# Response to referee #1 and referee #2 and corrected manuscript with marked-up changes

Dear referees,

We want to thank both of you for the detailed reviews, which helped to improve the manuscript a lot. We tried to include all you suggestions in the final manuscript, and answered your questions in the point-by-point response.

# Point-by-point response to referee #1

**Referee comment:**

general comments: This is an interesting and overall well written manuscript describing the physical and community characteristics of cryoconite holes. The manuscript further attempts to determine how both the physical features of the environment and tropic level interactions may affect the biology of the system. Few previous studies have treated cryoconite holes in this manner and this manuscript compliments these earlier works well.

**Author's response:**

Dear Referee #1, we want to thank you for the detailed feedback and comments, which helped to improve the manuscript a lot. We considered the comments and changed the new version of the manuscript accordingly. Please, find our specific responses below.

**specific comments**

Page 11752

Line 6

**Referee comment:**

Suggest at examples of the "grazers"

**Author's response:**

We agree, that these details are helpful here. We changed the sentence in the following way:

… relations to their potential grazers, such as tardigrades and rotifers …

Line 11

**Referee comment:**

Add comment mentioned in the conclusions that the positive relationship could be caused by similar environmental requirements of grazers and microalgae

**Author's response:**

We agree that more details are helpful here. A similar environmental requirement is one possibility, but a positive control via nutrient recycling is another one, mentioned in the discussion. We changed the sentence in the following way:

…not show any significant negative correlation with microalgal abundances, but a positive correlation with eukaryotic microalgae. Shared environmental preferences and a positive effect of grazing are the proposed mechanisms to explain these correlations.

Line 18

**Referee comment:**

Bird guano is a nutrient input not just a proxy.

**Author's response:**

We agree and changed the sentence in the following way:

… and a high impact of nutrient input by bird guano. ~~, as a proxy for nutrients.~~

Page 11753

**Referee comment:**

Suggest a comment on the life span of a cryoconite hole, i.e. do they form in the same location each year forming around the dark cryoconite on the glacier surface? Can they be considered a "semipermanent" habitat?

**Author's response:**

Thanks for the comment, we can add this information in the following way:.

Cryoconite holes are usually open and photosynthetically active for a few months in summer. During this time they are highly dynamic systems with occasional stripping events during which they can be cleared and the newly distributed sediment starts forming new cryoconite holes nearby (personal observations; MacDonell and Fitzsimons, 2008). During this time several cryoconite holes are connected hydrologically. Most of the year, they are sealed with an ice lid and covered by snow, which protects them from stripping events, but which also inhibits the photosynthetic activity (Jesamine Bartlett, personal communication).

However, the cited study relates to cryoconite holes in Antarctica, which are quite different from the cryoconites in our study. We couldn't find a specific study for the Arctic, but during our observations in the current study, we observed a rapid exchange of meltwater in the cryoconite holes and a few stripping events. Some of the data are given as the changing dimensions (depth,

diameter) of the cryoconite holes on Hørbyebreen and Nordenskiöldbreen in the supplement. We could also discuss these data in this manuscript, but we don't think that it adds much relevant information to the topic of this study and rather added it to the supplement.

Lines 16-18.

**Referee comment:**

Delete the truism that only organisms adapted to the cryoconite holes can survive there.

**Author's response:**

We agree that this statement is too generalized and not all organisms, living in cryoconite holes are specifically adapted to this habitat. We changed the sentence in the following way:

Cryoconite holes represent ultraoligotrophic environments (Hodson et al., 2008) inhabited ~~only~~ by microorganisms, which are able to cope with many environmental challenges associated with a life on the surface of glaciers.

Page 11754

Line 12

**Referee comment:**

Give some idea of sizes. Small is a relative term.

**Author's response:**

We agree that is information is important. We added the typically observed maximum size of the grazers in our study. Only very few tardigrades reached larger sizes. We changed the sentence in the following way:

… to consist of much smaller grazers, usually shorter than 200 µm (personal observations).

Line 18

**Referee comment:**

Expand on the "adaptation". In what way?

**Author's response:**

Thanks for the comment. We clarified the "adaptation" in the following way:

… enlarged colonies of a *Coenobium* species as possible adaptation to grazing. Larger colonies are proposed to outgrow the maximum food size of filtration feeders.

Page 11755

Line 3.

**Referee comment:**

Suggest beginning each "group" with a Roman numeral, i). . ..

**Author's response:**

We agree, that this helps to clarify the structure of this section.

microalgae can be classified into four dominant groups … . i) Filamentous cyanobacteria… . ii) Nostocales, … . iii) Chlorophyceae, … . iv) Zygnematophycea … .

Page 11756

**Referee comment:**

Include in the Site description something on the life span of the cryoconite holes. Are they formed new each year or does the cryoconite ensure they form in the same location each year? How many months of the year are they present? When does the surface snow clear from these glaciers?

**Author's response:**

Please, see also the comment about page 11753 in addition to the following comment.

Thanks for this comment. Unfortunately, it is hard to generalize the life span and the length of the summer season. To our knowledge, no study covered this life time dynamics for Arctic cryoconites. Hence, the following section is mainly based on personal observations during the current study. A series of pictures was taken during the present study and could demonstrate the explained dynamics. However, we do not think that this information would add much crucial information to the focus of this manuscript. Thus, we add one figure here in the method section, but won't focus on it in the results and discussion section. Most cryoconite holes form a dynamic system with hydrologically interconnected cryoconites holes. The dimensions are frequently changing and some cryoconite holes may experience stripping events, whereby the sediment content is transported downstream and builds a new cryoconite hole nearby. The time, when the surface snow clears from the glacier is highly depended on the altitude, thus we can only give the usual start of the snowmelt. Close to the equilibrium line altitude, the time of snow-free days can be as short as a few days. We tried to add the required information in the following paragraph:

The cryoconite holes are rather unstable habitats with a life span often shorter than one summer season. The closer the cryoconite hole to the glacier margin, the shorter the life span (personal observations). Hence, the cryoconite holes on the Plateau on Nordenskiöldbreen have the longest life span and the cryoconite holes near Retrettøya the shortest one. During the current study twenty cryoconite holes were monitored continuously with depth measurements and photography. We could show that three cryoconite holes experienced a complete stripping event and that nine of them drained, but regrew at the same place (Figure S3). Cryoconite holes on the present glaciers are only open for one to three months in summer, depending on their altitude. They remain rather stable after an ice lid gets formed in autumn until the snow starts melting in late June and the first parts of the glacier clear from the snow in July (personal observations). The current study focusses on the summer months, because only during the summer season, a significant photoautotrophic activity is expected.

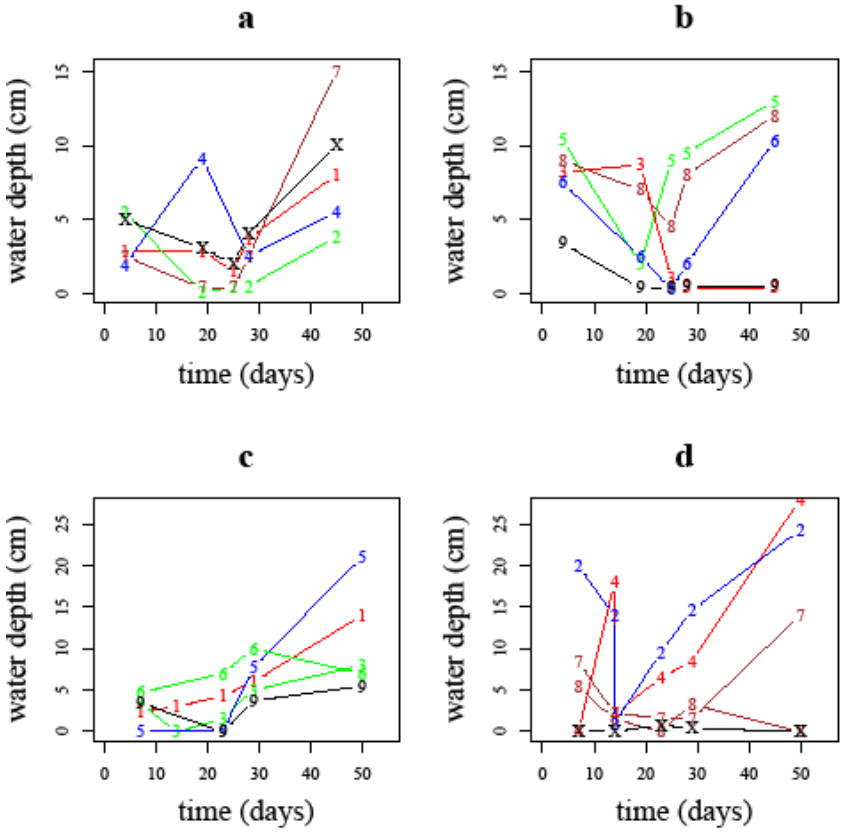


Figure S3. Water depth in cryoconite holes on Hørbyebreen (a,c) and on Nordenskiöldbreen (c,d). The numbers represent the ID of the continuously samples cryoconite holes. X refers to the ID number 10. A and c refer to the cryoconite holes that were sampled continuously throughout the season and b and d are the control cryoconite holes, which were only sampled in the beginning and in the end of the study.

We could add this figure in the supplement to show the life times of some of the studied cryoconite holes. However, it is not too closely related to the story of the current paper. Thus it appears in the supplement, rather than in the manuscript.

Line 10.

**Referee comment:**

Not very clear how many samples taken on the Ebbabreen.

**Author's response:**

We tried to clarify the total number of samples, taken on Ebbabreen in the following way:

On Ebbabreen, in total 6 samples were collected every 25–100 m in height.

Page 11757

Section 2.2.

**Referee comment:**

State where the lab work was undertaken. At field camp or were the samples returned to the mainland?

**Author's response:**

We added this information in the following sentence:

All density estimations were done in the field station in Petuniabukta. The species determinations were done on fixed cryoconite samples (4% Formaldehyde) back in the lab in the Czech Republic.

Line 5.

**Referee comment:**

State that there were no organisms in the supernatant. Was this examined?

**Author's response:**

We screened the supernatant in some of the samples to exclude the possibility of abundant planktonic grazers. We included this information in the following way:

The supernatant was screened randomly for planktonic individuals, but no grazers have been found.

**Referee comment:**

Please state what keys were used for the identifications. How were these ids performed? Where is the identified material deposited?

**Author's response:**

The missing information is added in the following section:

The rotifers have been identified, using the monograph of Donner (1965). Tardigrades were identified, using the key to world tardigrade by Ramazotti and Maucci (1983) and by comparisons with other original papers (Dastych, 1988; Miller et al., 2005). The identified material is deposited in the Biology Centre AS CR, Institute of Soil Biology in Ceske Budejovice in the Czech Republic.

Dastych, H.: The Tardigrada of Poland. Monografie Fauny Polski 17. Donner, J., 1965. Ordnung Bdelloidea (Rotatoria). Akademie-Verlag, Berlin, 1988.

Ramazzotti, G., and Maucci, W. 1983. II Philum Tardigrada (III. edizione riveduta e aggiornata). Memorie dell'Istituto italiano di idrobiologia, 41, 1-1016.

Section 2.3

Line 18.

**Referee comment:**

How was "wet supernatant" judged? Small differences in water content will have large differences

on the determined densities.

**Author's response:**

The wet sediment is defined as the sediment that settled after more than 30 minutes. The supernatant was removed completely with a syringe, and only the water saturated sediment was used for microalgae density estimations. The water content of this saturated wet sediment was measured later and the wet weight per area was calculated as the total weight of the wet sediment, which was collected in a defined area. We added this information in the following way:

After settling of the sediment for at least 30 minutes the supernatant was removed with a sysinge and kept for further dilutions. Due to the current of meltwater through cryoconite holes, the sediment is already well selected towards high sedimentation rates and the supernatant appeared clear and no remaining paricles have been observed. The remaining water saturated wet sediment was used for estimations of the microalgae densities and the water content. For the counting, 0.25 g of wet …

Line 19.

**Referee comment:**

Diluted with "meltwater"? Where did this originate? From collected ice?

**Author's response:**

The meltwater is the supernatant from the same cryoconite. See comment for 11757 Line 18.

Page 11758

Lines 1-4.

**Referee comment:**

Some references are required to support these divisions of filtering classes. Especially as these become a major point in the ms later.

**Author's response:**

The divisions of filtering classes are mainly based on measurements of the feeding apparatuses of the rotifers in our own samples.

The tardigrades (e.g. dominant genus *Hypsibius*) and bdelloid rotifers (e.g. dominant genus *Macrotrachella*) that were found in the cryoconite holes could both be herbivorous (Guil and Sanchez-Moreno 2013). They differ mainly in their feeding strategy. Tardigrades forage for single particles and ingest them completely or suck them out (Kinchin 1994). Rotifers are usually vortex feeders creating currents in the surrounding water by trochus cilia and are able to filter unicellular algae as well as small particles (Devetter 2009). Thus, we propose that single cellular microalgae are most favorable as food source.

However, due to the small size of the cyanobacteria, we introduced 25 µm filaments as the maximum food size that is ingestible for filtering rotifers. This value is mainly based on measurements of the feeding apparatuses in our own samples. 25 µm was also the most common size of unicellular green algae. For the rotifer *Brachionus pricatilis* Hino and Hirano (1980) found also a linear relationship between the maximum ingestible particle size and the body length. For 200 µm long specimens, which is on the upper end of the rotifers found in the current study, they found a maximum ingestible particle size of about 21 µm.

Thus, we propose 25 µm as an important threshold of filamentous microalgae. In our study, this applies mainly to cyanobacteria (e.g. Oscillatoriales). Additionally, we introduced the division of small (≤10 µm) and large (>10 µm) cells, in order to see whether differences in the size of single cells play an additional role (e.g. green algae). For non-filamentous colonies we propose a colony size of 10 cells to exceed the filtration size of the present rotifers.

Guil, N. & Sanchez-Moreno, S. (2013). Fine–scale patterns in micrometazoans: tardigrade diversity, community composition and trophic dynamics in leaf litter. Syst. Biodivers. 11, 181–193.

Hino, A., & Hirano, R. (1980). Relationship between body size of the rotifer Brachionus plicatilis and the maximum size of particles ingested. Bull. Jpn. Soc. Sci. Fish, 46(10), 1217-1222.

Kinchin I. M. 1994. The Biology of Tardigrades. Portland Press Ltd.

Line 6.

**Referee comment:**

Reference required for photosynthetic activity occurring only in the first few µm of the sediment.

**Author's response:**

For sediments oxygen profiles, measured with microsensors support this statements. For cryoconites one study by Telling et al. (2011) can support the idea. We added the following information.

General oxygen profiles in sediments, obtained with microsensors showed photosynthetic activities at sediment depths only below 0.5-1mm (E.g. Revsbech et al., 1986). For cryoconite sediments a study by Telling et al. (2011) showed that only in sediment layers <3 mm a net autotrophic system is maintained. …

Revsbech, N. P., Madsen, B., & Jørgensen, B. B. (1986). Oxygen production and consumption in sediments determined at high spatial resolution by computer simulation of oxygen microelectrode data. Limnol. Oceanogr, 31(2), 293-304.

Line 15.

**Referee comment:**

Has the work in 2012 been published? If not, some details on the sequencing of the 16S rRNA required.

**Author's response:**

No, it has not been published. We added the following method section for the sequencing details:

2.4 16S rRNA gene sequencing and sequence analysis

The highly variable V3/V4 region of the 16S rRNA gene was amplified with the bacterial primers S-D-Bact-0341-b-S-17 forward and S-D-Bact-0785-a-A-21 reverse, with overhang Illumina adaptors attached to the primer sequences, creating a single amplicon of about 460 bp (Klindworth et al., 2013). The reaction was carried out in 50 µl volumes, containing 0.3 mg ml-1 Bovine Serum Albumin, 250 mM dNTPs, 0.5 mM of each primer, 0.02 µl Phusion High-Fidelity DNA Polymerase (Finnzymes OY, Espoo, Finland) and 5x Phusion HF Buffer, containing 1.5mM MgCl2. The following PCR conditions were used: initial denaturation at 95°C for 5 min., followed by 25 cycles consisting of denaturation (95°C for 40 s=, annealing (55°C for 1 min.) and extension (72°C for 1 min.) and a final extension step at 72°C for seven minutes. The amplified DNA was sequenced using the Illumina MiSeq platform at Liverpool Centre for Genomics Research and generated 2 x 300 bp overlapping pairs-end reads.

The 16S sequences were further processed, using the mothur (v. 1.35) pipeline (Schloss et al., 200). Chimeric sequences were identified and removed using UCHIME (Edgar et al., 2011). Reads were clustered into operational taxonomical units (OTUs), based on at least 97% sequence similarity, and assigned taxonomically against the SILVA database (Quast et al., 2013).

The sequences are stored at NCBI and available under the accession number PRJNA296475.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. and Knight, R.: UCHIME improves sensitivity and speed of chimera detection, Bioinformatics, 27(16), 2194–2200, doi:10.1093/bioinformatics/btr381, 2011.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F. O.: Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies, Nucleic Acids Res., 41(1), 1–11, doi:10.1093/nar/gks808, 2013.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. a., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J. and Weber, C. F.: Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities, Appl. Environ. Microbiol., 75(23), 7537–7541, doi:10.1128/AEM.01541-09, 2009.

Line 26.

**Referee comment:**

When were these measured?

**Author's response:**

The measurements included in this study were done immediately after sampling. In the cryoconite holes, which were sampled more than one time the depth was measured continuously (See FigS3) to observe the overall stability of these cryoconite holes and to detect stripping events. We changed the sentence in the following way:

As proxies for the age and stability of the hole, water depth was measured with a ruler immediately after the sampling of the sediment.

Line 28.

**Referee comment:**

Please define "saturated sediment" more clearly. How was the excess water removed first?

**Author's response:**

See comment for 11757 Line 18.

Page 11768

Line 21.

**Referee comment:**

Please explain 'lateral thermal conductivity' and how this results in a thin grain layer.

**Author's response:**

We clarified it in the following way:

…by lateral thermal conductivity if time allows. Thereby, the absorbed solar radiation is conducted laterally to the ice walls of the cryoconite hole, resulting in an increasing area and a decreasing sediment thickness.

Page 11769

Line 2.

**Referee comment:**

Consider using full site names in the text rather than abbreviations (e.g. HC and NC). It is easier for the reader to follow.

**Author's response:**

Some of the site names are rather long (e.g. Plateau on Nordenskiöldbreen, main site on Nordenskiöldbreen), but we can changed it.

Page 11770

Lines 3-7.

**Referee comment:**

This is a rather awkward sentence.

**Author's response:**

We changed it in the following way:

Previous sentence:

The finding that all cyanobacteria identified have had heterocysts or close relatives with the *nif*H gene and their dominance in often nitrogen depleted cryoconites supports the hypothesis that sediment associated cyanobacteria act as drivers of this ecosystem in respect to inorganic carbon and nitrogen fixation in nutrient depleted areas.

Changed sentences:

All cyanobacteria found in the current study are known to have heterocysts or to have close relatives with the *nif*H gene for nitrogen fixation. These potential diazotrophs were often dominating in nitrogen depleted cryoconites. These findings indicate that sediment associated

cyanobacteria are highly important as ecosystem engineers in cryoconites in respect to inorganic carbon and nitrogen fixation, especially in nitrogen depleted areas.

Page 11771

Line 13.

**Referee comment:**

Define more clearly what the 'strong selective pressure' is to etc.

**Author's response:**

We defined it more detailed.

One possibility for this temporal homogeneity is the short summer season and the strong selective pressure, such as cold temperatures, high light intensities and unstable habitats which are rather constant over the summer season.

Section 4.6

**Referee comment:**

This is rather awkward to read and I suggest a re-write.

**Author's response:**

We re-wrote the whole section in the following way:

4.6 Microalgae size and grazing resistance

The formation of large cyanobacteria colonies (< 10 cells, or > 25 μm) observed in the studied cryoconite holes may have several benefits for the organisms.

Firstly, the colony size most likely becomes larger than the maximum prey size of the present filtration feeders (Sand-Jensen, 2014). A previous study by Vanormelingen et al. (2009) showed that the increasing colony size of a *Coenobium* species can be an effective defense strategy against filtration feeders. The habitat of closely connected freshwater ponds studied by Vanormelingen et al. (2009) is well comparable to cryoconite holes in regard to their size and connectivity. In the current study, the negative correlation between the average length of Oscillatoriales trichomes and the abundance of filtrating rotifers indicates that this may also be true for cryoconites. We propose that with increasing length of the trichomes, rotifers have a decreasing amount of ingestible food available in the system, which yields in a smaller density.

Secondly, a large colony size may be an adaptation to the typical environmental stressors in cryoconites. Previously, large colonies of *Nostoc* sp. have been shown to be more tolerant to freezing and desiccation than smaller colonies (Li and Gai, 2007). Also a nutrient storage mechanism via extracellular mucus has been proposed to be an effective strategy to cope with nutrient pulses in otherwise ultraoligotrophic environments (Li and Gao, 2007). Both mechanisms are good strategies to live with the environmental stressors in cryoconites. Another

indirect advantage of long filaments is their importance in stabilizing large granules, which are important for possibly symbiotic heterotrophic bacteria (Takeuchi et al., 2001). The overall reason for the formation of large colonies in cryoconites can be related to both, environmental and predation based stressors.

Ciliates are most likely unimportant as predators for microalgae due to their small size and usually bacterivorous diet. The positive relation between ciliate abundance and Oscillatoriales trichome length can be explained by several indirect effects. One possible explanation is that ciliates can act as food source for larger grazers. If the larger grazers are absent, the microalgae and ciliates have an advantage.

Another reason could be that a lack of competition for bacteria as diet with the filtrating rotifers increases the number of ciliates.

Green microalgae are, in general, relatively large and occur mainly as single cells. Grazer abundances were not correlated to their sizes (Table 7). Thus, it is proposed that grazing as a minor impact on the morphology of green microalgae.

Page 11772

Section 4.7

**Referee comment:**

This section feels a bit repetitive from earlier sections and would benefit from reducing or focussing more clearly.

**Author's response:**

We agree, that this section is rather repetitive. Thus, we removed the section and added the additional information to the sections about Microalgae distribution (4.1) and geographic properties (4.2), where appropriate.

Page 11773

Line 13.

**Referee comment:**

Grazer abundances are related to the impact of birds not impact of birds to grazers as the text currently implies.

**Author's response:**

We switched it in the following way:.

The latter is more likely … and grazer abundance and green microalgal densities are positively related to the impact of birds.

**technical corrections**

**Referee comment:**

The English is generally very good but there are some grammar errors that should be addressed. Here are a few examples**.**

**Author's response:**

Thanks for the grammatical corrections. We corrected all the errors and looked for additional errors.

Table 2

**Referee comment:**

It is unclear to me why site NR appears in the column but not the row and NL occurring in a row but not a column?

**Author's response:**

We assumed that NR and NR are per definition the same. But for clarification we made sure to add the NR to the rows. NL is already in the columns and in the rows.

Fig 1.

**Referee comment:**

Suggest a map locating Svalbard. Suggest simplifying the map, e.g. less detail, fewer contours, to enable the site locations and names to be more easily read.

**Author's response:**

Ok, we increased the font size and visible area of the surrounding mountains. We also added a general map of the location of Svalbard in the upper right corner and the location of Billefjorden on Svalbard in the lower right corner.

Figure 2

**Referee comment:**

can be deleted. This system is basically a large pooter and could be referenced to Southwood and P A Henderson 2000 Ecological Methods. Blackwell.

**Author's response:**

Thanks for the reference. We removed the figure and cited the method instead.

Cryoconite sediment was collected into a 0.5 l polyethylene bottle with a pooter (Southwood and Henderson 2000). Sediments in a defined area within a 4.5 cm plastic ring were taken. All sampling equipment was washed with meltwater from the sampling site prior to the sampling.

Southwood, T. R. E., & Henderson, P. A. (2009). Ecological methods. John Wiley & Sons p.269.

Figures 3 and 5a

**Referee comment:**

are only understandable in colour. Can these be adjusted to be clear in B&W?

**Author's response:**

We adjusted the figures and we changed the colors to avoid using the colors of green and red in the same figure.

# Point-by-point response to referee #2

We want to thank the anonymous referee #2 for the detailed review and comments.

The presented study investigates in good detail the control of microalgal communities in cryoconite holes on glaciers in Svalbard. The study is performed with great care at numerous sampling sites (62 cryoconite holes) on three different glaciers and provides new insights into the algal/cyanbacterial communities and is therefore recommended to be published in Biogeoscience. Most interesting is the lack of any significant negative correlation of grazers with the eukaryotic algal communities, more predictable the algal/cyanobacterial communities in relation to nutrient supply.

A few minor inaccuracies should be corrected before final acceptance of the study:

p. 11752, l 12: when talking about 'large colonies' a cell number of < 10 cells appears rather small. (the same again in the discussion on p. 11771)

With large colonies we refer to a colony size that exceeds the maximum food size for possible grazers in the system. But we agree that the term is a bit misleading in this context. We removed the term "large" in the corrected version.

p. 11757, l 2+10: avoid the term 'big' ciliates; rather 'large'

Thanks for the comment, changed it in the corrected manuscript.

p. 11757, l 12: give an explanation why only moving individuals were counted – as estimate for their viability?

Only moving individuals were counted because they were active and most important in a cryoconite food web. In this sense it was an estimate for their viability/ activity. We added this information in the corrected manuscript.

p. 11757, l 16: . . .estimated by epifluorescence microscopy for cyanobacteria and light microscopy for. . .

We changed this sentence accordingly.

p. 11758, l 15: the results on 16S rRNA sequencing come suddenly, they were obtained earlier (in 2012) and likely from similar, but not the same sampling sites. Have these studies been published before? if so give a citation, if not explain that they were used only as a comparison for genus distribution and give a citation for the methods used (MiSeq Illumina sequencing); as it stands no, the reader does not have enough information to judge on significance of this comparison.

We added a detailed description and the accession number of the 16S rRNA sequencing in the corrected version of the manuscript. The data are not unpublished. We added the required information why it was used and where the samples were taken.

p. 11761 l. 16: see above, if this is an integral part of this study, more information is needed – similar sampling sites etc. otherwise no direct comparison is possible.

The sampling sites for the 16S rRNA sequencing are near the other sampling sites. We added their coordinates ID of the closest cryoconite hole location for microalgae and grazer quantification.

p. 11765, l 10: should it not read: . . . bird colonies with high nitrogen levels?

Yes you are right. We corrected this mistake in the corrected version.

p. 11765, l 23: not sure if 'trichomes' of Oscillatoriales is correct, the author rather mean 'trichal' Oscillatoriales; (the same again in the discussion on p. 11771)

We corrected this term in the corrected version.

p. 11772, l 4 Green microalgae . . .occur mainly as single cells – this is likely too general e.g. filamentous Zygnemales (like *Ancylonema*) never occur as single cells.

We agree that this statement it too general in the context of this section and we changed it here. We did have quite some filaments of *Ancylonema*, but we also found a lot of single cellular Zygnemales. The Chlorococcales were never filamentous.

p. 11792 Fig. 3 c it is not clear which column is for Hørbyebreen (Hørbye.1) and Norden.1 (in the figure only the respective .2 are marked? what is the middle column??

The middle column refers to Nordenskiöldbreen. We agree that this is not clear from the plot. We corrected the plot for the corrected version of the manuscript.

p. 11795 Fig. 6 Rotifers were separated in bdelloid (rotifers). . . and *Encentrum* sp.: the latter not in the graph visible.

Only bdelloid rotifers occurred in high abundances and were considered for the shown rda. Due to the rare occurrence of *Encentrum,* their abundances were estimated with a rather large uncertainty and thus not used for statistical tests. We added this information in the corrected manuscript.

**Marked-up version of the corrected manuscript**

# Controls on microalgal community structures in cryoconite holes upon high Arctic glaciers, Svalbard

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## Abstract

Glaciers are known to harbor surprisingly complex ecosystems. On their surface, distinct cylindrical holes filled with meltwater and sediments are considered as hot spots for microbial life. The present paper addresses possible biological interactions within the community of prokaryotic cyanobacteria and eukaryotic microalgae (microalgae) and relations to their potential grazers, such as tardigrades and rotifers, additional to their environmental controls. Svalbard glaciers with substantial allochthonous input of material from local sources reveal high microalgal densities. Small valley glaciers with high sediment coverages and high impact of birds show high biomasses and support a high biological diversity. Invertebrate grazer densities do not show any significant negative correlation with microalgal abundances, but a positive correlation with eukaryotic microalgae. Shared environmental preferences and a positive effect of grazing are the proposed mechanisms to explain these correlations. Most microalgae found in this study form ~~large~~ colonies (< 10 cells, or > 25 µm), which may protect them against invertebrate grazing. This finding rather indicates grazing as a positive control on eukaryotic microalgae by nutrient recycling. Density differences between the eukaryotic microalgae and prokaryotic cyanobacteria and their high distinction in RDA and PCA analyses indicate that these two groups are in strong contrast. Eukaryotic microalgae occurred mainly in unstable cryoconite holes with high sediment loads, high N:P ratios, and a high impact of nutrient input by bird guano, as a proxy for nutrients. In these environments autochthonous nitrogen fixation appears to be negligible. Selective wind transport of Oscillatoriales via soil and dust particles is proposed to explain their dominance in cryoconites further away from the glacier margins. We propose that, for the studied glaciers, nutrient levels related to recycling of limiting nutrients is the main factor driving variation in the community structure of microalgae and grazers.

## 1 Introduction

Cryoconite holes are cylindrical cavities filled with meltwater and biological active sediments found on the surface of glaciers worldwide. Their diameter can range between a few centimeters and several meters (MacDonnel and Fitzsimons, 2008). They are mainly created by air-borne sediment inputs into small depressions, which result in an increased melt rate caused by a decreased albedo (McIntyre, 1984; Fountain et al., 2004). Even though they are ice-free only during the short Arctic summer, cryoconite holes can cover a large part of the ablation zone and contribute significantly to the glacier runoff (Hodson et al., 2008). Cryoconite holes are usually open and photosynthetically active for a few months in summer. During this time they are highly dynamic systems with occasional stripping events during which they can be cleared and the newly distributed sediment starts forming new cryoconite holes nearby (personal observations; MacDonell and Fitzsimons, 2008). During this time several cryoconite holes are connected hydrologically. Most of the year, they are sealed with an ice lid and covered by snow, which protects them from stripping events, but which also inhibits the photosynthetic activity (Jesamine Bartlett, personal communication). Recently reviewed studies also demonstrated that glacial ecosystems have a significant impact on the global carbon cycle (Stibal et al., 2012a). Common approaches tried to find environmental controls on the net ecosystem productivity, but the biotic controls have often been overlooked. We hypothesize that the biotic controls have similar dynamics to temperate lakes, where primary productivity is not solely controlled by environmental parameters (bottom-up), but also by grazing pressure (top-down) (Sterner, 1986).

Cryoconite holes represent ultraoligotrophic environments (Hodson et al., 2008) inhabited ~~only~~ by microorganisms, which are able to ~~adapt to~~cope with many environmental challenges associated with a life on the surface of glaciers. Filamentous phototrophic cyanobacteria and mostly coccal heterotrophic bacteria are shown to act as ecosystem engineers within the cryoconites, capable of forming distinct dark granules up to 3 mm thick in diameter (Takeuchi et al., 2001; Langford et al., 2010). These granules provide a substrate for growth of surprisingly high biomasses and diversities of bacteria, cyanobacteria, eukaryotic microalgae and protozoa (Mueller et al., 2001; Christner et al., 2003; Cameron et al., 2012). Additionally, invertebrates mainly comprised of tardigrades and rotifers have been found~~,~~ inhabiting cryoconite holes on glaciers worldwide (De Smet, and van Rompu, 1994; Groongard and McInnes, 1999; Säwström

et al., 2002; Porazinska et al., 2004; Zawierucha et al., 2014). The species diversity of these grazing invertebrates is relatively low and relatively well-known but their ecological role in the cryoconite community has not been addressed yet. It is believed that they act as top predators in a microbial food web consisting of both grazing and carnivorous species (De Smet and van Rompu, 1994).

In temperate freshwater systems grazing is known to have a substantial effect on microalgal communities (to avoid duplication of terms, "microalgae" in the text also includes Cyanobacteria, unless further specified). For example, Sterner (1986) described two effects of invertebrate grazing on microalgal communities. Firstly, selective feeding can suppress the population of the preferred food organisms. Secondly, invertebrate grazing is ~~suit~~able to release nutrients from microalgae biomasses and enhance the growth of otherwise nutrient limited organisms. In contrast to the crustacean dominated grazer communities in temperate ponds, preying on relatively large organisms, the cryoconite communities are known to consist of much smaller grazers, usually shorter than 200 µm (personal observations). Generally, Arctic freshwater ponds are characterized by a food web with a few trophic levels, dominated by crustacean grazers with short generation times, due to the short growing season (Rautio et al., 2011). The zoobenthos community is thought to obtain its carbon from benthic primary production and associated bacterial growth (Rautio et al., 2011). Another effect of grazing has been described by Vanormelingen et al. (2009), who observed enlarged colonies of a *Coenobium* species as possible adaptation to grazing. Larger colonies are proposed to outgrow the maximum food size of filtration feeders. Bdelloid rotifers are known as size selective filtration feeders for small cells (Ricci and Balsamo, 2000; Devetter, 2009) and are common in cryoconite holes (Zawierucha et al., 2014). Tardigrades, another part of the grazer community in cryoconite holes, are able to prey on much larger organisms (Nelson and Marley, 2000). Ciliates in cryoconite holes can generally act as grazers on microalgae and bacteria, or as prey for larger metazoans (Sinistro et al., 2006), but Mieczan et al. (2013) found that carnivorous and bacterivorous ciliates prevail in Antarctic cryoconites. Another difference between temperate and polar food webs is the slower growth rate of herbivores compared to microalgae in cold environments, which is known to lead to a weak and delayed top down control in habitats with low temperatures (Rose and Caron, 2007). So far,

none of the mechanisms described above has been studied in cryoconite holes and the significance of trophic interactions in cryoconite holes is yet unknown.

For the present study microalgae can be classified into four dominant groups differing in their adaptations to a life on glaciers. i) Filamentous cyanobacteria, usually consisting of Oscillatoriales (*Leptolyngbya* sp. and *Phormidium* sp.) (Mueller et al., 2001), are capable of stabilizing the cryoconite granules which, reversely, can protect the microalgae from physical stress (Takeuchi et al., 2001). Also a small amount of atmospheric nitrogen can be fixed by these non-heterocystous oscillatorian cyanobacteria (Bergman et al., 1997; Telling et al., 2011). ii) Nostocales, usually consisting of *Nostoc* sp. (Mueller et al., 2001) can form big colonies as protection against environmental stresses and act as storage for nutrients and carbon (Li and Gao 2007). They also form heterocysts capable of efficient atmospheric nitrogen fixation (Kumar et al., 2010). iii) Chlorophyceae, mainly consisting of *Chlamydomonas nivalis* (Mueller et al., 2001), are well adapted to high light intensities by the production and storage of photoprotective pigments (Bidigare et al., 1993). Furthermore, snow microalgae are known to migrate to favorable microhabitats (Kavecka, 1986). iv) Zygnematophycea are another group of eukaryotic microalgae capable of production and storage of photoprotective pigments in a moveable vacuole (Remias et al., 2012; Yallop et al., 2012). In summary, cyanobacteria on glaciers are well adapted to nitrogen limitations, whereas green microalgae are better adapted to high light intensities and environmental disturbances. Hence, the stability and nutrient levels should influence the ratio of green microalgae to cyanobacteria and competition is likely to occur.

The aim of the present study was to investigate the importance of environmental controls compared to biological interactions (grazing, competition) on the microalgal community structure and to discuss possible mechanisms involved. The community structures and densities of microalgae and their possible grazers are estimated and environmental parameters were measured. Correlation analyses were then applied to assess possible controls on the microalgal community structure and their relative importance.

## 2 Methods

### 2.1 Site description and sampling

Between July and August 2014, 62 cryoconite holes on the three valley glaciers Nordenskiöldbreen, Hørbyebreen (HC), and Ebbabreen (E) (Table 1, Figure 1) around Petuniabukta and Adolfbukta in Svalbard (76°30' - 80°30' N and 10° - 35° E) were sampled. The three glaciers were all valley glaciers. Nordenskiöldbreen was separated into 4 sampling sites: 1) close to the glacier margin and a bird colony on the peninsula Retrettøya (NR), 2) on the southern site of the glacier (NC), 3) on a central plateau (NI), and 4) on the bottom of a drained supraglacial lake (NL). On Hørbyebreen, 10 samples were taken from the central part and 6 samples in 25 - 100 m elevation intervals. On Ebbabreen, in total 6 samples were collected every 25 - 100 m in height. As will be described, the sites vary in some environmental factors, such as nutrient availability, stability (e.g. water depth), and isolation of the cryoconite holes. For an overview of the studied glaciers see Rachlewicz et al. (2007).

The cryoconite holes are rather unstable habitats with a life span often shorter than one summer season. The closer the cryoconite hole to the glacier margin, the shorter the life span (personal observations). Hence, the cryoconite holes on the Plateau on Nordenskiöldbreen have the longest life span and the cryoconite holes near Retrettøya the shortest one. During the current study twenty cryoconite holes were monitored continuously with depth measurements and photography. We could show that three cryoconite holes experienced a complete stripping event and that nine of them drained, but regrew at the same place (Figure S3). Cryoconite holes on the present glaciers are only open for one to three months in summer, depending on their altitude. They remain rather stable after an ice lid gets formed in atumn until the snow starts melting in late June and the first parts of the glacier clear from the snow in July (personal observations). The current study focusses on the summer months, because only during the summer season, a significant photoautotrophic activity is expected.

On the central part of Hørbyebreen and the southern site of Nordenskiöldbreen 5 cryoconite holes were sampled 4 times throughout the summer season (June - August) in order to test for seasonal variations. Five additional cryoconite holes on these sites were sampled at the beginning and the end of the season to test for possible impacts of the repeated sampling (Control). From all other

sites 6 samples were taken. The samples taken, and measurements done, are summarized in Table 1.

Cryoconite sediment was collected into a 0.5 l polyethylene bottle ~~equipped with a two-way lid and two siphons in order to produce underpressure (Figure 2~~with a pooter (Southwood and Henderson, 2000)). Sediments in a defined area within a 4.5 cm plastic ring were taken. All sampling equipment was washed with meltwater from the sampling site prior to the sampling.

## 2.2 Density estimations of invertebrates and ciliates

Densities of tardigrades, rotifers and large~~big~~ ciliates (> 25 µm) were estimated as the number of individuals per cm² of cryoconite sediment layer. The fresh sample was transferred into a clean 120 ml beaker. The sample was left for at least 30 minutes to settle and the supernatant was removed until 100 ml of the sludge remained. The supernatant was screened randomly for planktonic individuals, but no grazers have been found. The sample was then homogenized in the laboratory by shaking and a 10 - 20 ml subsample was taken and transferred into a 10 cm petri dish with parallel lines on the bottom with a distance of 5 mm. In this subsample, the number of 5 functional grazers or predators was counted (tardigrades, bdelloid rotifers (*Macrotrachella* sp., *Adineta* sp.), carnivorous monogonont rotifers (*Encentrum* sp.), and ~~big~~ large ciliates) with a stereomicroscope. All samples were analyzed within 24 hours after the sampling and stored in the dark at temperatures below 4°C. In all sampling sites, only actively moving individuals were counted as estimate for their viability. For tardigrades and rotifers, species level identification was carried out in 1 - 3 random sites per glacier. The rotifers have been identified, using the monograph of Donner (1965). Tardigrades were identified, using the key to world tardigrade by Ramazotti and Maucci (1983) and by comparisons with other original papers (Dastych, 1988; Miller et al., 2005). The identified material is deposited in the Biology Centre AS CR, Institute of Soil Biology in Ceske Budejovice in the Czech Republic. All density estimations were done in the field station in Petuniabukta. The species determinations were done on fixed cryoconite samples (4% Formaldehyde) back in the lab in the Czech Republic.

## 2.3 Density estimations of microalgae

Microalgal biovolumes were estimated ~~using an~~by epifluorescence microscop~~y~~e for cyanobacteria and light microscopy for eukaryotic microalgae as described by Kaštovská et al. (2005). After settling of the sediment for at least 30 minutes the supernatant was removed with a syringe and kept for further dilutions. Due to the current of meltwater through cryoconite holes, the sediment is already well selected towards high sedimentation rates and the supernatant appeared clear and no remaining particles have been observed. The remaining water saturated wet sediment was used for estimations of the microalgae densities and the water content. For the counting, 0.25 g of wet sediment was diluted with 3 ml of the meltwater from the analyzed sample and crushed in order to homogenize the granules. 40 µl of this suspension was transferred onto a microscopic slide and at least 200 cells were counted and measured. Basic geometric equations for cylinders with hemispherical ends and spheres were applied to calculate the biovolume per wet mass of sediment. After measuring the total sediment mass in the predefined area, it was possible to calculate the densities as biovolumes per area ($\mu m^3\ cm^{-2}$ of cryoconite sediment layer). Additionally, the biovolumes were separated into different size classes based on estimated limits for grazing by filtrating organisms. The estimations are based on the common size of grazers (100 - 200 µm) and their feeding apparati (buccal tube of tardigrades 5 - 10 µm, filtrating organ opening of rotifers 25 - 50 µm) in the samples of this study. The division of filtering classes is mainly based on measurements of the feeding apparatuses of the filter feeding rotifers in our own samples. Additionally, Hino and Hirano (1980) found a linear relationship between the maximum ingestible particle size and the body length in the rotifer *Brachionus pricatilis*. For 200 µm long specimen they found a maximum ingestible particle size of about 21 µm. Microalgal biovolumes of single cells ≤ 10 µm, single cells > 10 µm, colonies ≤ 10 cells, colonies > 10 cells, filaments ≤ 25 µm, filaments > 25 µm were separated in order to visualize the spectrum of possible food items. The mean and median sizes of the colonies and cells were estimated. All densities are given in $\mu m^3\ cm^{-2}$ of cryoconite sediment layer, since photosynthetic activity is thought to be limited to the first few µm of the sediment surface. General oxygen profiles in sediments, obtained with microsensors showed photosynthetic activities at sediment depths only below 0.5-1mm (E.g. Revsbech et al., 1986). For cryoconite sediments a study by Telling et al. (2011) showed that only in sediment layers <3 mm a net autotrophic system is

maintained. Errors of this method related to the dilution, determination, measurements and counting are described by Mueller et al. (2001). For the study of population dynamics, the microscopic approach is preferred to molecular methods since the taxonomic resolution is not as important as accurate density estimations of functional groups. A PCR-bias in genetic methods would, however, lead to a higher uncertainty in density estimations. Nevertheless, the cyanobacterial community structures of Hørbyebreen (HC) and Nordenskiöldbreen (NR) were compared with ~~previous~~ measurements of the prokaryotic community structure based on MiSeq Illumina sequencing of the V3-V4 regions of the 16S rRNA genes in 2012. This additional genetic method helps to validate the microscopy derived estimates and gives an estimate of the abundances of additional bacteria and cyanobacterial genera. It is mainly used to compare the genus distributions between the two glaciers. The sampling sites were located at 78.63°N 17.13°E on Nordenskiöldbreen and at 78.76°N 16.46°E on Hørbyebreen. The locations are near to the Hørbyebreen (HC) and Retretøya (NR) sampling sites. The most dominant genera were then compared to previously found *nif*H genes, important for nitrogen fixation, in the NCBI database (Gaer et al., 2010). The functional cyanobacteria groups in this study are; Nostocales as heterocystous cyanobacteria, and Oscillatoriales as filamentous cyanobacteria without heterocysts, but with the ability to stabilize cryoconite granules. The eukaryotic microalgal groups are; Chlorophycea and Zygnematophyceae. Diatoms and Chroococcales were excluded from the analysis due to their low abundances and the related inaccuracy of biovolume estimations in dilutions.

## 2.4 16S rRNA gene sequencing and sequence analysis

The highly variable V3/V4 region of the 16S rRNA gene was amplified with the bacterial primers S-D-Bact-0341-b-S-17 forward and S-D-Bact-0785-a-A-21 reverse, with overhang Illumina adaptors attached to the primer sequences, creating a single amplicon of about 460 bp (Klindworth et al., 2013). The reactionwas carried out in 50 µl volumes, containing 0.3 mg $ml^{-1}$ Bovine Serum Albumin, 250 mM dNTPs, 0.5 mM of each primer, 0.02 µl Phusion High-Fidelity DNA Polymerase (Finnzymes OY, Espoo, Finland) and 5x Phusion HF Buffer, containing 1.5mM $MgCl_2$. The following PCR conditions were used: initial denaturation at 95°C for 5 min., followed by 25 cycles consisting of denaturation (95°C for 40 s=, annealing (55°C for 1 min.)

and extension (72°C for 1 min.) and a final extension step at 72°C for seven minutes. The amplified DNA was sequenced using the Illumina MiSeq platform at Liverpool Centre for Genomics Research and generated 2 x 300 bp overlapping pairs-end reads.

The 16S sequences were further processed, using the mother (v. 1.35) pipeline (Schloss et al., 200). Chimeric sequences were identified and removed using UCHIME (Edgar et al., 2011). Reads were clustered into operational taxonomical units (OTUs), based on at least 97% sequence similarity, and assigned taxonomically against the SILVA database (Quast et al., 2013).

The sequences are stored at NCBI and available under the accession number PRJNA296475.

### ~~2.4~~2.5 Environmental variables

As proxies for the age and stability of the hole, water depth was measured with a ruler immediately after the sampling. The water content of the sediments was calculated as percentage of weight loss of water saturated sediments after drying at 50°C for 12 hours. The total organic matter (TOM) content was estimated as the weight loss of the dried sediments after dry combustion at 450°C for 5 hours. The sediment load was estimated as the total mass of cryoconite sediments within a defined area. The sediment coverage of Nordenskiöldbreen (NC) and Hørbyebreen (HC) was estimated using aerial pictures taken by a multicopter using ImageJ after Irvine-Fynn et al. (2010). The elevation and distance to the closest deglaciated land was measured using a hand held GPS and topographic maps from 1990 with an error of about 25 m related to the mapping, and an underestimation of approximately 75 m related to glacial retreats. The time of the sampling was calculated as summer degree days (sdd). Sdds are commonly used to model the surface runoff of glaciers (Braithwaite, 1995) and thus a good indicator of the environmental disturbance on the supraglacial system, related to time. As a proxy for nutrient inputs the impact of birds was estimated as ranks between 0 and 3 based on; 1) the presence of birds or bird remnants (excrements, carcasses), and 2) the distance to bird colonies. An impact of 0 refers to a site with no signs of birds or excrements, far away from any bird colonies, whereas an impact of 3 means a site with birds resting on the glacier with excrements around and a bird colony nearby. For the chemical analyses of cryoconite sediments, ammonium and ammonia ($NH_3$-N and $NH_4^+$-N ($NH_X$-N)) were measured by the gas diffusion method using a FIA LACHAT QC 8500 (Lachat Instruments, USA) after Karlberg and Twengstrom (1983)

(Application note ASN 50-0187, Tecator, ISO 11732), and the total mineralized phosphorous (TP) was measured after Kopáček and Hejzlar (1995), while bioavailable orthophosphate ($PO_4^{2-}$-P) was measured photometrically after Mehlich (1984). For the chemical analysis of the meltwater, total organic and inorganic carbon (TOC, TIC) were measured from a filter, using an elemental analyzer. Due to the stability of chemical properties in cryoconites, previously observed (Porazinska et al., 2004), all nutrients were measured once during the season and in a mix of sediments from different cryoconites of each site.

### ~~2.5~~2.6 Statistical analysis

In order to test for differences between the sites and possible controls, multivariate and univariate statistics were applied using different statistical programs. Log transformed data were used for all ordination analyses. Analyses of similarities (ANOSIM) were performed, using P~~p~~ast (Hammer et al., 2001), for comparing the community structures between the sites, controls and treatments, and different sampling times within the same cryoconite hole, using Bray-Curtis dissimilarities. The null hypothesis was rejected if $p<0.05$. p values of multiple tests were corrected after the false discovery rate. A one-way ANOVA followed by a Tukey honest significant difference test was applied, using R (R Development Core Team, 2008), to test for differences of environmental variables, and mean and median sizes of microalgae between the sampling sites. For direct correlation between grazer and microalgae, correlation analysis of log(x+1) transformed densities and standardized microalgal densities (x $10^{-6}$) were applied using R. Multiple linear regression models using untransformed (Oscillatoriales), log(x+1) transformed (other microalgae) data and assuming a poisson distribution were used to assess the effects of grazer densities on the mean and median sizes of the different microalgal groups.

For a more detailed analysis of possible biotic interactions, a principal component analysis (PCA) was performed using CANOCO 5.03. A partial redundancy analysis (RDA) was applied in order to test for environmental controls, using CANOCO 5.03., as a linear constrained ordination method. Prior to the ordination, a detrended correspondence analysis (DCA) was used to test whether a linear ordination is appropriate. A gradient length of 2.4 SD supported a linear model. Interactive-forward-selection-covariates was used in order to build a model, which only includes the best explanatory variables and to avoid the problem of colinearity. After the ordination, a

permutation test based on $r^2$ values with 999 permutations enabled testing the amount of variation explained by the model and the explanatory variables. In order to test for environmental controls, a model using the environmental variables as explanatory variables and the spatial variables as co-variables was used.

## 3 Results

### 3.1 Differences between sites

Differences between the sites were found in their environments and their community structures as shown in Figure 2~~3~~a,b,c and Table 2. Hørbyebreen (HC) shows the highest proportion and concentration of cyanobacteria (88%, 238 x $10^{-6}$ µm³ $cm^{-2}$) compared to eukaryotic green microalgae (31 x $10^{-6}$ µm³ $cm^{-2}$) and the highest densities of all microalgae based on the microscopic counts (270 x $10^{-6}$ µm³ $cm^{-2}$) (Figure 2~~3~~a). The Retrettøya (NR) community differs from all other sites because of a microalgal community dominated by green microalgae (209 x $10^{-6}$ µm³ $cm^{-2}$) (Figure 2~~3~~a). The sites Nordenskiöldbreen – Plateau (NI) and Nordenskiöldbreen – supraglacial lake (NL), which were furthest away from deglaciated land, have the highest proportion of Oscillatoriales (56 and 71%). The other sites are rather similar with a cyanobacteria dominated community (71 – 68 %).

16S rRNA sequence based abundances of cyanobacteria in 2012 show, overall, similar patterns as observed in 2014 via epifluorescence microscopy (~~Figure 3~~Figure 2a,c). Cyanobacteria constitute a substantial part of the prokaryotic community (21 and 26% on Nordenskiöldbreen, and 39% on Hørbyebreen of all 16S reads) (~~Figure 3~~Figure 2c). The most dominant cyanobacteria in the 16S reads were *Arthronema* sp., *Microcoleus* sp. and *Nostoc* spp., *Calothrix* spp., *Symploca* sp., and *Leptolyngbya* sp. were also abundant genera (~~Figure 3~~Figure 2c).

The rest of the bacterial diversity in the 16S reads is mainly represented by Proteobacteria, Bacteroidetes, and Actinobacteria. Other potentially diazotrophic bacteria included bacteria of the genera *Clostridium*, and *Ralstonia*. The only additional phototrophic bacteria found in the 16S reads was the green non-sulfur bacteria group of Chloroflexi (<1%). In a few samples of this study (1 - 3 per glacier), microalgae have been identified to genus level by microscopy.

Cyanobacteria of the genera of *Nostoc*, *Leptolyngbya*, *Phormidium*, and *Microcoleus* prevailed in the microscopic counts. The most abundant cyanobacteria genera in the 16S reads, *Arthronema* sp. and *Calothrix* sp., have not been recognized via microscopy. The most dominant green microalgae included *Chlamydomonas nivalis*, *Ancylonema nordenskioldii*, *Cylindrocystis brebissonii* and *Mesotaenium berggrenii*.

Regarding the grazers, in most sites tardigrades and rotifers were equally abundant (1 – 5 Ind. $cm^{-2}$) (~~Figure 3~~Figure 2b). Only on Ebbabreen (E) did the grazer community have higher densities of tardigrades (25 Ind. $cm^{-2}$) compared to the other sites (~~Figure 3~~Figure 2b). A seasonal change in the community structure was found between the first and last sampling dates on Hørbyebreen (HC) (p=0.0384), but no difference between the repeatedly sampled cryoconite holes and their controls, and no seasonal variation of the community structures were found.

~~As for the microalgae, i~~In a few samples, invertebrates were identified to genus or species by microscopy. The most dominant rotifers belonged to the *Macrotrachella insolita* group, ranging between 1 (NL) and 4 (HC) Ind. $cm^{-2}$. Particularly *M. musculosa* made up the largest proportion of this group. Also, a few individuals of *Adineta vaga* (0.4 (NR) – 0.9 (E) Ind. $cm^{-2}$), and *Encentrum* sp. (0 (NL, NR) – 0.3 (E) Ind. $cm^{-2}$) were found. The most frequent tardigrades found on all sampled glaciers were *Pilatobiotus recamieri* and *Hypsibius dujardini*. Rarely found were also *Hypsibius cf arcticus* and the genus *Isohypsibius* (Zawierucha et al., in prep.). Tardigrade species were not identified immediately in the field and were thus not quantified. Ciliates were not identified to species or genera.

A more precise description of differences in environmental variables for each site is given in Table 3. Overall, the variation in environmental factors and community structures within one glacier (Nordenskiöldbreen: NC - main site, NR, NI, NL) is often higher than the variation between the glaciers (Tables 2 and 3).

The sites NC and HC have similar nitrogen and phosphorus concentrations and ratios. The nutrient data for NR and NI showed generally higher N:P ratios. The TOC:TIC ratio on Hørbyebreen (HC) compared to Nordenskiöldbreen (NC) seems to be higher.

Hørbyebreen is characterized by the lowest water depth and highest sediment coverage, but Nordenskiöldbreen, and particularly the Retrettøya site (NR) had the highest sediments loads

(sediment thickness in cryoconite), the highest water content and the highest concentration of organic matter. The deepest cryoconite holes were found on the upper plateau of Nordenskiöldbreen (NI). The cryoconite holes next to Retrettøya are closest to deglaciated land and have the highest sediment load and impact of birds, since they were right next to a colony of Arctic terns. Also a high number of Black-legged Kittiwakes used to rest on the glacier when the low tide sweeps the icebergs out of the fjord. The supraglacial lake is the farthest from any deglaciated land and cryoconite holes in this area were particularly deep with the lowest sediment load and organic matter content.

### 3.2 Possible biotic interactions

Principal component analysis (PCA) (~~Figure 4~~Figure 3) was used to look for possible correlations between all groups and linear Pearson's correlation (Table 4) for the correlation between invertebrate grazer densities and their proposed prey. The abundance of grazers were significantly correlated with an increase in Zygnemales concentrations ($r^2$=0.29-0.31) (Table 4). Rotifers were positively correlated with both Zygnemales and Chlorococcales, and tardigrades only with the usually larger Zygnemales (Table 4). In contrast, both groups of cyanobacteria (Oscillatoriales and Nostocales) were not correlated with either tardigrades or rotifers.

The PCA shows that the first axis explains most of the variation for green microalgae and grazers, but both of the cyanobacterial groups are mainly explained by the second axis (~~Figure 4~~Figure 3). This indicates different controls on eukaryotic microalgae and grazers, in contrast to cyanobacteria. Besides the positive correlation between grazers and eukaryotic microalgae, the PCA suggests another positive correlation between the green microalgae and consumer groups (ciliates, rotifers and tardigrades).

The distribution of mean and median sizes of different microalgae as possible food sources for grazers (~~Figure 3~~Figure 2 and ~~Figure 5~~Figure 4a, b) show in general that most eukaryotic microalgae are larger than the suggested filtration limit for rotifers, and most cyanobacteria form colonies which are larger than 10 µm (cells) or longer than 30 µm.

An ANOVA showed that the communities of the supraglacial pond (NL) have significantly longer filaments of Oscillatoriales and a generalized linear model assuming a poisson distribution shows that the median length of Zygnemales is significantly different between the different sites.

Multiple linear regressions with log(x+1) transformed (Nostocales), and untransformed (Oscillatoriales) data and generalized multiple linear regressions assuming a poisson distribution (Zygnemales, Chlorococcales) show that the densities of the filtrating rotifers are negatively related to the average length of Oscillatoriales and the median length of Zygnemales (Table 5). Ciliates are positively correlated with the mean size of Oscillatoriales.

### 3.3 Environmental controls

Possible environmental controls were tested by redundancy analysis (RDA). Firstly, a RDA with temporal (time of sampling) and spatial (glacier, and place on glacier) variables as explanatory variables showed that these variables can only explain 10.7 % of the total variation. The spatial variables in this model explained 84.9 % of the variability. In total, it appears that the cryoconite communities are influenced by spatial and only to a smaller degree by temporal variation. The part of explained variation in the final model is shown in Table 6.

In a partial RDA, all environmental variables and time were used as explanatory variables and spatial variables were used as co-variables. After interactive-forward-selection-covariates, a model with three significant explanatory variables remained, as shown in Table 7. The impact of birds (bird) (17.5%), the elevation (14.1%) and sediment load (sedmass) (10.5%) explained most of the variation in the model (42.2%).

The RDA biplot (~~Figure 6~~Figure 5) shows that the sediment load strongly decreases with elevation. If no bird remnants are present, cyanobacteria dominated. Eukaryotic microalgae (Chlorophyceae and Zygnematophyceae) are positively related to the sediment load. The grazer abundances are positively related to possible fertilization by birds. All axes of the biplot explain a significant (p=0.02, F=2.9) part of the total variation.

## 4 Discussion

### 4.1 Microalgae distribution

The current study showed a high spatial variability of the abundance of different microalgal groups and a high distinction between green microalgae and cyanobacteria in the RDA and PCA.. High eukaryotic microalgae to cyanobacteria ratios were observed in environments close to the sea, deglaciated land, or bird colonies with ~~low~~ high nitrogen levels. Significantly higher proportions of cyanobacteria were found further away from possible nitrogen sources. Oscillatoriales dominated over Nostocales the furthest away from any deglaciated terrain~~land~~.

Three different selective mechanisms are proposed to explain the observed variation of

microalgal groups among different environments. The first selection mechanism is wind transport of dust and soil particles, including cyanobacteria and nutrients (Broady, 1996; Porazinska et al., 2004). This wind transport is proposed to be selective for certain cyanobacteria groups. We propose that selection occurs because polar cyanobacteria are often associated with dust in soil, and thus easily transported by wind (Broady, 1996). Furthermore, they are well adapted to desiccation and cryoinjuries which frequently occur during wind transport and on glaciers and could explain their usual dominance in polar freshwater habitats (Tang et al., 1997; Šabacká and Elster, 2006) and in our samples. Hence, thin ~~trichomes of~~trichal Oscillatoriales (Leptolyngbya, Arthronema eg.) are likely to be easily transported on glacial surfaces by this way. Nitrogen input by dust is proposed to be of rather low impact, if the dust originates from adjacent slopes, but having a relatively high impact if it originates from tundra soil (Stibal et al., 2006). The second selection criterion is the nitrogen input in the form of nitrate, nitrite and ammonia, or ammonium which selects for eukaryotic microalgae. In fact, green microalgae occurred mainly in cryoconite holes with a high input of bird guano and dominated in holes with higher $NH_X$-N concentrations and PN : TP ratios above Redfield (16 : 1). The most important inputs are most likely atmospheric inorganic nitrogen stored in snow and ice followed by sea spray or bird guano, tundra soil and moraine dust with the least hypothesized importance. While there are high inputs of tundra soil and bird guano, we propose an insignificant role of autochthonous $N_2$ fixation. The third selection mechanism is the stability of the environment, where eukaryotic microalgae are better adapted to quickly changing environments due to their quick growth, photoprotection by complex adaptation processes of their photosystems and mobility in the case of snow microalgae.

All three mechanisms together can explain the distribution described above. Namely, high eukaryotic microalgae concentrations occur in an unstable environment with high concentrations of bioavailable nitrogen and a high impact of birds. High Oscillatoriales proportions are found further away from the glacier margins, but still at low concentrations due to their less efficient pathways of $N_2$ fixation. Higher Nostocales proportions occur where nutrient inputs are limited to dust from adjacent moraines, which would transport Oscillatoriales, but much less bioavailable nitrogen for the growth of eukaryotic microalgae.

Another explanation could be that the green microalgae found in this study were accidentally imported to the cryoconite holes. Since these microalgal groups usually occur on glacial surfaces,

unstable cryoconite holes with thick sediment layers at lower elevations would accumulate more supraglacial organisms by meltwater inflow.

The dominance of *Arthronema* sp. and *Calothrix* sp. in the 16S reads was unexpected. Both genera are usually absent or rarely found in cryoconites (Mueller et al., 2001) and the microscopic identifications did not show high abundances of these genera in our samples. In fact, *Arthronema* sp. has not been found in cryoconites at all. *Arthronema gygaxiana* is known to be distributed globally in freshwater and soil habitats, including glacier forefields (Casmatta et al., 2005; Frey et al., 2013). Hence, the presence of this species in our analyses from 2012 is possible. However, sequence similarity analysis of previously analyzed 16S rRNA genes of *Arthronema* spp. and the other dominant species in our reads using ARB (Quast et al., 2013) showed a high heterogeneity between strains. One strain was more closely related to *Leptolyngbya antarctica* than to all other strains. Hence, we interpret the 16S reads of cyanobacteria only to the genus level. The ecological interpretations in the present paper focus on broader taxonomic levels of microscopically identified cyanobacteria.

### 4.2 Geographic properties

The valley glaciers on Svalbard typically have a substantial allochthonous input of sediment and nutrients from local sources due to their small size compared to larger ice sheets. Microalgal densities found in this study are between 1.8 (NI) and 7.8 (HC) times higher than previously measured on the Greenland ice sheet (GrIS) (Stibal et al., 2006; Stibal et al., 2011, Stibal et al., 2012b). It is clear that small valley glaciers with high sediment coverages and high impact of birds contain usually higher nutrient levels and thus a higher biomass and a higher biological diversity than larger ice sheets. However, the cyanobacterial proportion within the phototrophic cells (73%) is comparable with the findings from the GrIS (66%) (Stibal et al., 2006).

Eukaryotic microalgae contributed with biovolumes of 14 – 32 $\mu m^3\ cm^{-2} \times 10^{-6}$ (14 - 29 %) on most sites, except near Retrettøya (NR) ($209 \times 10^{-6}\ \mu m^3\ cm^{-2}$, 83 %) where the contribution was small. Similar values have been observed by Stibal et al. (2006) on the GrIS. In direct comparison, most sites in the present study are enriched in cyanobacteria compared to the GrIS, except for the exceptional site ~~NR~~near Retrettøya. Only 17% of the phototrophic cells at this site were cyanobacteria, which would rather fit to the values of medial moraines on the GrIS (24%)

measured by Stibal et al. (2006), but the general concentration of phototrophs at Retrettøya ~~NR~~ is two orders of magnitude higher compared to the medial moraines. This finding may indicate a system with high productivity due to sufficient nutrient input and sunlight compared to the moraines or more isolated cryoconites, but a different community structure. Most of the eukaryotic microalgae found are known as ice- or snow microalgae, and possible reasons for their accumulation ~~at the NR site will be explained later~~in unstable cryoconite holes have been described in the last chapter.~~.~~

Spatial variability between close glaciers has also been found. Our data indicate high variability in the community structure within various parts of one glacier. Stibal et al. (2012b) found different environmental controls on microbial communities in cryoconite holes at different altitudes on the Greenland ice sheet. Chemical variables were mostly explained by physical and/or geographic parameters. The altitude, slope, distance to the closest deglaciated land, debris coverage and suggested ecological zones (glacier margin, bare ice, slush) explained most of the variability within the microbial community structure and the measured chemical parameters. Since the present study did not cover a comparable range of slopes, no effect of the slope was found. For the debris coverage, elevation and distance to the closest deglaciated land, the proxies measured and used were elevation and sediment load for the habitat stability and age and bird impact for external nutrients. Each showed a significant impact on the microalgal community structure and on their proposed consumers (grazer). Similar environmental controls on grazer abundances have been observed in Antarctica (Porazinska et al., 2004) with significant effects of sediment load and elevation.

The low abundances of cyanobacteria on glacial surfaces (Lutz et al., 2014) also suggest a weaker adaptation to quickly changing and unstable environments. Such a negative relation between cyanobacteria presence and high sediment loads in lower elevations in cryoconite holes is clearly visible. It is well know that cyanobacteria are slow growing (Tang et al., 1997), which means that they are more sensitive to disturbance, as shown by the negative relation with the sediment load. On the contrary, eukaryotic microalgae are fast growing and more resistant to disturbance by sediment load. In fact, Cook et al. (2010) found that cryoconite granules usually form a single grain layer between 0.04 and 0.20 g $cm^{-2}$ by lateral thermal conductivity if time allows. Thereby, the absorbed solar radiation is conducted laterally to the ice walls of the cryoconite hole, resulting

in an increasing area and a decreasing sediment thickness. This means that a thick sediment layer indicates a younger, unstable cryoconite hole. The sediment load of the present study ranged between 0.161 g $cm^{-2}$ at NI and 0.396 g $cm^{-2}$ at NR. These values are, compared to Cook's et al. (2010) study, on the higher end and indicate rather unstable environments. Furthermore, some microalgal cells might be recently mixed into deeper layers of the sediment.

### 4.3 Nutrient inputs

The external nutrient inputs by birds together with the stability of the cryoconite holes play an additional role. The ~~N:P~~N:P ratio below 16:1 (Redfield 1958) on ~~HC~~ Hørbyebreen and Nordenskiöldbreen~~NC~~ suggest a nitrogen limited environment where cyanobacteria dominate, whereas the plateau of Nordenskiöldbreen~~NI~~ and Retrettøya ~~NR~~ with higher N:P ratios indicate, on the contrary, a phosphorous limited environment, where eukaryotic microalgae prevail. However, the number of replicates did not allow for reliable statistical tests on the exact nutrient levels. Also, Telling et al. (2011) found that phosphorous is generally the main limiting nutrient on glaciers and that nitrogen is usually introduced by snow and rain (atmospheric nitrogen) rather than by cyanobacterial nitrogen fixation. Previous research performed in Greenland by Stibal et al. (2006) did not show a clear effect of nutrient levels on cryoconite hole microbial diversity and organic matter production, either. This research rather proposed that physical factors influence the nutrient conditions on glacial surfaces. In fact, Stibal et al. (2006) showed that soil texture, water content and pH are the main factors, controlling microalgal community structures in supraglacial environments. In the present study, it appears that these factors did not play an important role in our small valley glaciers.

The cyanobacterial diversity seems to be controlled by completely different variables as indicated by the PCA (~~Figure 4~~Figure 3), in which eukaryotic microalgae and grazers were mostly explained by the first and cyanobacteria by the second axes, respectively. Considering the nitrogen fixation ~~cap~~ability of cyanobacteria, it is clear that these organisms are dominant in nitrogen limited environments. This is indicated by the negative relation to the impact of birds and a high N:P ratio on the site at Retrettøya (NR) with the highest impact of birds.

## 4.4 Nitrogen fixation

Microalgae, including cyanobacteria, are an important part of the microbial community in cryoconite sediments. In fact, in our samples cyanobacteria biovolumes represent about 49 - 250 $\mu m^3\ cm^{-2}\ x\ 10^{-6}$ of the cryoconite sediment layer. In the 16S rRNA reads, 20 – 39 % of the prokaryotic community are cyanobacteria and within the microalgae community mostly between 71 and 88 %. All cyanobacteria found in the current study are known to have heterocysts or to have close relatives with the *nif*H gene for nitrogen fixation. These potential diazotrophs were often dominating in nitrogen depleted cryoconites. These findings indicate that sediment associated cyanobacteria are highly important as ecosystem engineers in cryoconites in respect to inorganic carbon and nitrogen fixation, especially in nitrogen depleted areas. ~~The finding that all cyanobacteria identified have had heterocysts or close relatives with the *nif*H gene and their dominance in often nitrogen depleted cryoconites supports the hypothesis that sediment associated cyanobacteria act as drivers of this ecosystem in respect to inorganic carbon and nitrogen fixation in nutrient depleted areas.~~

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In particular, the high abundances of cyanobacteria in the cryoconite community suggest that the nitrogen limitation in these ultraoligotrophic environments may be compensated by atmospheric nitrogen fixation. In fact, all cyanobacteria identified via microscopy and 16S sequencing are known to have relatives with *nif*H genes for nitrogen fixation in their genome (Isojarvi et al.,, unpublished; Paul et al., 2014; Starkenburg et al., 2011; Steward et al., 2004; Taghavi et al., 2013). Only for *Arthronema* sp. is previous genome analysis lacking. However, in several studies it has also been proposed that allochthonous atmospheric nitrogen inputs is two orders of magnitude higher than autochthonous nitrogen fixation, which would mean that cryoconites are mainly phosphorous limited (Hodson et al., 2010; Telling et al., 2011; Žárský et al., 2013). Nevertheless, in ultraoligotrophic samples far from the impact of nesting or resting birds, cyanobacteria are thought to be crucial for atmospheric nitrogen storage on the glacier. Telling et al. (2012) already showed the importance of cyanobacterial nitrogen fixation on the Greenland Ice Sheet (GrIS). Eventually, nitrogen fixation may enhance the production of organic matter and expolymeric substances, which is proposed to support higher trophic levels, such as the metazoan grazers (Telling et al., 2011; Žárský et al., 2013) and heterotrophic bacteria (Decleyre et al., 2015).

### 4.5 Temporal variability

Temporal variability in the microalgal community structures has been measured for the first time in this study. An ANOSIM analysis did not show any seasonal variation, but the RDA suggests a small temporal variability within one season, which is masked by environmental and spatial factors. The temporal impact is statistically significant, but the explanatory importance is negligible. A similar study by Musilova et al. (2015) also found no temporal trend in the microbial community structure on the Greenland ice sheet. However, their method was solely based on 16S tag sequencing, replicates were lacking and their results should be treated carefully. Also, the cyanobacterial proportion in the microbial community was smaller (3 - 29 %), compared to our study, which may be caused by a different system on the Greenland ice sheet, or by different primers used. The overall community structure is fairly similar. The fact that both studies used different methods, different taxa and different habitats and still came to the same conclusion highly supports a cryoconite community of eukaryotes and prokaryotes which is not considerably influenced by temporal factors. One possibility for this temporal homogeneity is the short summer season and the strong selective pressure, such as cold temperatures, high light intensities and unstable habitats which are rather constant over the summer season.

### 4.6 Microalgae size and grazing resistance

The formation of large cyanobacteria colonies (< 10 cells, or > 25 µm) observed in the studied cryoconite holes may have several benefits for the organisms.

Firstly, the colony size most likely becomes larger than the maximum prey size of the present filtration feeders (Sand-Jensen, 2014). A previous study by Vanormelingen et al. (2009) showed that the increasing colony size of a Coenobium species can be an effective defense strategy against filtration feeders. The habitat of closely connected freshwater ponds studied by Vanormelingen et al. (2009) is well comparable to cryoconite holes in regard to their size and connectivity. In the current study, the negative correlation between the average length of Oscillatoriales trichomes and the abundance of filtrating rotifers indicates that this may also be true for cryoconites. We propose that with increasing length of the trichomes, rotifers have a decreasing amount of ingestible food available in the system, which yields in a smaller density.

Secondly, a large colony size may be an adaptation to the typical environmental stressors in cryoconites. Previously, colonies of Nostoc sp. have been shown to be more tolerant to freezing and desiccation than smaller colonies (Li and Gai, 2007). Also a nutrient storage mechanism via extracellular mucus has been proposed to be an effective strategy to cope with nutrient pulses in otherwise ultraoligotrophic environments (Li and Gao, 2007). Both mechanisms are good strategies to live with the environmental stressors in cryoconites. Another indirect advantage of long filaments is their importance in stabilizing large granules, which are important for possibly symbiotic heterotrophic bacteria (Takeuchi et al., 2001). The overall reason for the formation of colonies in cryoconites can be related to both, environmental and predation based stressors.

Ciliates are most likely unimportant as predators for microalgae due to their small size and usually bacterivorous diet. The positive relation between ciliate abundance and Oscillatoriales trichome length can be explained by several indirect effects. One possible explanation is that ciliates can act as food source for larger grazers. If the larger grazers are absent, the microalgae and ciliates have an advantage.

Another reason could be that a lack of competition for bacteria as diet with the filtrating rotifers increases the number of ciliates.

Green microalgae are, in general, relatively large and occur mostly as single cells. Grazer abundances were not correlated to their sizes (Table 7). Thus, it is proposed that grazing as a minor impact on the morphology of green microalgae.~~Most cyanobacteria found in this study form large colonies (< 10 cells, or > 25µm), which may protect them against grazing by filtration (Sand-Jensen, 2014). In fact, we found a negative correlation between the average length of trichomes of Oscillatoriales and filtrating rotifers. A similar effect has been found on the colony sizes and dimensions of a *Coenobium* species in interconnected freshwater ponds and has been found to be an adaptation to grazing pressure (Vanormelingen et al., 2009). Ciliates are positively related to the mean length of Oscillatoriales, which may be explained by a shared positive effect for Oscillatoriales and Ciliates if the filtrating grazers are less abundant. Perhaps it is caused by a lack of competition for their bacterivorous diet with the filtrating feeding strategy of rotifers. Regarding the environmental factors, it is known that filamentous cyanobacteria in cryoconite holes act as ecosystem engineers by stabilizing relatively large granules, which are more stable and can support mutualistic relationships with heterotrophic bacteria (Takeuchi et al., 2001). For~~

this function, a certain size would be necessary, considering average diameters of cryoconite granules above 1 mm. The large colonies of Nostocales can be an adaptation to typical environmental stresses, such as freezing and nutrient limitation. Li and Gao (2007) showed that larger colonies of *Nostoc* sp. can be more tolerant to freezing and desiccation and can be capable of storing nutrients. Green microalgae are, in general, relatively large and occur mainly as single cells. Grazer abundances were not correlated to their sizes (Table 7).

### 4.7 Cyanobacteria vs eukaryotic microalgae

Differences between the eukaryotic microalgal and cyanobacterial densities at the studied sites and their high distinction in the RDA and PCA analyses indicates that these two groups are in strong contrast. Green microalgae occurred mainly in cryoconite holes with high sediment loads and a high impact of bird guano, as a proxy for nutrients. Furthermore, green microalgae are most dominant in habitats with higher NHx-N and PN/TP ratios above the Redfield ? (16:1). This indicates that green microalgae prefer habitats with high nitrogen levels and can survive in unstable environments, where the sediment thickness does not yet reach an equilibrium depth (Cook et al., 2010). This is usually the case in glacial ablation zones at lower elevations, as was proved by the lower sediment load at the sites furthest away from the glacier margin (NI, NL), compared to the site close to the margin (NR)(Table 3). The eukaryotic microalgae found in this study consisted of taxa which are referred to as ice- (Zygnemales), and snow- (Chlorococcales) microalgae, respectively. These two groups are well adapted to living on the fast changing glacial ice and melting snow. This adaptation is connected with high light intensities, survival in unstable conditions, and an efficient use of nutrient pulses by quick growth, which has recently been shown by Telling et al. (2014). All these adaptations are most likely also favorable in unstable cryoconite holes with higher nutrient levels, where green microalgae can compete with the usually more dominant cyanobacteria (Stibal et al., 2006). Tang et al. (1997) and Šabacká and Elster (2006) suggested that cyanobacteria are, despite their slow growth, usually dominant in polar freshwater systems, due to their adaptation to freezing and desiccation. However, eukaryotic microalgae may become dominant in unstable environments, due to their higher growth rate. Another explanation could be that the green microalgae found in this study were accidentally imported to the cryoconite holes. Since these microalgal groups usually occur on

~~glacial surfaces, unstable cryoconite holes with thick sediment layers at lower elevations would accumulate more supraglacial organisms by meltwater inflow.~~

### ~~4.8~~4.7 Invertebrate grazing

Grazer densities did not show any significant negative correlation with microalgal abundances, but only a positive correlation with green microalgae. This can either indicate that grazing has a positive impact on green microalgal densities, perhaps by nutrient recycling, which should have the same effect as the impact of birds, or by shared environmental preferences. The latter is more likely, since the PCA (~~Figure 4~~Figure 3) showed very similar environmental gradients for green microalgae and cyanobacteria, and the ~~impact of birds~~grazer abundances and microalgal densities ~~is~~ are positively related to ~~grazer abundances~~the impact of birds~~, and green microalgal densities~~. Hence, nutrient availability seems to impact both green microalgae and grazers. One explanation could be that those grazers are mainly feeding on smaller heterotrophic bacteria, and only to a lesser extent on microalgae. In this case, high nutrient levels would support, besides the higher densities of green microalgae, also high densities of heterotrophic bacteria. The bdelloid rotifer species and genera found in this study are, indeed, known to be bacterivorous (Devetter, 2009). The tardigrades found in this study are expected to be bacterivorous or algivorous based on the morphology of their buccal tube. A few grazers found during epifluorescence microscopy had cyanobacterial cells in their stomach. In order to clarify this open question, future studies should include the densities of heterotrophic prokaryotes and an extended study of the stomach contents of grazers.

Trophic interactions between grazers are also possible, as pointed out by Cameron et al. (2012) and Zawierucha et al. (2014), but only positive correlations have been found between the major groups. The same positive correlation between tardigrade and rotifer abundances has been observed in Antarctica (Porazinska et al., 2004). This indicates in general shared food sources and low competition. In fact, the genera found in this study include grazers with different feeding strategies, including filtration feeders (*Macrotrachella* sp.), grasping feeders (*Adineta* cf. *vaga*), carnivores (*Encentrum* sp.), and omnivorous grasping tardigrades (*Hypsibius* sp., *Isohypsibius* sp.), which may reduce competition. Some organisms, such as small rotifers and ciliates, can act as a food source for larger omnivorous or carnivorous species. Correlation analyses of these

genera were not possible due to the low abundances of rare species and the related inaccuracy in estimation of their densities in diluted samples.

## 5 Conclusions

The spatial and temporal variability in microalgae and grazer community structures in cryoconite holes on central Svalbard has been studied. Environmental parameters, such as sediment load, elevation (proxy for cryoconite stability and age), and the impact of birds (proxy for nutrient inputs), explained most of the variation in the community structure. Different adaptations of various microalgae groups to ultraoligotrophic or unstable habitats are proposed to explain these effects. Grazer abundances were not found to be negatively correlated to any microalgae densities, but to some of their sizes. We propose that grazing pressure by filtrating rotifers probably led to longer cells and colonies as adaptations to size selective feeding.

A positive correlation between rotifers and green microalgae densities has also been found. A mainly bacterivorous diet for most of the grazers is suggested to explain this positive correlation. In fact, shared environmental preferences of green microalgae and bacteria for high nutrient levels are hypothesized to explain this correlation. Further experiments including bacterial abundances and the stomach contents of grazers could help to test this novel hypothesis. Microalgae have been found to occur in very high abundances with cyanobacteria making up a substantial part of the prokaryotic community, indicating their importance as ecosystem engineers. Also, the high abundances of tardigrades, rotifers, and ciliates, including genera with different feeding strategies, have been found and suggest a complex food web between more trophic levels than measured in the present study. Feeding experiments and analysis of stomach contents may help to bring a more detailed picture of this yet hardly known food web.

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## Authors contribution

T.R. Vonnahme, J. Elster, J.D. Žárský, M. Devetter, and M. Šabacká contributed to the preparation of the manuscript, analysis of the data, and experimental design. J. Elster coordinated the study. The community structures were assessed by T.R. Vonnahme, M. Devetter (microscopy), and M. Šabacká (16s rRNA). Environmental parameters were measured by T.R. Vonnahme and J.D. Žárský. Nutrient analyses were performed by J. D. Žárský and M. Šabacká.

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Table 1. Sampling and analysis design. Sampled sites and their abbreviations are used throughout the paper. Number of sampled cryoconite holes for different analyses.

| Site | Abbreviation | sample size | repeated sampling (4x) | Nutrients |
|---|---|---|---|---|
| **Ebbabreen** | E | 6 | - | - |
| **Hørbyebreen** | HC | 16 | 5 | 3 |
| **Nordenskiöldbreen** | | | | |
| main site | NC | 10 | 5 | 4 |
| Retrettøya | NR | 6 | - | 1 |
| supraglacial lake | NL | 6 | - | - |
| Plateau | NI | 6 | - | 1 |

Abbreviation: Abbreviation for the sampling site, used in the text; Sample size: Number of sampled cryoconite holes; repeated sampling (4x): Number of cryoconite holes that were sampled 4 times over the season; Nutrients: Number of cryoconite holes, where nutrient analysis were performed.

Table 2. Statistically significant (corrected $p<0.05$) differences between the sites in their community structures (ANOSIM results) and environments (ANOVA results).

| | HC | NC | NI | NL | NR |
|---|---|---|---|---|---|
| **community** | | | | | |
| E | A | G | G | G | A |
| HC | | A | A | A,G | A |
| NC | | | A | - | A |
| NI | | | | - | A |
| NL | | | | | A |
| NR | | | | | - |
| **environment** | | | | | |
| E | De | Om | - | - | - |
| HC | | Om | - | Sm | - |
| NC | | | De | Sm,Wc,Om,De | - |
| NI | | | | Om | De |
| NL | | | | | - |
| NR | | | | | - |

A: Microalgae, G: Grazer

De: Depth, Om: Organic matter, Sm: Sediment mass, Wa: water content

Table 3. Environmental variables for each site as ranges or averages ± the standard error. Bold numbers indicate particularly high values and underlined numbers low values. n indicates the samples size for the different kind of analysis. Abbreviations for the different parameters are given in brackets.

| Site | | HC | NC | NI | NR | E | NL |
|---|---|---|---|---|---|---|---|
| n | | 16 | 10 | 6 | 5 | 6 | 6 |
| Elevation (e) | m.a.s.l. | 170-230 | 150 | 200 | 20-50 | 160-525 | 200 |
| Sediment load (sedmass) | mg w.w. $cm^{-2}$ | 238 ± 15 | **277** ± 23 | 195 ± 27 | **396** ± 162 | 212 ± 18 | <u>161</u> ± 15 |
| Water content (water) | M [% ] | 48 ± 2 | **51** ± 4 | 50 ± 5 | 47 ± 2 | 51 ± 3 | <u>39</u> ± 6 |
| Organic matter (om) | mg $kg^{-1}$ | 434 ± 14 | **1184** ± 498 | 607 ± 83 | 603 ± 62 | 293 ± 81 | <u>207</u> ± 134 |
| Water depth (depth) | Cm | <u>0.4-14.5</u> | 0.1-28 | **15.8-49** | 1.7-33 | 8-43 | 8-43 |
| Distance to deglaciated land | m | 20-400 | 850 | 2800 | <u>50-150</u> | 50-1400 | **3300** |
| Sediment coverage (sediment) | A [%] | **12.69** ± 0.53 | 8.79 ± 0.39 | | | | |
| Impact of birds (birds) | Rank | 0-1 | 2 | 1 | 3 | 0-2 | 1 |
| Particulate nutrients in sediments | | | | | | | |
| N | | 3 | 4 | 1 | 1 | | |
| Bioactive-$PO_4^{2-}$-P | mmol $kg^{-1}$ | 0.21 ± 0.02 | 0.15 ± 0.02 | 0.19 ± NA | 0.20 ± NA | | |
| Total P (TP) | mmol $kg^{-1}$ | **6.81** ± 0.43 | 6.11 ± 0.86 | 4.88 ± NA | 5.46 ± NA | | |
| NHx-N | mmol $kg^{-1}$ | 90.31 ± 12.38 | 77.46 ± 21.43 | 89.76 ± NA | **110.36** ± NA | | |
| NHx-N /TP | | 13.56 ± 2.47 | 14.56 ± 4.56 | **18.40** ± NA | **20.20** ± NA | | |
| PO4-P /TP | | 0.03 ± 0.00 | 0.03 ± 0.01 | 0.04 ± NA | 0.04 ± NA | | |
| Dissolved carbon in water | | | | | | | |
| N | | 1 | 5 | | | | |
| Total organic carbon (TOC) | ppb | **4287** ± 45 | 2420 ± 238 | | | | |
| Inorganic carbon (TIC) | ppb | 622 ± 2 | **946** ± 262 | | | | |
| Total carbon (TOC+TIC) | ppb | 4907 ± 45 | 3365 ± 122 | | | | |

Table 4. Correlation table with Pearson's $r^2$ values and corrected p values between microalgae and invertebrate grazers. Significant values are marked in bold.

| | | **Tardigrada** | **Rotifera** |
|---|---|---|---|
| Chlorococales | $r^2$ | 0,141 | 0,232 |
| | P | 0,471 | 0,075 |
| Zygnemales | $r^2$ | **0,3118** | **0,2885** |
| | P | **0,0171** | **0,0196** |
| Oscillatoriales | $r^2$ | -0,044 | -0,063 |
| | P | 0,796 | 0,796 |
| Nostocales | $r^2$ | 0,044 | -0,108 |
| | P | 0,796 | 1.00 |

Table 5. Regression table for linear regression models with median and mean sizes of microalgae as dependent variables and grazer densities as explanatory variables.

| | | Rotifera | Tardigrada | Ciliates |
|---|---|---|---|---|
| Nostocales colony size | p | 0.9622 | 0.9622 | 0.9622 |
| | Estimate | 0.005 | -0.001 | 0.002 |
| Oscillatoriales length | p | **0.0083** | 0.9622 | **0.0149** |
| | Estimate | **-0.016** | 0.0004 | **0.0136** |
| Chlorococcales diameter | p | 0.6072 | 0.9622 | 0.9622 |
| | Estimate | -0.011 | 0.0004 | -0.005 |
| Zygnemales length | p | 0.1032 | 0.9622 | 0.6072 |
| | Estimate | -0.0158 | -0.0001 | 0.0093 |

Table 6. Results for an RDA with spatial and temporal variables as explanatory factors and the explained variability of each variable on the final model. The glacier variable represents the three sampled glaciers.

| | explained variability % | F | P |
|---|---|---|---|
| Glacier | 58.6 | 5.2 | 0.003 |
| place on glacier | 55.8 | 4,9 | 0.008 |
| time of sampling | 37.3 | 3.2 | 0.015 |

Table 7. Results for the partial RDA with glacier and place as co-variables, after interactive-forward-selection-covariates. 14 environmental (physical, morphological and chemical) variables are tested, only significant results are shown. Explained variability means proportion of variability explained by each variable in the total model (variability explained by all variables is 64.3 % including non-significant ones). Contribution to explained variability means the proportion of a selected variable in variability explained by selected variables.

| Name | Explained variability % | Contribution to explained variability % | pseudo-F | p |
|---|---|---|---|---|
| Birds | 17.5 | 27.3 | 7.7 | 0.001 |
| Elevation | 14.1 | 21.9 | 7.2 | 0.009 |
| Sediment load | 10.5 | 16.4 | 6.2 | 0.023 |

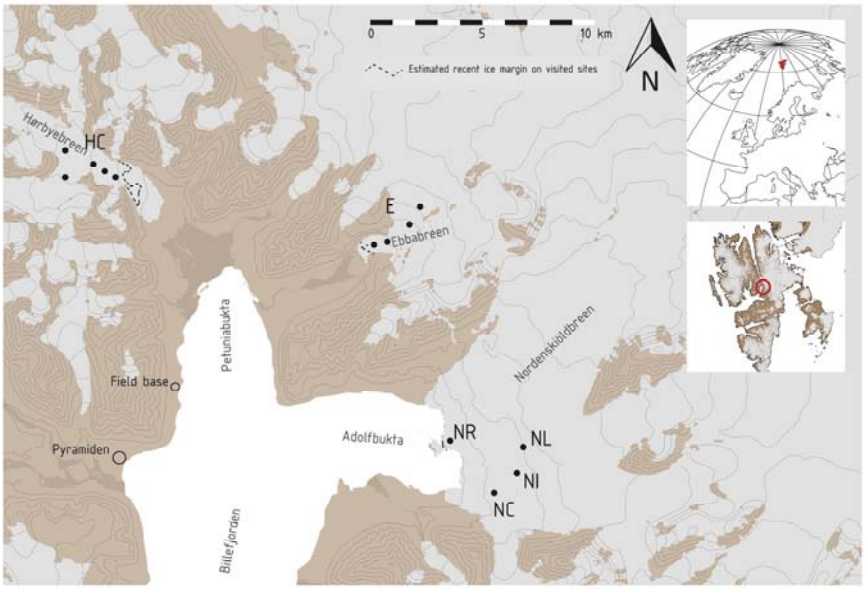
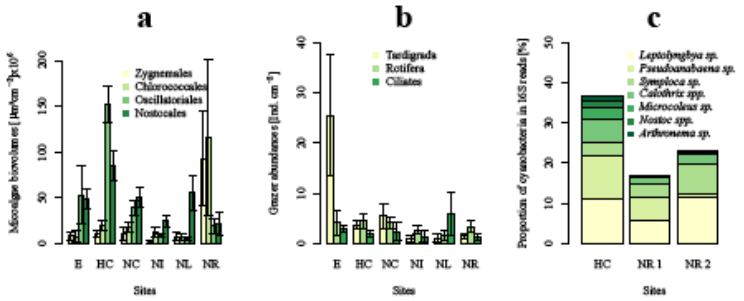


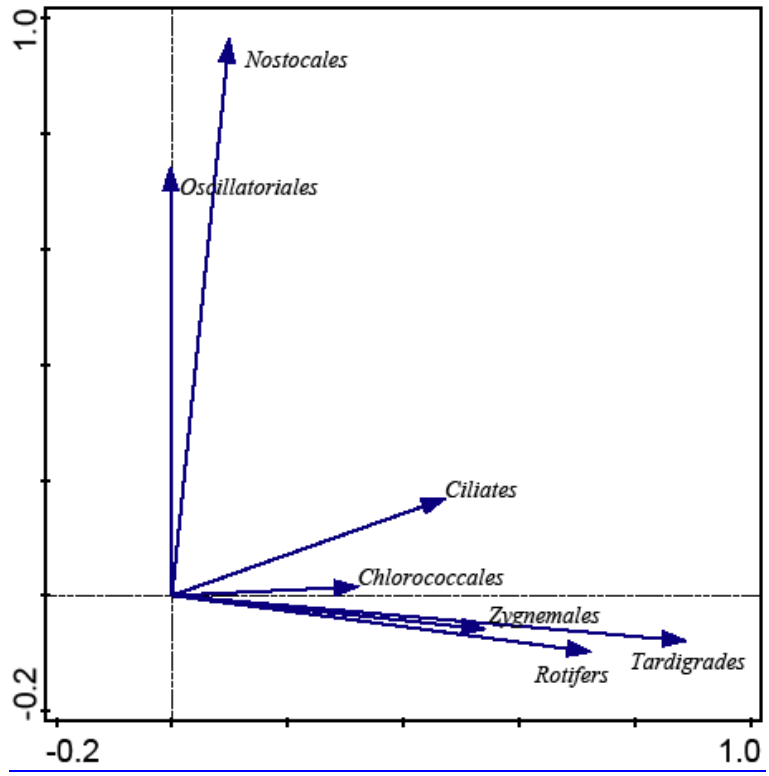

Figure 1. Sampling sites of the cryoconites. The abbreviations used are: HC for Hørbyebreen, E for Ebbabreen, NC for the main site on Nordenskiöldbreen, NI for the plateau on Nordenskiöldbreen, NL for the supraglacial lake on Nordenskiöldbreen, and NR for the part of Nordenskiöldbreen next to Retrettøya. The map is modified from the geographic data of the Norwegian Polar Institute (2014).

~~Figure 2. Sampling procedure for the sediment collection in cryoconite holes. An underpressure is produced by suction with the mouth. The underpressure is then refilled with the cryoconite sediment via a second tube. In order to avoid contamination, the two different tubes were labelled in different colors.~~




~~Figure~~ ~~3~~

Figure 2. Mean densities in cryoconite sediment layers of microalgae (a) in ($\mu m^3$ $cm^{-2}$) x $10^{-6}$ and grazer (b) in individuals per $cm^2$ for the different sites (E: Ebbabreen, HC: Hørbyebreen, NC: Nordenskiöldbreen, NI: Nordenskiöldbreen plateau, NL: supraglacial pond on Nordenskiöldbreen, NR: Retrettøya). The error bars indicate the standard errors. (c) shows the proportion of different cyanobacterial genera within all 16S sequences from 2012 on Hørbyebreen (~~Hørbye.1~~HC) and Nordenskiöldbreen (~~Norden~~NR.1, ~~Norden~~NR.2).




~~Figure 4~~Figure 3. PCA biplot of all organisms collected in this study. Euclidean dissimilarities were used. The data are log transformed and microalgal biovolumes were standardized by multiplication by $10^{-6}$.

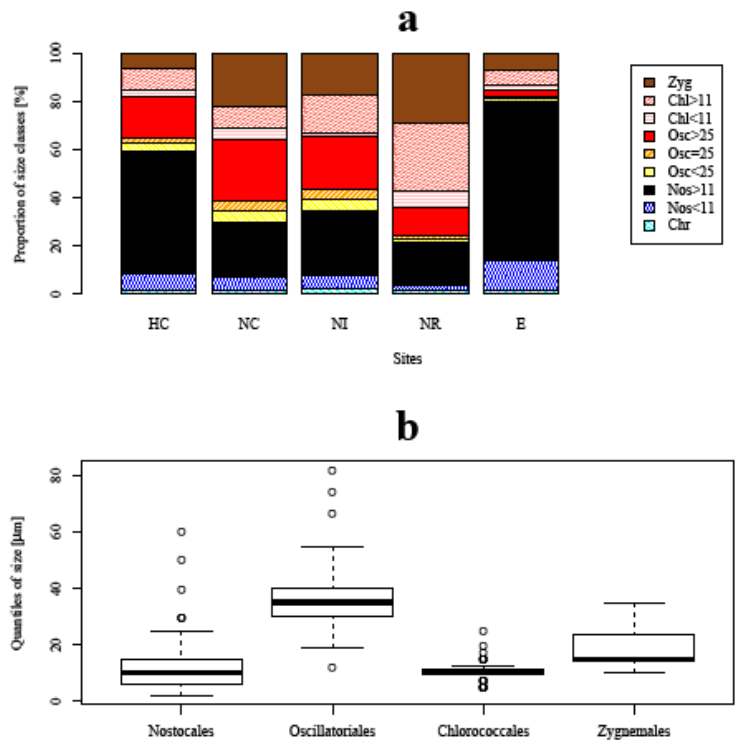
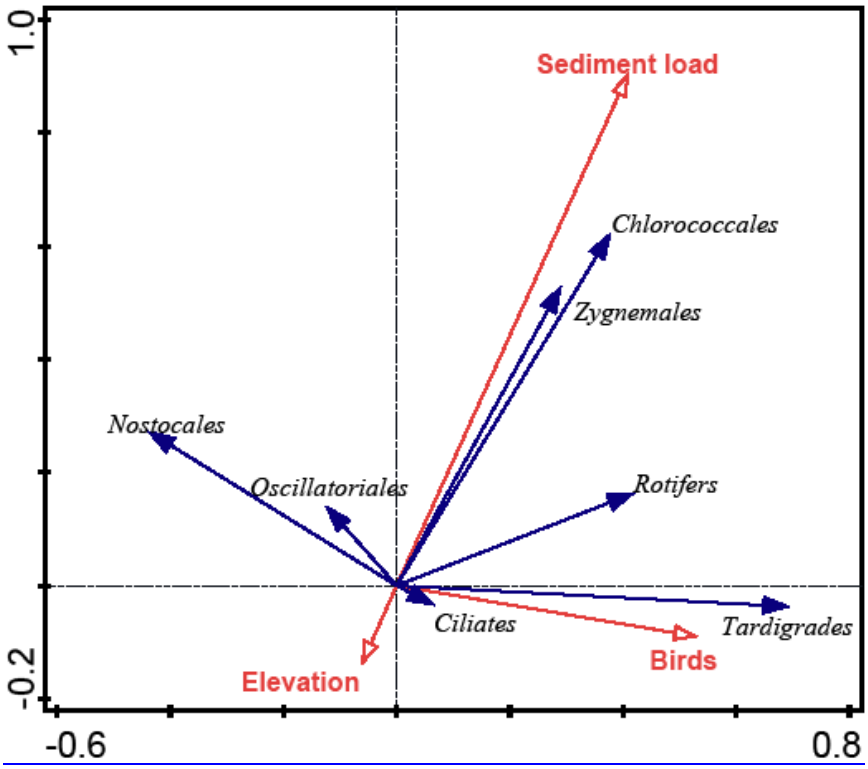



Figure 5Figure 4. (a) median, and quantiles of the biovolume proportion of suggested size classes and (b) the cell number proportion of the median length (Zygnemales), diameter (Chlorococcales), colony size (Nostocales), and mean length (Oscillatoriales) as smaller (<) or bigger (>) than a certain threshold in µm. The abbreviations used in plot a refer to Chroococcales (may include single cell Nostocales)(Chr), Nostocales (Nos), Oscillatoriales (Osc), Chlorococcales (Chl), and Zygnemales (Zyg).




~~Figure 6~~Figure 5. Biplot for the partial RDA with glacier and place as co-variables, after interactive-forward-selection-covariates. ~~Rotifers were separated in bdelloid rotifers (Rotifers) and the monogonont *Encentrum* sp..~~ Rotifers in this figure do not include *Encentrum* sp. due to their low abundances.