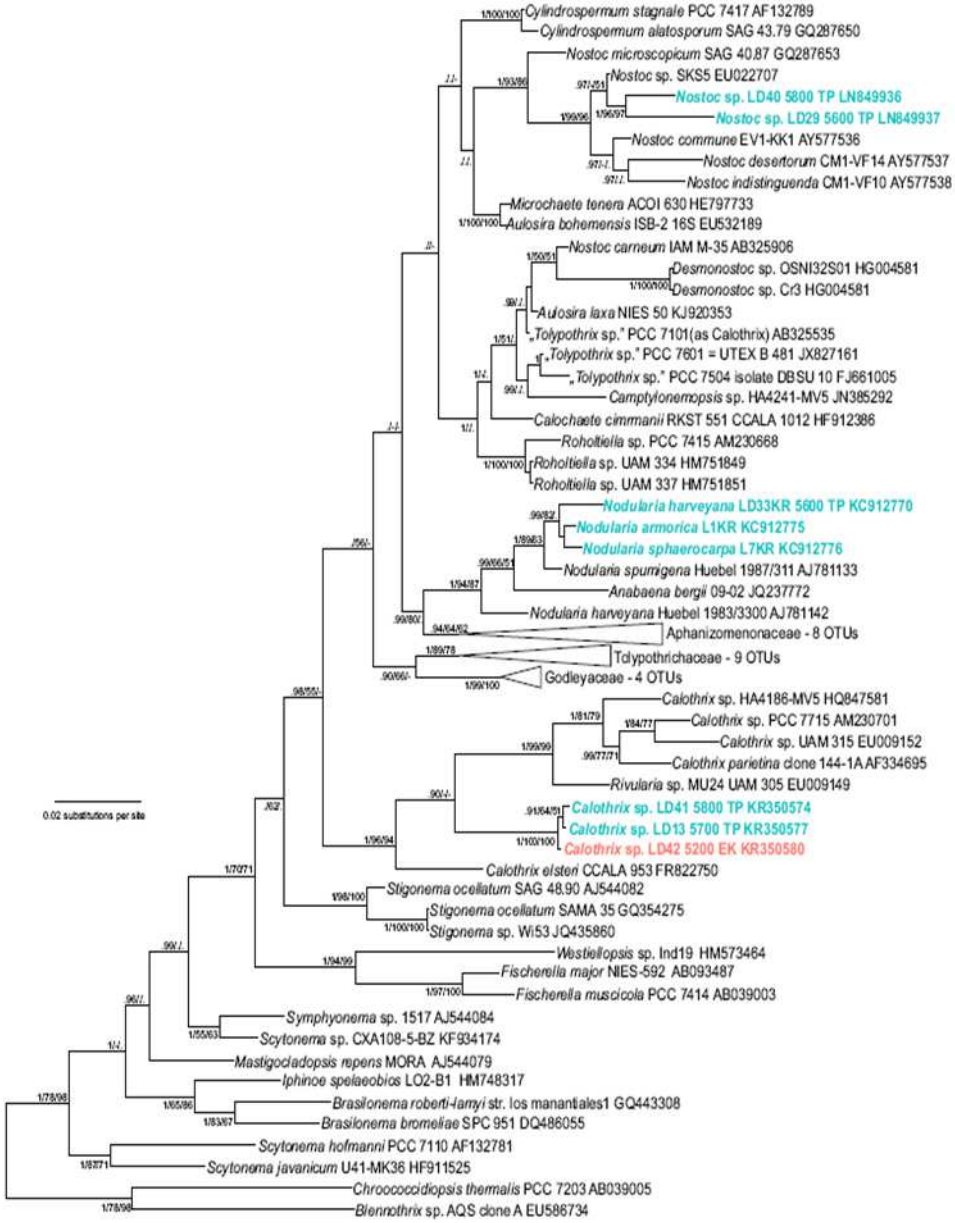

Fig. 2 Phylogenetic relationships of heterocytous cyanobacteria dominant in soil crusts in Ladakh using topology given by Bayesian analysis. OTUs typed in orange are from Eastern Karakoram, OTUs typed in blue are from Tibetan Plateau. The given support values are given for Bayesian posterior probabilities >0.9 and for Maximum likelihood and Maximum parsimony ≥50 %

Environmental Condition With Relationship to Phototrophic Diversity

Phototrophic microorganisms were found in all examined samples of BSCs, with cyanobacteria being the dominant component of the communities. Phototrophic communities contained 16 moiphotypes: 15 for cyanobacteria, one for green algae. The composition of morphotypes was identical for the E Karakoram and Tibetan Plateau transects (Table 1). The most abundant cyanobacterial order was Oscillatoriales, taxa *Microcoleus vaginatus* and *Phormidium* spp. accounted for most of the bio-mass. The total biovolume of phototrophs in BSCs at E Karakoram was lower than in crusts on Tibetan Plateau but increased significantly with elevation at both localities (Fig. 3). The biovolume of cyanobacterial order Oscillatoriales increased significantly with altitude at both mountain ranges, as well as the biovolume of *Nosloc* genera, while the biovolume of order Chroococcales and of the genera *Nodularia* decreased with elevation significantly (Fig. 3). This trend in the abundance of main cyanobacterial orders along altitudinal gradient was the same in E Karakoram as well as on Tibetan Plateau.

The combined effect of elevation, mountain range, and soil chemistry on the composition of soil phototrophic communities explained 52 % of the total data variation and was statistically significant (RDA: F= 2.6, P=0.002). Variance partitioning revealed that 8.4 % was explained solely by mountain range (F=3.6, F=0.004), 14.5 % by elevation (F=6.5, F=0.002), and 38.7 % by soil chemical properties (F=1.6, P=0.002), mostly by organic carbon (10.2 %), potassium (8.7 %), nitrate (8 %), magnesium (7 %), and phosphate (4.2 %). Forward selection of variables showed that phosphate and magnesium are the most important predictors of soil phototrophic communities in the East Karakoram transect (4600-5200 m, Fig. 4a). The soil phosphate concentration is higher at lower elevation (Fig. 5), and is associated with the main

Table 1 The biovolume of pholotrophs in pm^3 mg"DW(means) in soil crusts at fourelevation sites on the western slope of Saser Kangri, East Karakoram (4600, 4800, 5000, 5200 m) and between four elevation sites on the western slope of Chamser Kangri, Tibetan Plateau (5300, 5500, 5700,5900 m a s 1). An *upward* or *downward pointing arrow* indicates a positive or negative relationship between the dependent variable and altitude (| =increase in crust), and *ns* indicate that the relationship is not significant.The numbers in parenthesis are mean value of length and width of cyanobacterial strains in µm

	East Karakoram			Tibetan Plateau		
Morphotype	Mean	SDEV	Elevation	Mean	SDEV	Elevation
Nostoc sp. (5x5)	77.67	52.12	ns	137.23	89.23	↑ 0.000
Phormidium sp. (5x10)	261.69	162.58	↑ 0.046	297.89	193.05	↑ 0.006
Microcoleus vaginatus (5x10)	356.50	410.45	↑ 0.002	490.17	379.74	↑ 0.007
Microcoleus sp. (2,5x10)	3.09	8.35	ns	109.51	294.43	ns
Nodularia sp.(10x10)	30.09	31.01	↓ 0.007	40.42	56.64	↓ 0.000
Leptolyngbya sp.(2x10)	35.31	45.00	ns	28.57	18.93	ns
cf. *Calothrix* (5x10)	0.35	0.92	ns	0.84	3.24	ns
Cyanothece (5x10)	8.21	21.64	ns	5.95	9.81	ns
Cyanothece (10x15)	0.51	1.68	ns	1.35	4.08	↓ 0.047
Chroococcus 1 (2,5x5)	0.82	2.49	ns	1.47	3.73	ns
Chroococcus 2 (5x5)	6.58	12.54	↓ 0.038	0.04	0.09	ns
Chroococcus 3 (5x7,5)	0.18	0.79	ns	0.02	0.10	ns
Chroococcales (2,5x2,5) G1	0.20	0.45	↓ 0.048	0.59	0.63	ns
Chroococcales (5x5) G2	6.19	5.08	↓ 0.059	6.17	8.02	↓ 0.023
Chroococcales (10x10) G3	1.52	3.25	ns	0.77	2.17	ns
coccal microalgae (15x15) B1	0.56	1.86	↓ 0.069	2.92	13.04	ns
Chroccocales	24.21	26.76	↓ 0.002	16.36	13.28	↓ 0.021
Oscillatoriales	656.59	526.83	↑ 0.004	926.14	501.50	↑ 0.022
Nostocales	108.10	54.31	ns	178.49	79.28	ns
eukaryotic algae	0.56	1.86	↓ 0.049	2.92	13.04	ns
Total biovolume	789.46	540.86	↑ 0.013	1123.91	499.84	↑ 0.012

compositional changes along the first RDA ordination axis (Fig. 4a), clearly separating the soil cmst communities at lower elevation with higher biovolume of Chroococcales (Fig. 3), from those at higher elevation with low phosphate content and higher biovolume of Oscillatoriales, Nostocales, and in particular *Microcoleus vaginatus* and *Phormidium.* Higher elevation soils in the East Karakoram transect had more organic carbon, total nitrogen, and calcium, and higher pH compared to lower elevation sites (Fig. 5).

The same altitudinal pattem of compositional changes in soil phototrophic communities and soil chemistry as in the East Karakoram was found along the Tibetan Plateau transect (5300-5900 m, Fig. 4b). The combined effect of all environmental variables explained 29.2 % variation in the soil phototrophic composition (RDA: *F*— 1.7, P=0.006). Forward selection of variables showed that elevation and soil phosphate are the most important predictors of soil phototrophic communities in the Tibetan Plateau transect (5300-5900 m), accounting for 21.7 % variability. In the RDA ordination diagram (Fig. 4b), the main compositional changes along the first ordination axis are associated with elevation and soil phosphate content, clearly separating cyanobacteria from the orders Oscillatoriales and Nostocales predominating at the higher elevation sites (5700 and 5900 m), from Chroococcales that prevail at the lower elevation sites (5300 and 5500 m). Higher elevation sites had more soil organic matter due to higher soil moisture and lower phosphate and nitrate content and higher total phototrophic biovolume. The second RDA axis corresponds to the gradient of soil reaction and total nitrogen content, with higher biovolume of *Cyanothece* in soil with higher pH and lower soil Ca, Na, and total N content.

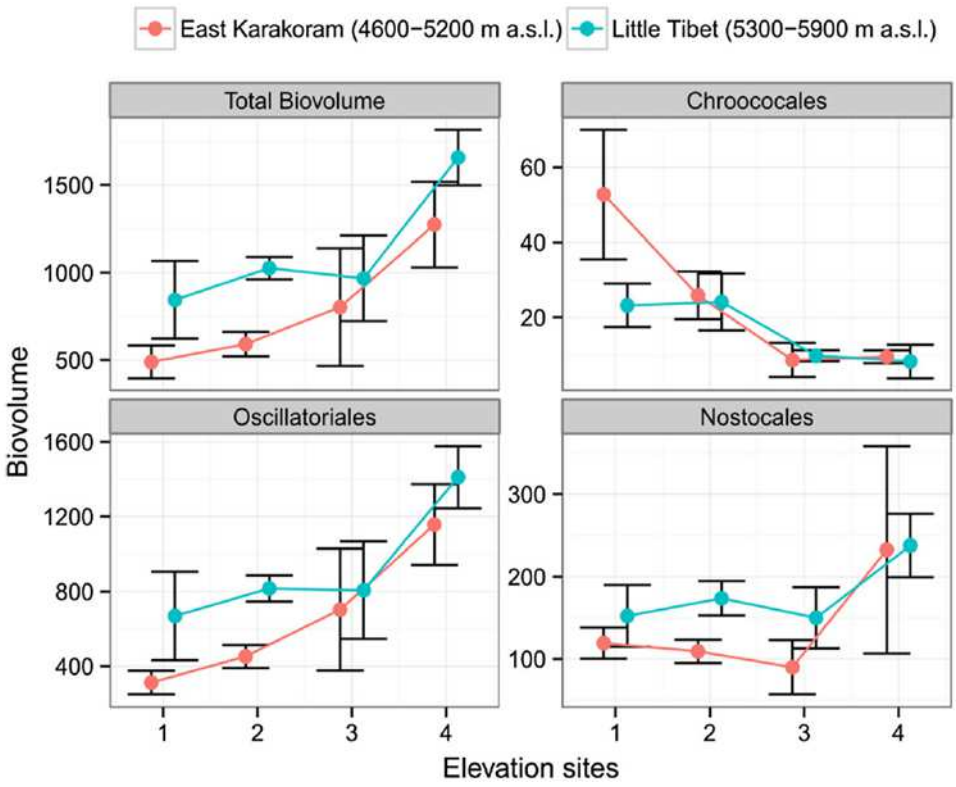

Fig. 3 Comparison of soil phototrophic biovolume (pm3 mg'1 dry soil) between four elevation sites at E Karakoram (reddots, 4600,4800, 5000, 5200 m) and between four elevation sites at Tibetan Plateau {green dots, 5300, 5500, 5700, 5900 m a.s.l.), Ladakh, NW India

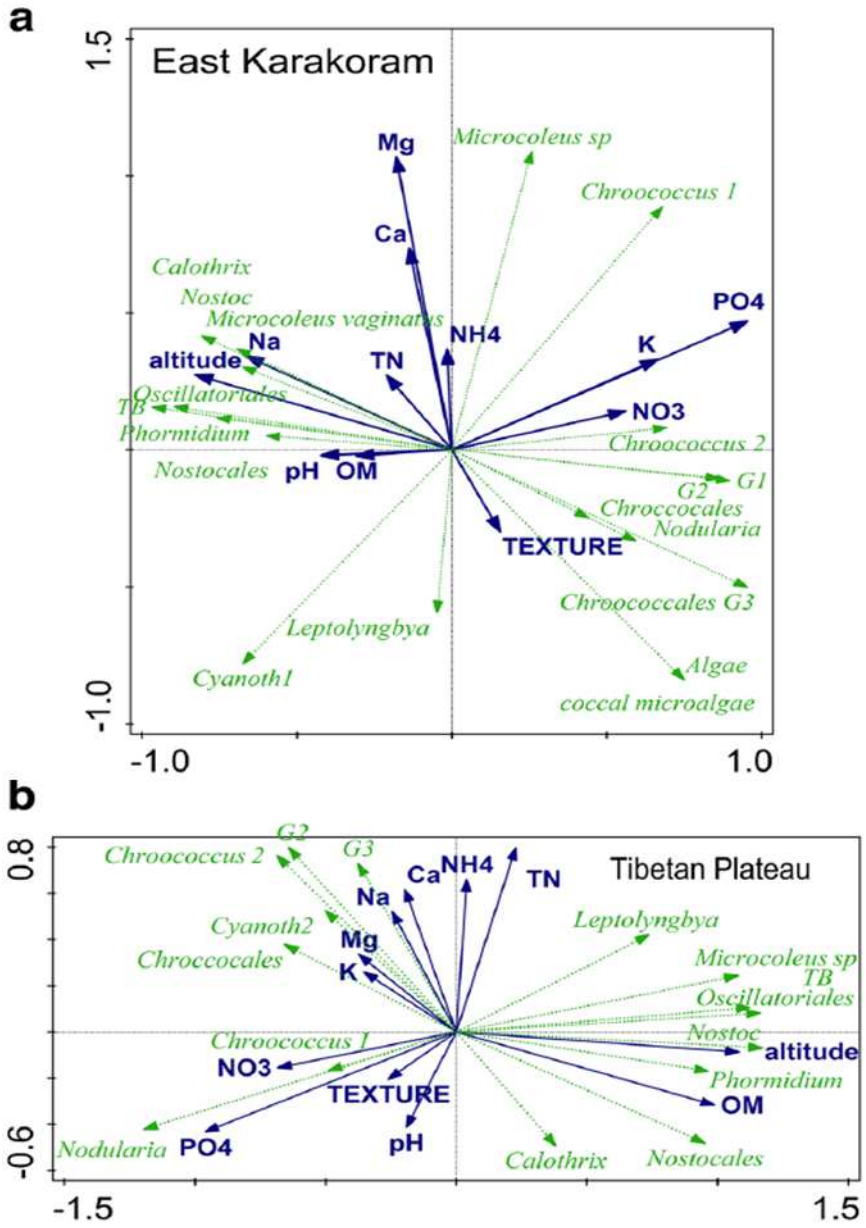

Fig. 4 Two redundancy analysis biplots (RDA) of soil phototrophs (response variables) in relation to elevation and soil nutrients at E Karakoram (4a) and at Tibetan Plateau (4b). Response variables are represented by *vectors {green arrows)* and are related to elevation physico-chemical characteristics of soil *{blue arrows).* The *angles between arrows* indicate correlations between variables. Legend: "OM"—organic matter, "TB" total biovolume.

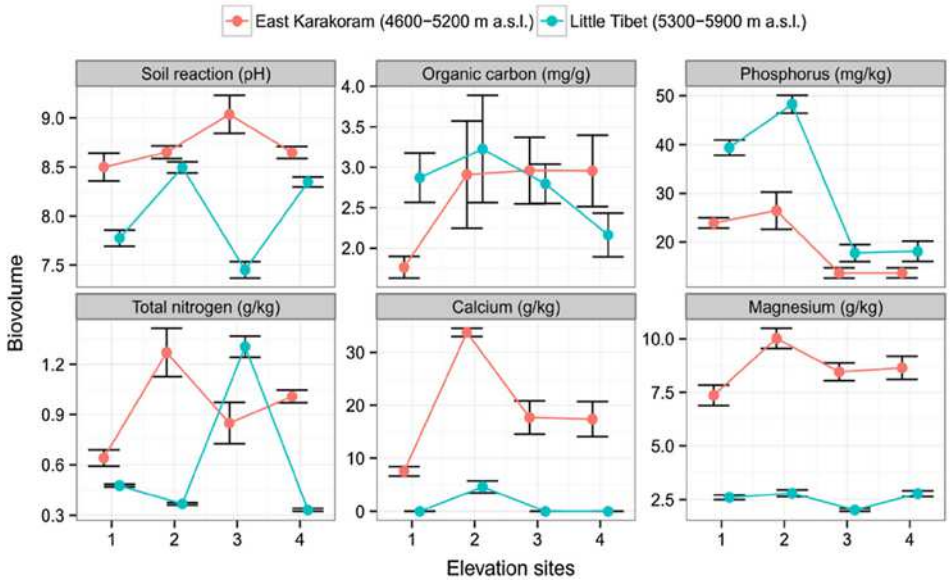

Fig. 5 Comparison of concentration of selected nutrients between four elevation sites on the western slope of Saser Kangri, East Karakoram (red dots, 4600, 4800,5000,5200 m) and between four elevation sites on the western slope of Chamser Kangri, Tibetan Plateau (green dots, 5300,5500, 5700, 5900 m a.s.l.)

Discussion

For the first time, the soil cyanobacterial diversity from elevations 5000-6000 m was investigated using microscopic and molecular approaches together. The harsh environment of this arid area is characterized by extensive development of BSCs, which form the crucial component of studied habitats. BSCs are dominated by cyanobacterial communities making them one of the most important ecosystem engineers here. Our previous studies reported the presence of phototrophs in subnival zones of Western Himalaya, but the species composition was investigated by traditional light microscopy only to cover generic identity of dominant types [11, 38]. In the present study, our research of

cyanobacterial diversity was improved by the 16S rRNA gene sequencing of isolated uni-algal strains to precise their determination. It allows us to see cyanobacterial diversity in broader ecosystem perspective.

We used the phylogenetic analyses of individual-cultivated strains because the proper placement of cyanobacteria into a taxonomic construct requires combination of morphology and sequence information. This is needed especially with respect to the aspects of ecosystem function, as the value of sequence data lacking morphological and ecological background is strongly limited.

Despite recent increase of interest for soil environments, the link between local diversity of microorganisms and environmental factors is still not well understood. It is supposed that microbial biogeographical patterns are shaped by environmental factors [39,40]. Our studied mountains ranges differ in geological origin and bedrock, as well as their local environmental conditions, such as temperature and humidity [41]. However, we found out that the overall diversity of cyanobacterial communities is highly similar at the Eastern Karakoram and Tibetan Plateau transects. The molecular analyses showed that also the phylogenic diversity of cyanobacteria from BSCs is highly similar between both localities.

Phototrophic communities are composed mainly by orders Oscillatoriales, Nostocales, and Chroococales, represented by common soil genera, e.g., Microcoleus, Nodosilinea, or Nosloc. These genera have extremely broad ecological valence, they occur in a wide range of habitats all over the world including extreme ecosystems. Large filamentous cyanobacteria, such as Microcoleus spp., Nodosilinea spp., or Nodularia spp. are responsible for an initial process in the origin of BSC [6]. After the filaments bound the soil particles together, the smaller taxa such as Nostoc spp. or Calothrix spp. followed them. These genera are crucial for origin of BSCs, so they are mentioned as a part of BSCs from all over the world [6] [20] [42]. The high similarity of

cyanobacterial community of BSCs of E Karakoram and Tibetan Plateau is principally given by the inherent process of BSC formation, which is species-dependent rather than environmental condi- tions-dependent.

Our study confirmed the previous results concerning the cyanobacterial community structure with molecular data and increased our knowledge about cyanobacterial types present in BSCs. Phylogenetic analysis of 16S rRNA gene revealed strains belonging to at least five genera. Besides clusters of common soil genera, e.g., Microcoleus, Nodosilinea, or Nosloc, two distinct clades of simple trichal taxa occurred. Even though the cold deserts of W Himalayas are extremely arid areas, several close relatives have been found from aquatic habitats. One of them is the clade formed by unidentified members of family Leptolyngbyaceae (Fig. 1, clade A). The two original sequences are strains isolated from two different localities, third sequence in this cluster comes from microbial mat of calcareous river in Spain. The Leptolyngbyaceae has been found to be a very genetically diverse group [3]. Our clade represents probably a new taxon. Their life cycle, morphology, and ultrastructure will be studied in more detail in near future. The second cluster with aquatic relatives is formed by strains of soil *Nodularia* types which match together with planktonic types. Furthermore, according to Komárek [43] no *Nodularia* from arid soils has been recorded by now. The phylogenetic analysis revealed also non-relatedness of *Geitlerinema* types. As noticeable in Fig. 1, the type of the genus, i.e., *Geitlerinema splendidum* is in a distant cluster to *G. pseudacutissimum* and other sequences including our original ones (Fig. 1, cluster B), which probably means, that these types represent a new genus. However, this must be proven by further analyses.

The number of heterocytous types is lower than simple trichal types mentioned above. However, there are more important facts regarding heterocytous types. The *Calothrix-Uke* strains

isolated from Ladakh form a separate cluster outside other Rivulariaceae with p-distance 0.051 or higher, which means, that they represent a new genus according to limits published by Stackebrand & Goebel [44]. Recently, the taxo- nomical revision of this group was finished by Berrenedro et al. 2015 [45]. Although members of the genus *Nostoc* are present in every desert soil all over the world [3, 46], and the abundance of *Nostoc* in our samples is relatively high, we managed to obtain only few *Nostoc* strains sequences. This is caused by problematic DNA extraction due to thick mucilage and relatively problematic strains cultivation.

Detailed view on the cyanobacterial diversity distribution among elevational gradient shows very similar trends at both studied localities. The total biovolume of phototrophs in BSCs at E Karakoram were lower than in cmsts on Tibetan plateau but increased significantly with elevation at both localities. This increase of cyanobacterial biomass within soil crust with increasing elevation is in sharp contrast to the commonly observed trend of a decreasing biomass of organisms with more severe environmental conditions [47, 48]. The reason of different abundance of cyanobacterial biomass between E Karakoram and Tibetian Plateau can be caused by various covers of vascular plant vegetation. The vegetation cover along the lower altitudinal gradient at E Karakorum (4600-5200 m) varied between 10-40 %, while the plant cover along the transect on Tibetan Plateau in higher altitudes (5300-5900) was considerably lower (5-15 %) [17, 18]. Less competition with vascular plants for resources such as nutrients, water, and light open the gap for extensive development of phototrophic communities in the BSCs. The temperature profile during the vegetation season provides better growing conditions for microorganisms than for higher plants. Short growing season and water shortage are conditions, which microorganisms are able to resist [49]. Phototrophic microorganisms have a much faster meta- bolic rate and a shorter generation time than vascular plants have. Furthermore, discovered genera of cyanobacteria are highly

adapted to extreme conditions. They possess thick mucilaginous sheets with pigments, which are resistant to water shortage, high UV radiation, and to the high temperature oscillations. All these circumstances allow occurrence of abundant phototrophic microbial biomass in areas with sparse vegetation cover.

The same explanation holds for the trend of increasing biomass with elevation recorded at both localities. The higher elevations at both sites have lower vegetation cover than lower elevations. The highest elevation of Tibetan Plateau area has negligible plant cover of less than 10 % [17] in contrast to BSCs, which cover more than 40 % of ground.

Comparison of the overall taxonomic diversity of phototrophs between two mountain ranges showed that on the both altitudinal transects Oscillatoriales and Nostocales prevailed in subnivalsoils, while Chroococcales was dominant in the soil crusts of alpine steppes and screes at lower elevations. By testing the effect of soil physicochemical parameters together with altitude on the composition and biovolume of cyanobacterial communities, we found out that despite of different bedrocks and origin of studied mountain ranges, the same altitudinal pattem of compositional changes in soil phototrophic communities and soil chemistry was found along the Tibetan Plateau transect as well as along the East Karakoram transect Results showed that altitude and soil phosphate concentration are the most important predictors of soil phototrophic communities at both sites. Both factors are very likely connected together. Phosphate concentration decreases with increasing altitude as a soil temperature and moisture regime change. The main source of phosphate for soil comes from bed-rock weathering. Weathering would be slower at high cold altitudes and faster at lower altitudes, where can be furthermore accelerated by organic acids production by plants or soil microbes or coming from OM decomposition. Natural phosphate gradient along altitudinal transect brought the main constrain especially for phototrophic microorganisms in BSCs. Phototrophic organisms

gain carbon and nitrogen from atmosphere, where both elements are abundant. However, their gain via photosynthesis and N-fixation is energetically demanding and manufacture demanding. In microbial cells, two most P- rich compounds are ATP and ribosomal RNA. First, one provides energy, whereas, second one provides machinery for protein synthesis. Both compounds make most of the P of whole microbial cell. Therefore, phosphate availability in soil is crucial for cyanobacterial C and N uptake and thus growth. In our study, we found increasing dominance of filamentous cyanobacteria within BSCs with decreasing phosphate concentration. Filamentous microorganisms are known to have greater affinity to nutrients. They are able to take up nutrients that are at very low concentrations [6]. This ability gave filamentous organisms competitive benefits above the others and thus they dominate in higher altitudes, where phosphate concentration is low. Even though, Ca and Mg concentrations differ largely among localities, they were not shown to be a significant predictor of cyanobacterial diversity. The reason for that is that both elements are considered to be micronutrients. That means, their demand by cyanobacteria is small and hence their unlimited amount is reached at all localities.

The investigation of cyanobacteria in BSCs around the world revealed relatively small and similar taxonomical diversity of phototrophs in the studied BSCs. It is very likely that this fact is connected to unique and extreme environmental conditions, where BSCs usually occur. These conditions are inherently highly selective for the best-adapted species as the conditions of the high-mountain mineral soils are supposed to be more limiting to life than other conditions on the surface of the Earth [50]. Obviously, there are only few species able to survive high-mountain conditions and in opposite there are much more species unable to survive them. Therefore, open and almost competition-free niche for few well-adapted species is available in high mountains. Cyanobacteria could prosper here in unexpected extent.

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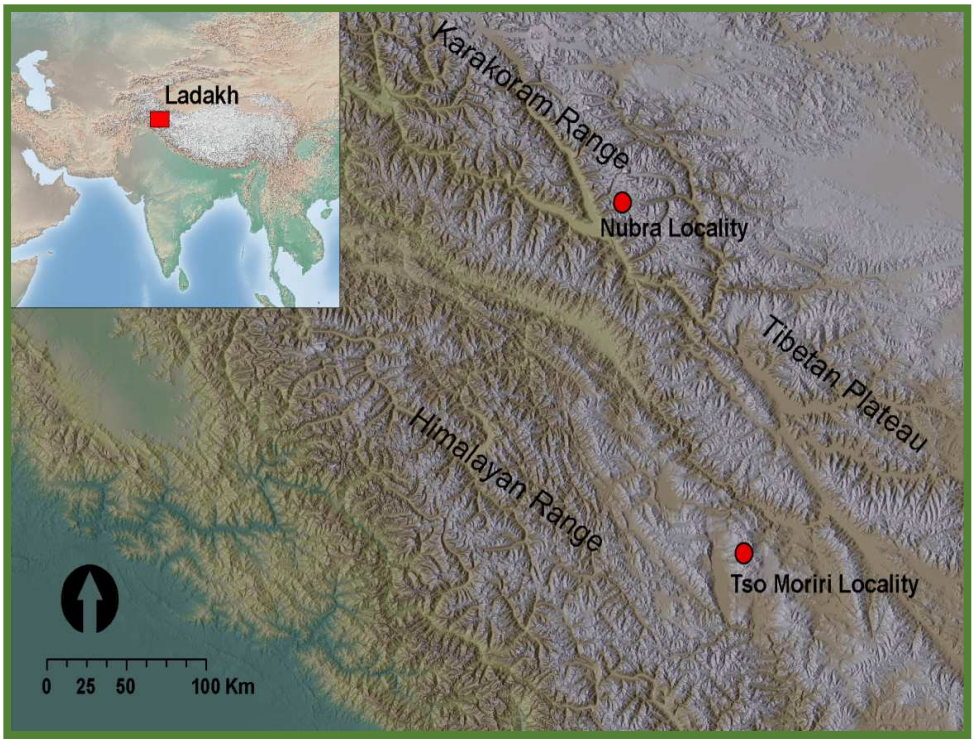
APPENDIX



Study localities. Nubra is situated within E Karakoram Range, Tso Moriri belongs to Tibetan plateau.

The area of cold deserts of Ladakh is in rain shadow – monsoons are stopped by the Great Himalaya range . (Wiev from the flight from Delhi to Leh).

Biological Soil Crust from subnival zones of Ladakh.

The uppermost elevation of the Nubra Valley locality, E Karakorum Range (5200m a.s.l.) (photo J. Doležal)

The Chamser Kangri site in the southwestern extension of the Tibetan Plateau above Tso Moriri Lake. Studied sites at 5600 m a.s.l. ↑ and on the plateau (5700-6000m a. s.l.). ↓

Well developed biological soil crusts at the plateau above the Tso Moriri lake

„Soil crust gardens“

Saussurea gnaphalodes, Stellaria decumbens, Saxifraga nanella, typical species of high altitudes growing in soil crust. Tibetan Plateau, Tso Moriri. (photo M. Dvorský)

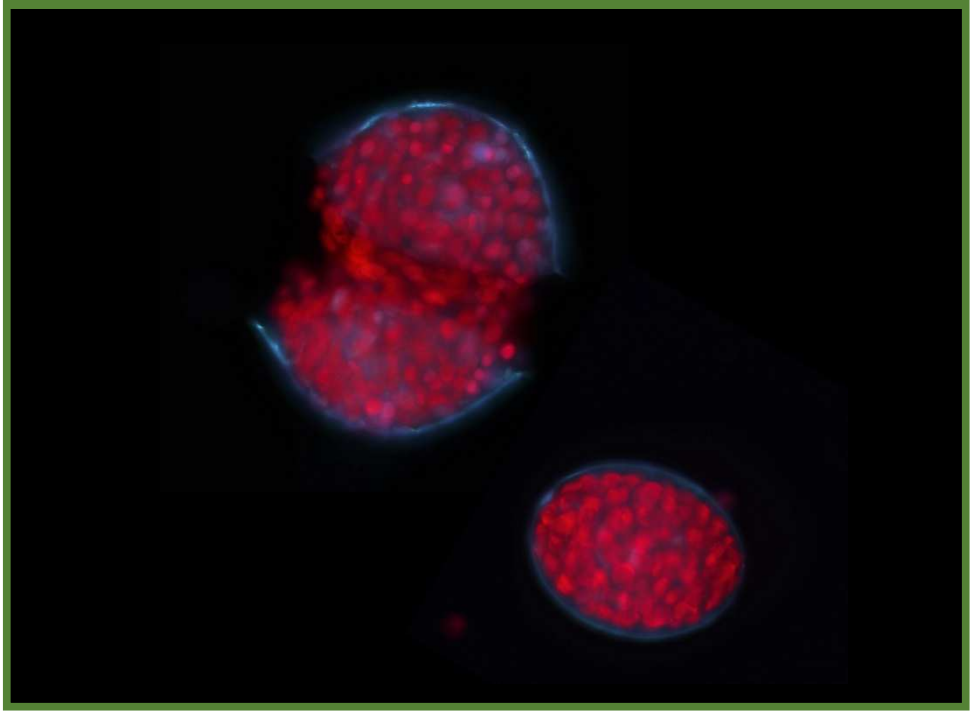
Thylacospermum caespitosum – dominant plant in subnival and nival zones: from the Tibetian plateau growing in the patch of BSCs.

Sampling of the BSCs samples and drying them in the field.

Field measuring of the metabolic activity of BSCs in dark/light conditions using the EGM4 Gas Analyser (Tibetian plateau, 5900m a.s.l.).

↑ *A strain of Calothrix-like heterpolar filamentous cyanobacterium isolated from BSC from Tibetan plateau which belongs to a new genus under description (Berrendero et al. submitted)*

↓ *The colony of Nostoc sp. in the epifluorescence microscope.*



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