**Response to referees letter**

**Referee #1 (Comments to the Author):**

**Title:**

5   You can trim the title simply into: Carbon content and structure of intact Arctic, Antarctic

> We would be pleased if we could keep the original title because it makes a point out of the novel and unique visualization technique. The first part of the title also helps a broader audience to conceive the intention of the study.

10  **Introduction:**

Page 1 Lines 25-26. The sentence is not well written. Please modify.
Page 1 Lines 29. Place a sentence connector. For example: Thus, plant cover is
sparse as well as decomposition rates and biodiversity are generally low. Plant cover
can be also moss and lichens which are sometimes very abundant. Here, you probably
15  want to say vascular plant cover.
Page 1 Lines 29 - Page 2 Line 1. This sentence is too complex. It is better to divide it
into two.

> The section has been redrafted, see page 1 lines 25 to 29.

Page 2 Line 1. I don't think it is correct to write that instead of the vascular plants BSCs
occur in polar and alpine regions. You can see vascular plants there and vice versa
you can see BSCs in other types of environment.
Page 2 Line 2. The word "algae" is too complex because this term includes macro-,
25  microalgae and cyanobacteria. Write something like eukaryotic microalgae.

> The section has been changed as follows at page 2 line 1 to 2:  Conglomerations of soil particles, cyanobacteria, bacteria, green algae, microfungi, lichens and bryophytes create a skin known as biological soil crusts (BSC) that dominate these ecosystems (Belnap et al., 2001; Williams et al., 2017).

Page 2 Line 3. You wrote that "cyanobacteria especially are important players within
these intimate associations." However, the explanation why are they so important to
compare to other organisms is very poor. For example, if you write about EPS, you
can add that production of EPS promotes the stabilization of the soil surface, moisture

retention, and protection against erosion.

Additional explanations with references were added such as soil stabilization by EPS (Belnap J (2003) Biological soil crusts and wind erosion. In: Belnap J, Lange OL (eds) Biological soil crusts: structure, function, and management. Springer, Berlin Heidelberg New York, pp 339–347), and nitrogen fixation by heterocytes (Fay, P., Stewart, W. D. P., Walsby, A. E., & Fogg, G. E. (1968). Is the heterocyst the site of nitrogen fixation in blue-green algae?. *Nature*, *220*(5169), 810-812.), please see page 2, line 3 to 5.

Page 2 Line 13. Cryoturbation is a process, but not an environment. You can write:
e.g., cryoturbated soils.

Changed to cryoturbated soils (page 2 line 13).

Page 2 Lines 8-9. It cannot be similar for green algae, because they have different functions to compare with cyanobacteria. Either write what is similar exactly or delete it.
Removed

Page 2 Lines 15-17. What's about other cyanobacteria contributing to C- and N- cycling? Nostoc, for example.

Response to reviewer: *Chroococcidiopsis* and *Scytonema* are named as well-known examples. For sure there are more taxa that contribute to C- and N- cycles.

*Scytonema* was replaced by *Nostoc*, to use a more common example on page 2 line 16.

Page 2 Lines 15-18. Provide references for 2d and 3d.

Response to referee: Reference for the whole paragraph is given in line 9 (Weber et al., 2016).

The following reference was added: Potts, Malcolm. "Desiccation tolerance: a simple process?." *Trends in microbiology* 9.11 (2001): 553-559.

Page 2 Lines 20-21. Modify the sentence into: Therefore, a large proportion of important ecosystem services, such as erodibility (Belnap and Gillette, 1998; Bowker et al., 2008), soil formation (Rillig and Mummey 2006), soil moisture (Belnap, 2006) and C and N- cycling (Shively et al., 2001; Tiedje 1988; Kowalchuk and Stephen 2001), are influenced by cyanobacterial communities.

The section has been redrafted as suggested at page 2 lines 20 to 21.

Page 2 Line 24. Whose biomass?

Response to reviewer: Cyanobacteria provide an initial structural integrity and accumulate biomass by growth.

Page 3 Line 1. I don't understand this sentence. Please clarify which carbon you mean here.

Sentences were redrafted at page 3 line 1: Soil is the largest pool for carbon in general. Three times the amount of carbon that is accumulated in above ground biomass, as well as double the amount of carbon fluctuating in the atmosphere as $CO_2$ is stored in soil (Schlesinger and Andrews 2000).

**Methods:**

Page 3 Line 27. and from 2 to 4 ∘C in July. Can you clarify in brackets what are frost days and what are ice days?

Explanations are added in brackets, page 3 line 27: Frost day: Minimum day temperature below 0°C. Ice day: Maximum temperature below 0°C.

Page 3 Line 33. with a polar tundra climate in both?

Response and to reviewer: Yes. Geopol and Ny-Alesund are only 8 km apart from each other. The term tundra usually refers to the continuation of permafrost.

Page 4 Line 2. "of" instead of "off"
Page 4 Lines 7-9. Either divide the sentence into several sentences or place the sentence connector.

All corrections inserted

Page 4 Line 19. Indicate the type of samples you collected: soil, soil crust or vegetated

soil. How many samples did you collect.

Page 4 Lines 19-26. How did you choose the samples? Randomly?

Page 4 Line 27. It is not clear which part of BSC you chose. Surface or part below surface?

Additional information was inserted in this section of the manuscript: 20 samples were randomly selected from areas where BSC dominated, a 9 cm petri dish was pressed 1 cm into the BSC surface and excess soil was removed with the petri dish lid. However, due to the heterogeneous nature of BSCs the thickness of the BSC itself varied from 1-2 mm (in Geopol) to up to 1 cm (in Hochtor).

Page 5 Line 2. What type of chlorophyll?

Added at page 5 line 2: chlorophyll-a

Page 5 Line 8. You should explain what is 530/30?

Response to referee: This is a common expression that is used to explain the width of the bandpass filter. This filter is set to 530 nm with a width of 30 nm, meaning that it ranges from 515 nm to 545 nm.

Page 5 Lines 10-11. How many centimetres?

Response and to reviewer: The sample collection part (page 4, line 22) was redrafted, all samples were taken with a Petri-dish with a thickness of 1 cm. All samples had the equal thickness of 1 cm but with varying relations of BSC to soil.

Page 5 Lines 13-15. Either divide the sentence or place the sentence connector.

Sentence was divided

Page 5 Line 18. How the solution was prepared?

Paragraph was added at page 5 lines 18-20 as follows: Macroscopic cyanobacterial thalli and green algae mats were picked from the surface of BSC samples from Hochtor and transferred to a drop of water at an objective slide.

Page 5 Lines 22-23. Modify the sentence, it is hard to understand in the current state.

Page 5 Line 26. although they are BSC organisms.

Page 6 Line 1. and available online.

Page 6 Lines 1-3. Use the past tense as you started to use in Method section.

Page 6 Lines 6-7. Correct the sentence. It is not well written.

Page 6 Line 6. You have already used the word voxel in previous sentences

Page 6, Line 2). You should put explanation of abbreviation at first time you use this word.

Page 6 Line 7. Take care of the tenses you use throughout the text. If you use past then use it everywhere in the methods.

Tenses have been changed, sentences redrafted and all corrections were inserted. Please see page 6 lines 1 to 7.

The explanation of voxel (value on a regular grid in three-dimensional space) was added in brackets at page 6 line 2 and was removed from page 6 line 6.

Page 6 Line 11. How do you visually estimate it? Sometimes you cannot see small microalgal or cyanobacterial cells by eyes.

Page 6 Lines 11-13. Again, the sentence is not well written. Modify it.

The paragraph has been changed as follows:

The EPS and dead cells created a dense matrix together with the soil. This texture changed where the BSC structure ended and the pure soil started. This point could be estimated by the scale bar and is therefore indicated as the end of the graphs.

Page 6 Line 23. Normally distributed data or data with normal distribution.

**Results**

Check the tenses you use here. It should be either past or present tense.

Page 6 Lines 28-29. Check punctuation throughout the manuscript including these two lines.

Punctuation and grammar was carefully checked and corrected throughout the manuscript.

Page 6 Line 30. Since you write about EPS here, maybe it would be useful to indicate

it (for example with arrow) in the Figure 1.

Arrow was added to indicate EPS and the reflectance as suggested.

Page 7 Lines 1-2. Divide the sentence into two.

The section has been redrafted

Page 7 Line 3. You write that Nostoc is found on top. I see on the figure 3 that white
10    triangles are in the upper layer but not on the top. Also from the figure it is very hard
to understand where exactly the surface of BSC is. Maybe you can show that. Also on
the figure 3 you indicate Nostoc. However I don't understand the way you decided that
it is these cyanobacteria. Especially Fig 3b.

15        Response and to reviewer: Figure 3 shows cross sections of the BSC. The beginning of the fluorescence
signals indicates the BSC surface. The dark black background above this is the agarose matrix. Soil
surfaces are rough and therefore the surface is not a smooth line. If you zoom into the images you can
see morphological features such as rounded heterocytes within the characteristic arrangements of
*Nostoc*-like trichomes (especially in Fig. 3a). Due to further light microscopy based investigations (paper in
20        preparation) we know that also the structure indicated in figure 3b is *Nostoc gelatinosus*.

The following sentence was added to the figure caption of figure 3: The beginning of the fluorescence
signals indicates the BSC surface. The dark black background above this is the agarose matrix.

25        The following sentence was changed in section 3.1, line 2: Macroscopic thallus forming *Nostoc* species
were found on top and within the BSC and their identity was checked by light microscopy.

A scale was added to figure 3 to clarify where the surface is as well as bars that indicate PIL and PAL
proportions for each panel. Additionally, a zoom was added to figure 3 as figure 3e where the *Nostoc*
30        thalli, bryophytes, filamentous cyanobacteria and green algae are highlighted.

Page 7 Lines 7-8. Delete this sentence. You have already mentioned it.

Removed

35    Page 7 Lines 11-12. Correct the sentence. For example: The highest values range
between 25 and 40 mg C cm-3 in all soil crust samples.
Page 7 Lines 18-19. Divide the sentence into two sentences.

Page 7 Line 20. The sentence is too complex and it is hard to read.

The section has been redrafted, please see page 7 line 11 to 20.

Page 7 Line 22. Here you talk about bryophytes but I don't see bryophytes in the Fig. 6 where you show who is responsible for total organic C.

Response to referee: Total organic carbon was obtained by loss on ignition. During that process BSC samples are burned at 500 °C. All biomass gets lost without discrimination between heterotrophic bacteria, plant litter, lichens, cyanobacteria, bryophytes etc. Therefore bryophytes are included in total organic carbon. The CLSM technique allows only a discrimination between cyanobacteria and green algae based on phycobillins. Chlorophyll a is shared by green algae, cyanobacteria and bryophytes.

Page 7 Lines 23-24. I don't understand the sentence. Cyanobacteria occupy 7-23% of BSC in general? Or BSC organisms?

Response to reviewer: With this technique, the total cross section area (from the top of the BSC up to 1 cm depth) was estimated that is occupied by green algae or cyanobacteria within the BSC. Across all four sites only cyanobacteria occupied between 7 and 23 percent of the total area.

Sentence at page 7 lines 23 to 24 was changed as follows: Cyanobacteria with their EPS and thallus structure occupy between 7 (Livingston) and 23 % (Ny-Ålesund) of the total area of BSC that was visualized in the 2D biomaps. In comparison, green algae contribute with 0.5 to 2 % as a minor group (Fig. 6).

Page 7 Line 25. accounted instead of differ

Replaced

**Discussion:**

The discussion can be trimmed. Some information is not so important for this manuscript. For example, the paragraph is too long and could be trimmed. I also feel that discussion is mostly focused on the results obtained from Hochtor. Maybe you should also discuss a bit more arctic and Antarctic

BSCs.

Response to reviewer: The discussion section is shortened. This paper will serve as an introduction to future work in these ecosystems and therefore the discussion includes ideas for further investigation WHERE Hochtor plays a major role.

Page 7 Line 27. You applied CLSM, to measure what?! Add in this sentence.
Page 7 Line 30. Which is instead of this is.
Page 8 Line 1. You have already mentioned in the beginning of the discussion that you
used CLSM for the first time. No need to repeat it again.
Page 8 Lines 9-13. This sentence is too long. It is hard to read.

Corrections are inserted and sentences have been redrafted at page 7 line 27 to 30 and page 8 line 1 and 9 to 13.

Page 8 Lines 19-20. Was it detected somewhere else or only in Hochtor? This sentence
is confusing. Was it found in your study? Or it was found only in Budel et al.,
2014 and Peer et al., 2013?

Response to reviewer: Eukaryotic green algae are rarely dominant in BSC and none were found exclusively in BSC. We could detect only a minor proportion of eukaryotic green algae within BSC. This is confirmed by the named references which stated the same for Hochtor.

Page 8 Lines 22-24. Write something like this: Interestingly, Büdel et al., (2014)
showed by Illumina sequencing that within the microbiome of BSC from Hochtor, cyanobacteria contributed
only 1.6 % to the total bacterial diversity, whereas we show that cyanobacteria occupy
20 % of space within the crust.

Correction was included as suggested at page 8 line 22-24.

Page 8 Line 24. Can you write a sentence with a small conclusion coming out of this
finding.

Sentence was added at page 8 line 24: This shows that the role of cyanobacteria within microbiome studies that are based on DNA proportions might be underestimated.

Page 8 Lines 26-27. Which literature exactly? Put references.

References added at page 8 line 26 to 27: Belnap, J., Lange, O. L.: Structure and functioning of biological soil crusts: a synthesis. In: Biological soil crusts: structure, function, and management. Springer Berlin Heidelberg, Germany, 471-479, 2001

Page 8 Lines 27-28. You write that light regime could be a responsible factor for the differences in the crusts thickness. However, in these sentences you point that light conditions are similar for all studied crusts.

Response to referee: Light regimes are comparable across the four sites but at Hochtor situations are fluctuating strongly.

Light regime parameters were added to the description of the sampling sites and highlighted again at page 8 line 28:

Hochtor: 600-1500 PAR ($\mu$mol m$^{-2}$ s$^{-1}$) with strong fluctuations (Büdel, Burkhard, et al. "Improved appreciation of the functioning and importance of biological soil crusts in Europe: the Soil Crust International Project (SCIN)." *Biodiversity and conservation* 23.7 (2014): 1639-1658.); (Colesie, Claudia, et al. "Summer activity patterns of Antarctic and high alpine lichen-dominated biological soil crusts—Similar but different?." *Arctic, Antarctic, and Alpine Research* 48.3 (2016): 449-460.)

Antarctica: 1200 PAR ($\mu$mol m$^{-2}$ s$^{-1}$) (Xiong, Fusheng S., and Thomas A. Day. "Effect of solar ultraviolet-B radiation during springtime ozone depletion on photosynthesis and biomass production of Antarctic vascular plants." *Plant Physiology* 125.2 (2001): 738-751.)

Svalbard: 1200 PAR ($\mu$mol m$^{-2}$ s$^{-1}$) (Barták, Milos, Peter Váczi, and Josef Hájek. "Photosynthetic activity in three vascular species of Spitsbergen vegetation during summer season in response to microclimate." *Polish Polar Research* 33.4 (2012): 443.)

Page 8 Lines 29-30. Reference?

The sentence following sentence was added at page 8 line 30: This is a suggestion which ongoing investigations will attempt to clarify.

Page 8 Line 34. Therefore

Page 9 Line 13. cyanolichen, chlorolichen and bryophyte crusts

Page 9 Line 16. Better not to use a colon. Rather start a new sentence.

All corrections were included

Page 9 Lines 21-24. Reference?

Reference added: (CANNONE, N.; GUGLIELMIN, M. Relationships between periglacial features and vegetation development in Victoria Land, continental Antarctica. *Antarctic Science*, 2010, 22. Jg., Nr. 6, S. 703-713).

Figure 2. I don't see the point to show Fig 2a and 2c. It is clear from the text that the green algae don't have phycobillins.

We agree with this suggestion and combined figure 1a-d with figure 2b and 2d. The new figure 1 contains now figure 1a-d (*Nostoc*) with arrows that indicate the EPS, 1e showing the red panel of the green algae and 1f showing the light microscopy image of the green algae. Wavelengths of the applied lasers for each panel have been added to the caption of figure 1 and appropriate paragraphs in the text have been fitted.

Figure 5. Can you present Depth scale in mm. It would be easier for the reader. The legend of the figure is too long. I see this text already in the results.
Figure 6. The same here. The legend is too long and should be trimmed. Inorganic carbon instead of anorganic.

Corrections were included and the scale was changed from μm to mm in figure 5.

**Response to referee letter**

**Referee #2 (Comments to the Author):**

**Statement:**
The present manuscript has the value of providing information regarding biological soil crust communities from poor studied locations. At the same time, the manuscript introduces new methodologies that can be used to further understand biocrust structure and organization. Although I like the approach the authors used, I believe that

a further effort in identifying cyanobacterial and microalgae species from the studied samples (by light microscopy or molecular survey) would have provided more insights and would have been helpful in supporting some of the points the authors make in the discussion and conclusion.

5    Overall, there is a need to improve redaction, grammar and the flow of the manuscript (mostly in the discussion). This can be achieved by removing no relevant information (discussion), splitting and shortening sentences, and using connectors and punctuation (overall).

10        Response to reviewer: We are pleased to inform you, that a second study regarding cyanobacterial diversity of the same habitats is already in process.

**Abstract:**

Line 12: main primary producers instead of main producers

15        The section has been redrafted as suggested at page 1 lines 12.

**Introduction:**

Page1 Line 2: Change by cyanobacteria, bacteria, microalgae.
Page 2 Line 3: missing a connector between (BCS), dominate these ecosystems.

        The section has been changed as follows at page 2 line 1 to 2:  Conglomerations of soil particles, cyanobacteria, bacteria, green algae, microfungi, lichens and bryophytes create a skin known as biological soil crusts (BSC) that dominate these ecosystems (Belnap et al., 2001; Williams et al., 2017).

        Page 2 Line 13: I do not think that Johansen 1993 is the most relevant/precise reference for the two previous sentences, mostly when referring to hot and cold deserts worldwide. It should include other citations as well.

30        The following references were added:
        -Lacap-Bugler, D. C., Lee, K. K., Archer, S., Gillman, L. N., Lau, M. C., Leuzinger, S., ... & de los Rios-Murillo, A. (2017). Global diversity of desert hypolithic cyanobacteria. *Frontiers in microbiology*, *8*.
        -Jungblut, Anne D., and Warwick F. Vincent. "Cyanobacteria in Polar and Alpine Ecosystems." *Psychrophiles: From Biodiversity to Biotechnology*. Springer, Cham, 2017. 181-206.
35        Johansen 1993 was removed.

Page 2 Lines 15-19 Citations are missing.

Reference added: Potts, Malcolm. "Desiccation tolerance: a simple process?." *Trends in microbiology* 9.11 (2001): 553-559.

**Methods:**

5   Page 3 Line 27 Can you clarify, within the given T/ frost and ice days, when biological
activity is expected/have been predicted? Can you provide for all locations an approximation
of current expected biological activity?

Response to referee: We support the idea of adding activity periods and provide appropriate references
10   for each section / habitat as far as possible.

Addition of page 4 line 17: Lichens assigned to BSC from Antarctica are known to show photosynthetic activity patterns after snow melt and during fog but only low rates of photosynthetic productivity and growth rates are expected throughout the year (Colesie et al., 2016).

Addition of page 3 line 30: Cyanobacteria dominated BSCs and lichens of Hochtor are known to be continuously active in terms of photosynthesis throughout the year, activated by fog, dew, rainfall and after snow melt (Colesie et al., 2016; Büdel et al., 2014)

20   Addition of page 4 line 9: At least for lichens of Spitsbergen it is known that they seem solely active during ice and snow free times where they are activated by rainfall and snow melt with low contributions to carbon fixation throughout the year. (Uchida, Masaki, et al. "Estimation of the annual primary production of the lichen Cetrariella delisei in a glacier foreland in the High Arctic, Ny-Ålesund, Svalbard." *Polar Research* 25.1 (2006): 39-49)

Page 4 Lines 19-15 Please provide number of samples collected/analyzed per location.
Were samples collected randomly or within a given transect?

Additional information was inserted in this section of the manuscript: 20 samples were randomly selected
30   from areas where BSC dominated, a 9 cm petri dish was pressed 1 cm into the BSC surface and excess soil was removed with the petri dish lid. However, due to the heterogeneous nature of BSCs the thickness of the BSC itself varied from 1-2 mm (in Geopol) to up to 1 cm (in Hochtor).

35   Page 5 Line 2 What type of Chlorophyll was targeted?

Added at page 5 line 2: chlorophyll-a

Page 5 Line 5-6 You are saying that chlorophyll a from green algae was excited using a 555nm beam and that chlorophyll a from cyanobacteria was excited by using a 639nm beam. It is not clear to me, how an excitation at 55nm will have no effect on chlorophyll a from cyanobacteria and vice versa.

Response to referee: For sure, there are other wavelengths that are also suitable but they often excite minerals or particles in the soil which create background noise. For this reason, we tried a variety of possible wavelengths (for chlorophyll a and phycobilli proteins) and found this to be the most adequate for the different soil types of all four locations. A high proportion of quartz in the soil for example, can

10      make it difficult to apply CLSM because quartz interferes with the excitation wavelengths. To demonstrate that the chosen wavelengths are sufficient to discriminate chlorophyll a and the phycobilli proteins (cyanobacteria versus green algae) we included figure 1 and especially figure 2.

Page 5 Line 18 Explain what do you mean by cyanobacteria and green algae were

15   isolated. Also, from which solution?

Paragraph was added at page 5 lines 18-20 as follows: Macroscopic cyanobacterial thalli and green algae mats were picked from the surface of BSC samples from Hochtor and transferred to a drop of water at an objective slide.

Page 5 Lines 24-25 Sentence difficult to follow

Sentence at page 5 line 24 to 25 was redrafted as follows: Thallus structures and excreted EPS by cyanobacteria and green algae were also taken into consideration. This was possible because at least the

25      periphery of the EPS reflects the fluorescence signal that is coming from the cells.

Page 5 Line 25-28 Does it mean that cyanobacteria associated to lichens were also neglected? How did you discriminate chlorophyll a fluorescence from mosses?

30      Response to referee: For this measurements lichens and their photobionts as well as bryophytes were neglected. It was not discriminated between fluorescence of chlorophyll a from bryophytes and chlorophyll a from green algae / cyanobacteria. The applied technique does also visualize bryophytes. Their rectangular cells form a pattern that is visible and makes it possible to identify them.

Page 6 Line 2 move meaning for the abbreviation for voxels from line 6 to line 2
Page 6 Line 11 Use past tense

Tenses have been changed, sentences redrafted and all corrections were inserted. Please see page 6 lines 1 to 11.

The explanation of voxel (value on a regular grid in three-dimensional space) was added in brackets at page 6 line 2 and was removed from page 6 line 6.

Page 6 Line 11-13 Sentence difficult to follow/understand

The paragraph has been changed as follows:
The EPS and dead cells created a dense matrix together with the soil. This texture changed where the BSC structure ended and the pure soil started. This point could be estimated by the scale bar and was therefore indicated as the end of the graphs.

Page 6 Line 23 Normally distributed data.

Correction was included at page 6 line 23.

**Results**

Page 6 Lines 27-30 Define a tense (past or present). Recurrent change in tense in the manuscript.

Punctuation and grammar was carefully checked and corrected throughout the manuscript.

Page 7 Lines 18-21 Improve sentences flow.

Paragraph at page 7 line 18 to 21 was redrafted as follows: The total organic carbon content of soils varies between 7 % and 17 % (Fig. 6), with Hochtor and Geopol being significantly different to Ny-Ålesund ($p \leq 0.05$). Based on CLSM–IA This total organic C can be divided into carbon evaluated from active photosynthetic organisms (apC), and non-photosynthetic carbon (npC). The latter includes dead organic material and remaining EPS.

Page 7 Line 22 How did you measure bryophytes contribution to apC and npC. Can you

please explain how this differentiation was made? I also do not see them (bryophytes) marked in any of your biomaps, neither in figure 6. Being this the case, please use arrows to show them in your biomaps.

Response to referee: To estimate total organic carbon, your sample (including soil, cyanobacteria, lichens, bryophytes, green algae, fungi, heterotrophic bacteria etc.) gets burned in a muffle oven at 500 °C. During that process all biomass gets lost. The difference in weight in percent is the total organic carbon content. This total organic carbon can be split into carbon coming from dead organisms and carbon coming from living organisms. The fraction of carbon coming from active organisms was measured by CLSM, based on fluorescence (apC). This includes cyanobacteria and green algae (as well as lichens and bryophytes <200μm) without any discrimination between the organisms. Subtracting apC from total organic carbon reveals the amount of carbon coming from dead proportions of the BSC (npC).

Page 7 Line 24 Please clarify what you mean by cyanobacteria occupy between 7 and 23 %. Is there any difference among locations?

Response to reviewer: With this technique, the total cross section area (from the top of the BSC up to 1 cm depth) was estimated that is occupied by green algae or cyanobacteria within the BSC. Across all four sites only cyanobacteria occupied between 7 and 23 percent of the total area.

Sentence at page 7 lines 23 to 24 was changed as follows: Cyanobacteria with their EPS and thallus structure occupy between 7 (Livingston) and 23 % (Ny-Ålesund) of the total area of BSC that was visualized in the 2D biomaps. In comparison, green algae contribute with 0.5 to 2 % as a minor group (Fig. 6).

**Discussion:**

Page 7 Line 28 -30 Revise sentences. Either add punctuation/connectors or split into more sentences. Revise this throughout the manuscript.

Page 8 Line 8 named instead of called

Corrections are inserted and sentences have been redrafted at page 7 line 27 to 30 and page 8 line 1 and 9 to 13.

Page 8 Line 9-10 I do not understand this sentence/what you are aiming to communicate

Response to reviewer: The group of Ranaaan (2016) also used a technique to visualize BSC. This study is discussed here because it is recent, only a few studies provide visualization techniques that are applicable

to BSC and they found similar structures. Additionally, it shows the need for such techniques and demonstrates the benefits of our study.

Page 8 Line 18 "hard to detect" by what means? Please explain

Response to reviewer: Cryptic stages of green algae and cyanobacteria are often impossible to detect by light microscopy because they occur in low abundances, have atypical morphologies or can be very small.

Page 8 Line 19-20 What and how is supported by Budel et al., 2014 and Peer et al.,

2013?

Response to reviewer: Eukaryotic green algae are rarely dominant in BSC and none were found exclusively in BSC. We could detect only a minor proportion of eukaryotic green algae within BSC. This is confirmed by the named references which stated the same for Hochtor.

Page 8 Lines 22-24 Which technique was used in Budel et al., 2014, was it the same time of the year? These sentences need a better flow to communicate better the point the authors are trying to make. A take home message from this finding is missing.

Sentence was changed at page 8 lines 22 to 24 as follows: Interestingly, Büdel et al., (2014) showed by Illumina sequencing that within the microbiome of BSC from Hochtor, cyanobacteria contributed only 1.6 % to the total bacterial diversity, whereas we show that cyanobacteria occupy 20 % of space within the crust. This shows that the role of cyanobacteria within microbiome studies that are based on DNA proportions might be underestimated.

Page 8 Line 28 Which literature? Add references and some comparisons

Light regime parameters were added to the description of the sampling sites and highlighted again at page 8 line 28:

Hochtor: 600-1500 PAR ($\mu$mol m$^{-2}$ s$^{-1}$) with strong fluctuations (Büdel, Burkhard, et al. "Improved appreciation of the functioning and importance of biological soil crusts in Europe: the Soil Crust International Project (SCIN)." *Biodiversity and conservation* 23.7 (2014): 1639-1658.); (Colesie, Claudia, et al. "Summer activity patterns of Antarctic and high alpine lichen-dominated biological soil crusts—Similar but different?." *Arctic, Antarctic, and Alpine Research* 48.3 (2016): 449-460.)

Antarctica: 1200 PAR (µmol m$^{-2}$ s$^{-1}$) (Xiong, Fusheng S., and Thomas A. Day. "Effect of solar ultraviolet-B radiation during springtime ozone depletion on photosynthesis and biomass production of Antarctic vascular plants." *Plant Physiology* 125.2 (2001): 738-751.)

5    Svalbard: 1200 PAR (µmol m$^{-2}$ s$^{-1}$) (Barták, Milos, Peter Váczi, and Josef Hájek. "Photosynthetic activity in three vascular species of Spitsbergen vegetation during summer season in response to microclimate." *Polish Polar Research* 33.4 (2012): 443.)

Page 8 Lines 28-30. These sentences are hard to follow, please re-write. How different in thickness were your biocrusts at the studied locations? Do your results agree with
10   your light regime explanation?

The sentences at page 8 line 28 to 30 have been changed as follows: Light regime could be a responsible factor, because all four sites share similar daylight times with PAR exceeding 1200 µmol m$^{-2}$ s$^{-1}$ (Colesie et al. 2016; Xiong et al. 2001; Barták et al., 2012), but with the strongest fluctuations at Hochtor (Büdel et al, 2014). The appearance of photoautotrophic organisms up to these depths may be possible due to a
15   diverse community composition of organisms with different adaptions regarding light regime. This idea supports a previous study that states a continuous year around photosynthetic activity of the cyanobacteria dominated BSC of Hochtor (Büdel et al., 2014).

Page 8 Line34 Page 9 Line 4 I do not see the point of adding the dark and light crust classification.

Sentence at page 8 line 34 and page 9 line 4 have been removed.

Page 8 Line34 Page 9 Line 16 Therefore is missing the last e

Correction has been included at page 8 line 34 and page 9 line 16.

30   Page 9 Lines 20-41 Please provide references

Reference added: (CANNONE, N.; GUGLIELMIN, M. Relationships between periglacial features and vegetation development in Victoria Land, continental Antarctica. *Antarctic Science*, 2010, 22. Jg., Nr. 6, S. 703-713).

35   Page 10 Line 5 demonstrated instead of could demonstrate

Correction has been included at page 9 line 5.

**Figures:**

Figures 1 Add used wavelength for each channel. Add arrows to show fluorescence
5  from EPS. Include either here or as a supplementary a similar panel showing a filamentous
cyanobacteria.
Figure 2 Add used wavelength for each channel. I am not sure you need to show
figures 2 a and c.

10      Used wavelengths were added to the figure caption, as well as arrows indicating the EPS in figure 1. We
agreed to remove figure 2a and 2c and combined figure 1a-d with figure 2b and 2 d to a new figure 1a-f.

Figure 3 Add arrows to indicate filamentous and single coccoidal organisms. Also show
differentiation between cyanobacteria and green algae. Indicate profile depth for each
15  panel. Indicate biocrust position in the profile. In the results, you mentioned that Nostoc
is on top as well as within the biocrust (Page 7 Line 3). I only see what you identified
as Nostoc within but not on top of the biocrust. Clarifying the biocrust position in the
profile may help with this. I also do not clearly understand how you concluded that
what white triangles are showing is Nostoc. Maybe a zoom in will help. Also, Nostoc
20  from figure 3b looks different from Nostoc in figure 3a, especially color wise. Add PAL,
PIL layers to figure 3 and provide measurements (profile depth).

We added a zoom to the figure 1 as 1e. In this new image bryophytes, Nostoc, coccoidal and filamentous
cyanobacteria, as well as green algae are highlighted. We also indicated where PIL and PAL is placed for
25      each panel and added a scale to clarify where the top is.

Response to referee: *Nostoc* was identified by picking it from the BSC surface and light microscopy
investigations. Based on a second study that is in preparation, we know that different *Nostoc* species are
present. The arrow in figure 1b for example shows *Nostoc gelatinosus*.
30
Figure 4. Provide layers measurements (profile depth). Optional since already asked
in figure 3. Although I acknowledge the effort and recognized its beauty, I do not see
the need to include Figure 4 in the main text. It could be supplementary. I leave it to
the authors to decide.
35
Figure 4 represents a schematic illustration, to demonstrate and simplify BSC structures and the PIL-PAL
proportion in general. With this study possibly being a part of a special issue of biogeoscience we want to
introduce BSC related content to a broad audience. Figure 4 makes it possible to understand complex

relationships between different types of cyanobacteria and green algae with soil and their role as ecosystem engineers in extreme habitats. For this reason we would be pleased to keep this figure.

Figure 6. I do not see bryophytes represented in the figure, however, their contribution to the apC and npC was mentioned in the results.

Response to referee: No discrimination between different organism took place during the estimation of the total organic carbon content by loss on ignition, because the whole sample with all biomass is burned. The CLSM technique allows a discrimination only between green algae and cyanobacteria, because chlorophyll a in green algae is the same as in bryophytes. For this reason, bryophytes are included in the total organic carbon content.

# Uncovering biological soil crusts:
# Carbon content and structure of intact Arctic, Antarctic and alpine biological soil crusts

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**Abstract.** Arctic, Antarctic and alpine biological soil crusts (BSCs) are formed by adhesion of soil particles to exopolysaccharides (EPS)~~,~~ excreted by cyanobacterial and green algal communities, the pioneers and main primary producers in these habitats. These BSCs provide and influence many ecosystem services such as soil erodibility, soil formation and nNitrogen- (N) as well as carbon- (C) cycles. In cold environments degradation rates are low and BSCs continuously increase ~~continuously~~ soil organic C, therefore ~~whereby~~ these soils are considered to be~~as~~ $CO_2$ sinks. This work provides a novel, non-destructive and highly comparable method to investigate intact BSCs with a focus on cyanobacteria and green algae and their contribution to soil organic C. A new terminology arose, based on confocal laser scanning microscopy (CLSM) 2D biomaps, dividing BSCs into a photosynthetic active layer (PAL), made of active photoautotrophic organisms and a photosynthetic inactive layer (PIL), harbouring remnants of cyanobacteria and green algae glued together by their remaining EPS. By the application of CLSM image analysis (CLSM–IA) to 3D biomaps, C coming from photosynthetic active organisms could be visualized as depth profiles with C peaks at 0.5 to 2 mm depth. Additionally, the

$CO_2$ sink character of these cold soil habitats dominated by BSCs could be highlighted, demonstrating that the first ~~cubic centimetre~~cm³ of soil consists of~~is made of~~ between 7 and 17 % total organic carbon, identified by loss on ignition.

# 1 Introduction

Antarctica, the Arctic and the Alps are dominated by a range of extreme environmental conditions which impose severe pressure on biological life, particularly for vegetation: They begin where trees no longer dominate the vegetation, usually have temperatures below 10 °C in the warmest month (Körner 1998), and are characterized by snow fall, at least in winter. Permafrost, long periods of darkness, continuous irradiance, short growing seasons, stable snow covers and rocky sites with low nutrient supply represent other common challenges of these ecosystems (Bayard et al., 2005; Forman and Miller 1984; Thomas et al., 2008a). Thus, vascular plant cover is sparse as well as~~,~~ decomposition rates and biodiversity being~~are~~ generally low~~.~~; For example,~~e.g.~~ only the grass *Deschampsia antarctica* ~~Desv.~~ and the Antarctic pearlwort *Colobanthus quitensis* ~~(Kunth) Bartl~~ as autochthonous flowering plants occur in Antarctica ~~which are solely~~ distributed solely in a few suitable areas (Thomas et al., 2008b). ~~Instead, C~~Conglomerations of soil particles, ~~(cyano-)~~bacteria, bacteria, microalgae, microfungi, lichens and bryophytes create a skin known as biological soil crust (BSC), that dominate these ecosystems (Belnap et al., 2001; Williams et al., 2017). Cyanobacteria especially are important players within these intimate associations. They bind~~adhere~~ the subsurface and surface, because their secreted extracellular polysaccharides (EPS) form a mechanical structure, surrounding the bacterial cells that together with the soil particles form a visible organic matrix on top and within the first millimetres of soil (Mazor et al., 1996; Breen and Lévesque 2008; Garcia-Pichel and Belnap~~,~~ 1996; Garcia-Pichel et al., 2003). Additionally, the excretion of EPS stabilizes the soil against erosion (Belnap 2003), and their ability to fix nitrogen (N) ~~with their heterocytes~~ increases the N content of soils (Fay et al., 1968).

These frequently diverse cyanobacterial communities within a BSC can be categorized into three major groups ~~(similar for green algae)~~, based on functional traits (Weber et al., 2016), found in hot and cold deserts worldwide (~~Johansen 1993~~Lacap-Bulger et al., 2017; Jungblut and Warwick 2017; Belnap and Lange 2001): (1) Filamentous cyanobacteria, such as *Microcoleus* or *Leptolyngbya* stabilize soils due to the presence of extracellular matrix made of EPS. Those cyanobacteria cause crust formation and are also the most abundant cyanobacteria species in BSCs (Johansen 1993). Building filaments is an essential feature that enables cyanobacteria to colonize physically unstable environments, e.g.~~,~~ cryoturbated soils~~ion~~, and ~~to~~ act as successful pioneers in the bio-~~-~~stabilization process against erosion (Garcia-Pichel and Wojciechowski 2009). Remaining EPS retains these features over many years after the trichomes have either moved out of their sheath envelopes or died (Potts 2001). (2) Cyanobacteria such as *Chroococcidiopsis* and ~~Scytonema~~ *Nostoc* prefer to live in the BSC environment, enhancing the ecological role of BSCs, e.g., through their contribution to C- and N- cycling. (3) Some cyanobacteria like *Phormidium* or *Chroococcus* are known to occur only stochastically in BSCs and may originate from other habitats, such as an aquatic environment or through lichen symbiosis.

Regarding their extremophile character in areas limited by temperature and/or water availability, cyanobacteria and green algae play key roles as ecosystem engineers if other photoautotrophic clades are absent (Belnap 2003). Therefore, a large proportion of important ecosystem services ~~are influenced by cyanobacterial communities~~, such as erodibility (Belnap and Gillette~~,~~ 1998; Bowker et al., 2008), soil formation (Rillig and Mummey 2006), soil moisture (Belnap~~,~~ 2006), C- and N-

5  cycling (Shively et al., 2001; Tiedje 1988; Kowalchuk and Stephen 2001), are influenced by cyanobacterial communities. Providing an initial structural foundation, they physically modify, maintain, or create habitats for other organisms and accumulate biomass. Hence, they may form the nutritional basis for higher trophic levels, such as reindeers in the Arctic which feed on lichens that are a part of the BSC communities (Cooper and Wookey 2001, Elster et al., 1999).

Currently, these high altitude and latitude ecosystems are experiencing effects of human induced environmental changes that

10  are expected by many prospective predictions to be both large~~r~~ in magnitude and have great impacts (Bálint et al., 2011). BSCs have recently been shown to be vulnerable to the potential impact of climate change as well as to shifting meteorological conditions since their activity and structure is strongly affected (Escolar et al., 2012; Kuske et al., 2012; Wertin et al., 2012; Lane et al., 2013; Maestre et al., 2013). Therefore, it is likely that an invasion of foreign species will alter the BSC composition during warming events in the Arctic and the Antarctic Peninusla (Pushkareva et al., 2016). In

15  terms of climate change their microbiota is a focal point of scientific interest because in contrast to their local habitat function they also provide atmospheric overall services by fixing and storing substantial amounts of C (Evans and Lange 2003), whereby they can increase the total surface soil C by up to 300 % (reviewed in Belnap et al., 2003). Soil ~~in general~~ is the largest pool of C in general: ~~in the biosphere, storing T~~three times the amount of C that is accumulated in above ground biomass as well as ~~and two times~~double the amount of carbon fluctuating in the atmosphere as $CO_2$ is stored in soil

20  (Schlesinger and Andrews 2000). BSCs are considered a major source of soil organic C in for example, semiarid ecosystems (Evans and Lange 2003; Housman et al., 2006), accumulating C as carbohydrates in EPS and as energy reserves for cells through their photosynthetic mechanisms (Bertocchi et al., 1990).

Most parts of the earth are now 10,000 to 18,000 years removed from the last major glacial episode (Schlesinger 1990), and lands once covered by continental glaciers are now accumulating soil organic C at a rate possibly between 0.075 and 0.18 Gt

25  of C year$^{-1}$ (Harden et al., 1992). Hence, these soils may be sinks for $CO_2$ within the atmospheric $CO_2$ balance (Amundson 2001). The large carbon pools of the biosphere can lead to accelerated emissions of greenhouse gases into the atmosphere, if destabilized through changes in climate and land use (Gruber et al., 2004). Tak~~en~~ing into consideration that BSCs are valuable ecological indicators for abiotic factors, ecological health and climate change (Belnap et al., 2001, Pushkareva et al., 2016), these communities will certainly help improve ~~the~~ prediction~~s~~ regarding~~of~~ future climate change (Pushkareva et

30  al., 2016). Thus, it is necessary to improve our knowledge regarding complex BSC interactions. Fluorescence microscopy has been applied repeatedly to cryptogamic organisms since photoautotrophic organisms naturally emit ~~naturally~~ auto fluorescence (Schallenberg et al., 1989; Kuwae and Hosokawa 1999; Solé et al., 2009; Raanan et al., 2016). The confocal laser scanning microscopy (CLSM) technique applied in this work gives novel insights into the complex architecture of BSCs, avoiding the need to either manipulate or stain the samples. Moreover, it allows accurate and non-destructive optical

investigations of BSC cross sections that generate high resolution images where out-of-focus is eliminated. As a highly comparable approach, it is possible for the first time to calculate C values and partitioning patterns of green algae and cyanobacteria within intact BSCs by applying the CLSM image analysis (CLSM–IA) procedure developed by the group of Solé et al., (2009). Additionally, these results were set in relation to organic C values obtained by loss on ignition to highlight the $CO_2$ sink character of the first cubic centimetre$cm^3$ of these cold soils dominated by BSCs.

## 2 Material and methods

### 2.1 Study sites

Hochtor, near the Großglockner High Alpine Road, Hohe Tauern National Park in Austria represents the aAlpine site. The site is placed in the high mountains of Hohe Tauern (Austria), close to the Grossglockner High Alpine Road at 47°50' N and 12°51' E. The elevation ranges from 2,500 to 2,600 m a.s.l. The climate is aAlpine with a mean air temperature ranging from -10 to -8 °C in January and 2 to 4 °C in July. On average, there are 250 frost days (minimum day temperature below 0 °C), 150–200 ice days (maximum day temperature below 0 °C) and 80 to 90 frost alternation days each year. Light conditions show strong fluctuations in photosynthetic active radiation (PAR), ranging from 600 to 1500 µmol m$^{-2}$ s$^{-1}$ (Büdel et al., 2014). Mean annual precipitation is between 1,750 and 2,000 mm, with more than 70 % falling as snow. Snow cover lasts for 270–300 days. Under these climatic conditions development of soil and the subsequent establishment of higher plants is extremely slow but high coverage of BSCs are recorded (Büdel et al., 2014). Cyanobacteria dominated BSCs and lichens of Hochtor are known show photosynthetic activity during 86 % of time during the snow free growing season from August to October (Raggio et al. 2017). They are expected to be activated by fog, dew, rainfall and after snow melt (Colesie et al., 2016; Büdel et al., 2014).

The Arctic region is represented by two localities in Spitsbergen, Svalbard: Ny-Ålesund (78°55'26.33''N, 11°55'23.84''E) and Geopol (78°56'58.38''N, 11°28'35.64''E), with a polar tundra climate (Peel et al., 2007, Vogel et al., 2012). Ny-Ålesund is an international research platform on the Brøgger peninsula at the coast of Kongsfjorden. Geopol lies roughly 8 km north-west off Ny-Ålesund and is a rocky site, dominated by skeletal soils and permafrost polygons. The temperature is low year-round with an annual average of -4.5 °C, the highest and lowest monthly temperatures range between 5.8 °C (July) and -12 °C (March), respectively (Maturilli et al., 2013). However, longer cold periods (-20 to -35 °C) are possible. High light conditions around 1200 µmol m$^{-2}$ s$^{-1}$ PAR are reached throughout the year (Barták et al., 2012). The annual precipitation averages 471 mm at both habitats with 70 % falling between October and May, when snow cover is usually complete (based on data from the Norwegian Meteorological Institute). The surface soil of the A-horizon (0–5 cm) has a predominantly sandy texture. Svalbard is covered by less than 10 % of vegetation, current information includes ca. 170 vascular plants, ca. 350 bryophytes (Bengtsson 1999), and ca. 600 lichen species (Elvebakk and Hertel 1997), including intact BSC communities. The latter , occupying more than 90 % of the soil surface in many areas (Williams et al., 2017). Regarding BSC activity in Spitsbergen the only available information is in respect to lichens, which have been shown to be

solely active during ice and snow free times, the whenre they are activated by rainfall and snow melt and therefore have with low contributions to carbon fixation throughout the year (Uchida et al., 2006).

The Antarctic habitat lies around the Juan Carlos I base (62°39'46.00''S, 60 °23'20.00''W), which is located in the South Bay of Livingston Island, Antarctica. Livingston Island belongs to the South Shetland archipelago in the Southern Ocean which is situated near the Antarctic Peninsula. It ranges from 61° to 63° south latitude and from 54° to 63° west longitude. Mean annual temperatures are -2.8 °C with summer mean temperatures above freezing, and maximum mean temperature is 4.3 °C (Bañón et al., 2013). High light conditions around 1200 µmol $m^{-2}$ $s^{-1}$ PAR are reached throughout the year (Xiong and Day 2001). Mean annual precipitation is 444.5 mm, with 75 % falling in summer and autumn (Bañón et al., 2013). The surface soil of the A–horizon (0–5 cm) of the test sites was characterized as sandy loam as the dominating texture class. Numbers of lichen (110) and bryophyte (50) species were reported from the vicinity of Juan Carlos I base in Livingston by Sancho et al., (1999), as well as large proportions (43 %) of BSC coverage (Williams et al., 2017). Lichens assigned to BSC from Antarctica are known to show photosynthetic activity patterns after snow melt and during fog but only low rates of photosynthetic productivity and growth rates are expected throughout the year (Colesie et al., 2016).

## 2.2 Sample collection

Samples from Hochtor (Austria, alpine) were taken during the Soil Crust International Project (SCIN) in July, 2012 (Büdel et al., 2014). Samples from Livingston Island (Antarctica) were collected during February 2015 and samples from Svalbard, Spitzbergen (Geopol and Ny-Ålesund, Arctic) in August 2014 (Williams et al., 2017). 20 samples were randomly selected from areas where BSC dominated (including bryophytes, lichens, cyanobacteria, green algae), where a 9 cm Petri dish was pressed 1 cm into the BSC surface and excess soil was removed with the Petri dish lid. However, due to the heterogeneous nature of BSCs the thickness of the BSC itself varied from 1-2 mm (Geopol) up to 1 cm (Hochtor).
Samples were taken by pressing a sterile 94 mm diameter Petri dish into the crust to remove the top 1 cm of the photic zone from the surrounding BSC. Excess soil was removed with the Petri dish lid from the samples, whichSamples were left to air dry in the field immediately after collection for 2–3 days, until no condensation formation occurred any more. The dry and sealed crust samples were preserved at -20 °C until further processing. For this study the samples were slowly defrosted under air-tight conditions and used for investigations.

## 2.3 Sample preparation

Representative, intact, 1 cm thick parts of the BSC with soil substrate were completely embedded in a 0.9 % agarose pre-chilled solution in glass Petri-dishes. Therefore, 12 hours of cooling and hardening at room temperature in daylight was sufficient, and water available from the agarose matrix, reactivated the BSC organisms. The block was cut with a razor blade to obtain cross sections of the intact, activated and fixed BSC.

**2.4 CLSM and biomaps**

Cross sections were examined with a CLSM (LSM 700, Carl Zeiss) equipped with diode lasers. Photomultiplier parameters were adjusted to achieve the maximum signal from the chlorophyll-a and phycobiliproteins of present and active photoautotrophic organisms, while simultaneously keeping the noise signals generated by the soil particles and the agarose

5 matrix to a minimum. Auto-fluorescence of green algae (chlorophyll--a) and cyanobacteria (chlorophyll--a and phycobiliproteins) were excited by beams of 555 nm and 639 nm wavelengths, respectively. Using two laser beams made it possible to differentiate between green algae and cyanobacteria whereby superimposed images were generated by the outputs of two channels. Emitted wavelengths were collected using a band-pass filter 530/30 and cyanobacterial natural fluorescence with a 590 nm long pass filter. Z-stacks were scanned through the BSCs with a 10x objective (n.a. 0.7 Achroplan, colour

10 depth 8 bit). Stack series of the cross sections were taken along the depth of the sample, from the surface of the crust to where the pure soil without fluorescence signals started and the BSC structure ended. Each image frame per depth was chosen to contain a small overlap section to align the single images, showing the structure of the BSC. Each stack contained between 40 and 140 single images (512x512 pixel resolution), with three replicates per site and depth (6012 single images). Complete stacks were used to calculate C contents (3D biomap), their maximum projection was converted into 2D pictures

15 to present the BSC structure and to obtain area patterns of green algae and cyanobacteria (2D biomap) by the procedure of CLSM–IA with the software *ImageJ 1.47v*.

**2.5 CLSM micrographs**

Macroscopic cyanobacterial thalli and green algae mats were picked from the surface of BSC samples from Hochtor under athe binocular stereoscope and transferred to a drop of water onat an objective slide.

20 Cyanobacteria and green algae were isolated from an aqueous solution of BSC material under the binocular stereoscope. The CLSM micrographs were obtained as indicated above except a 63x objective was used and immersion oil as well as light microscopy was utilized in the same way.

**2.6 CLSM–IA: Partitioning estimation**

Maximum projections of the 2D biomaps were used to calculate the total area of the soil crust in mm² and to differentiate

25 between percentages occupied by algae or cyanobacteria based on their specific auto-fluorescence traits with *ImageJ 1.47v*. Thallus structures and excreted EPS surrounding by cyanobacteria and green algae were also taken into consideration. This was possible because at least the outer periphery of the mucilage EPS reflecteds the fluorescence signal that was coming from the cells.light, coming from fluorescent pigments of the cell itself. Bryophytes and lichens were neglected although they belong within BSC organisms, because they grow up right out of the soil in different developmental stages, shifting the

30 relative thickness of the BSC.

**2.7 CLSM–IA: Carbon estimation**

Image stacks of the 3D biomaps were imported in their original image format (8 bit 512x512 tiff image sequences) to *ImageJ 1.47v* and transformed into black and white binary images. Subsequently, a plugin called *Voxel Counter*, created by Wayne Rasband and~~which~~ is available online (http://rsb.info.nih.gov/ij/), was applied to the *ImageJ 1.47v* software. This plugin calculate~~d~~s the ratio of threshold voxels (the abbreviation for "volume element"), as cyanobacterial/green algae volumes to all voxels, which represent~~ed~~ the total volume of sediment from each binary image in every single stack. The obtained biovolume percentage was multiplied by a conversion factor of 310 fg C $\mu m^{-3}$ to convert it to biomass (Fry, 1990; Bloem et al., 1995), which has also been used previously for cyanobacteria (Solé et al., 2001; 2003; 2007; 2009). Biomass results were therefore expressed in carbon units (mg C $cm^{-3}$) of sediment. CLSM–IA is very precise, given that the minimum biomass that it can detect corresponds to a voxel ~~(the abbreviation for "volume element")~~, equivalent to $1.183 \times 10^{-3}$ mg C $cm^{-3}$ of sediment (Sole et al., 2008). The obtained carbon value was~~is~~ based on the auto–fluorescence signal only of the living and active green algae and cyanobacteria (bryophytes and lichens <200 µm on top of the crusts were included) and was~~is~~ therefore called active photosynthetic carbon (apC). Nevertheless, their EPS which contain~~ed~~s massive amounts of carbon was~~is~~ only considered as a minor proportion due to the missing fluorescence signal, therefore apC should be higher. The EPS and dead cells created a dense matrix together with the soil. This texture changed where the BSC structure ended and the pure soil started. This point could be estimated by the scale bar and was therefore indicated as the end of the graphs. ~~Graphs are ended where the BSC visually ends to indicate the thickness of each crust. Beyond the value at the deepest point pure soil starts, which does not contain photosynthetic active organisms, this is not shown to improve the visualization of the BSC structure.~~

**2.8 Total organic carbon**

Total organic C content was determined by pre-heating four samples per site for 12 h at 105 °C to obtain dry weight and volume values before manually grinding and mass loss after ignition for 2 h at 550 °C (Black, 1965). Remaining material contained only inorganic C. ~~and N~~non-photosynthetic C (npC) values were calculated by subtracting active photosynthetic C (apC) from total organic C. The latter therefore contain~~ed~~s dead cells, EPS and heterotrophic macro- and microfauna of the BSC, that ga~~i~~ve no fluorescence signals. All values ~~are~~ were expressed in percentage and~~,~~ applied to the volume of 1 cm³ dry matter.

**2.9 Statistical analysis**

Statistics for loss on ignition data were completed by using the software *Statistica* (Version 9.1; StatSoft Inc. 2010). Those data were tested for normal distribution with a Shapiro-Wilk-Test. One-way ANOVA with a following Tukey posthoc test was used to look for differences between groups for normally distributed data.

# 3 Results

## 3.1 Biomap structure and graphic scheme

Cyanobacteria ~~were found to~~ dominate~~d in their abundance~~ over green algae across all four sites, which was detected based on the different fluorescent features of the photoautotrophic organisms in the 2D biomaps: Chlorophyll-~~-~~a (red channel) of green algae was excited separately from the phycobillins (green channel) of cyanobacteria, (Fig. 1~~+~~2). The EPS showe~~d~~s a minor fluorescence signal at its periphery as an artefact of reflectance from the main cells (Fig. 1c, d, white arrow). BSCs from all four sites were~~are~~ densely packed with a variety of cyanobacteria and green algae, ranging from single coccoidal to filamentous organisms (Fig. 2e), of which ~~whereby~~ Hochtor shows the highest morphological diversity ~~(Fig. 3a)~~. Macroscopic thall~~ius~~ forming *Nostoc* species (Fig. 2a–e, white triangles) were found on top and within the BSC and their identity was confirmed~~hecked~~ by light microscopy. ~~Different thallus forming Nostoc species are shared between three of the four sites (white triangles, Fig. 3a–c), and are found on top of the BSC as well as within.~~

Additionally, the BSC can vertically be divided into an upper photosynthetic active layer (PAL), where fluorescence signals from cells with active chlorophyll-a and phycobillins accumulate, and a lower photosynthetic inactive layer (PIL) that represents inactive cells, glued together with stones through EPS (Fig. 3~~4~~). This layer does not give a fluorescence signal but belongs to the crust itself. Both compartments can be removed from the soil as a single entity. ~~To visualize this concept a graphical scheme was drafted, presenting these structures more easily (Fig. 4).~~

## 3.2 Active photosynthetic carbon (apC) depth profiles

The apC depth profiles (Fig. 4~~5~~) reveal specific patterns for each of the four sites, with C peaks in the upper part of the crust in Livingston and Geopol samples or located between 1 and 2 mm in Hochtor and Ny-Ålesund samples. ~~Within the depth of these peaks, soil contains~~ The highest values ranged between 25 and 40 mg C cm$^{-3}$. Light grey backgrounds indicate the soil stratum made of active photosynthetic organisms (PAL), whereas the dark grey parts mark the layer (PIL), containing inactive cyanobacteria and green algae (limit set to <5 mg C cm$^{-3}$) according to the concept explained previously. Values at the deepest position show the mean thickness of each crust, with Hochtor having the thickest crust reaching almost 4 mm and Livingston showing the thinnest crust with a depth of 2.8 mm.

## 3.3 Carbon and partitioning

The total organic carbon content of soils varies between 7 % and 17 % (Fig. 5), with Hochtor and Geopol being significantly different to Ny-Ålesund (p≤0.05). Based on CLSM–IA t~~T~~his total organic C can be divided into carbon evaluated from active photosynthetic organisms (apC), and non-photosynthetic carbon (npC). The latter includes dead organic material and remaining EPS. ~~Soils of the study sites contain 7 % (Hochtor, Geopol), 14 % (Livingston) and 17 % (Ny-Ålesund) of total organic C, obtained by loss on ignition (Fig. 6), whereby Hochtor and Geopol differ significantly from Ny-Ålesund (p≤0.05). This total organic C can be divided into carbon coming from active photosynthetic organisms (apC), based on CLSM-IA~~

~~and non-photosynthetic carbon (npC), including dead organic material, remaining EPS and macro-/micro faunal elements.~~ Only 1.5 to 3 % of the total organic carbon is provided by active cyanobacteria, green algae and bryophytes as apC (without EPS) as well as 4–11 % npC.

Cyanobacteria with their EPS and thallus structure occupy between 7 (Livingston) and 23 % (Ny-Ålesund) of the total area of BSC that was visualized in the 2D biomaps. In comparison, green algae contribute with 0.5 to 2 % as a minor group (Fig. 5).

~~Cyanobacteria with their EPS and thallus structures occupy between 7 and 23 % within the BSC structure, in comparison algae contribute with 0.5 to 2 % only as a minor group (Fig. 6).~~ Non-fluorescing EPS as the main matrix of the BSC together with stones, gravel, dead material and faunal elements accounted for ~~differ~~ between 76 and 92 %, grouped as adhering material.

## 4 Discussion

For the first time, CLSM was applied to visualize intact BSC, collected from the Arctic, Antarctica and the Alps. Together with carbon estimations we provide new insights into BSC structure. The crusts themselves show compartmentation with a PAL stratum, containing the active fraction of mainly cyanobacteria and a few green algae and the PIL fraction~~,~~ with predominantly dead material~~.~~, ~~T~~this is additionally reflected in the carbon contents and visualized in a graphic scheme (Fig. 3~~4~~).

Embedding BSC in an agarose matrix allowed visualization ~~for the first time~~ of intact and active BSCs with CLSM. The biomaps provide insights into structural features of the different BSCs, such as spatial distribution patterns related to depth, community composition of morphological and taxonomic groups, as well as discrimination between green algae and cyanobacteria.

~~-~~The different crusts have several structural features in common:

Their crust layers are divided into a top layer characterized by high densities of active photoautotrophic organisms, which is therefore defined as PAL (0–2.5 mm) and a sublayer (2–4 mm) with a strongly decreased abundance of photoautotrophic organisms, named~~called~~ PIL. Both layers are part of the BSC and can be removed from the soil as a single entity~~,~~ because~~,~~ the EPS adheres the microorganisms and~~with~~ sediment. In most of the investigated BSCs the ~~cases investigated,~~ different ~~BSC~~ structures were found to be very pronounced between~~at~~ all sites, however, these structural differences ~~but~~ have additionally been identified with this technique from crusts in other habitats (Szyja; in preparation). The layered structure is similar to what has been recently described for arid desert BSCs of Israel, where three layers were defined (Ranaan et al., 2016): A top crust layer, that is about 1–2 mm thick, which is composed of small packed particles and most of the organic material (Drahorad et al., 2013), followed by a vesicle layer of approximately 0.3–0.7 mm thickness, filled with trapped air and a sub crust layer made of dead material, EPS and soil particles. The formation of a vesicle layer is maybe prevented at

the cold sites of this study because of the freeze-thaw dynamics and continuous water runoff into the soil, during the active season, transporting and shifting small particles that fill potential air vesicles.

Additionally, eukaryotic green algae represent only a minority in terms of occupied space at all sites. In general, it is known that there are no eukaryotic algae exclusively found in BSCs (Büdel et al., 2016). Green algae are rarely the dominant crust-forming organisms and they occur in low abundance or may be present as dormant resting stages, which are hard to detect and to identify. This is supported by other studies that detected, at least from Hochtor, Austria, only a minor proportion of green algae (Büdel et al., 2014; Peer et al., 2013).

Furthermore, cyanobacteria constitute the dominant photoautotrophic spatial unit at all sites, probably due to their thallus structures being composed of EPS and their cell densities. Interestingly, Büdel et al., (2014) showed by Illumina sequencing that within the microbiome of BSC from Hochtor, cyanobacteria contributed only 1.6 % to the total bacterial diversity, whereas we show that cyanobacteria occupy 20 % of space within the crust. This shows that the role of cyanobacteria within microbiome studies that are based only on DNA proportions might be underestimated. This becomes even more interesting when other findings are considered, where Büdel et al., (2014) showed that within the microbiome of BSC from Hochtor, cyanobacteria contributed only 1.6 % to the total bacterial diversity, whereas we show here that they occupy 20 % of space within the crust.

Differences among the crusts based on CLSM 2D biomaps were reflected in their thicknesses and the morphological groups that could be identified. The alpine site Hochtor for example, harbours the thickest crust that is known throughout available literature with a thickness that exceeds 4 mm. Light regime could be a responsible factor, because all four sites share similar daylight times with PAR exceeding 1200 µmol m$^{-2}$ s$^{-1}$ (Colesie et al., 2016; Xiong et al., 2001; Barták et al., 2012), but with the strongest fluctuations at Hochtor (Büdel et al, 2014). The appearance of photoautotrophic organisms up to these depths may be possible due to a diverse community composition of organisms with different adaptions regarding light regime. This idea supports previous studies that state high rates of photosynthetic activity at least during the snow free growing season for BSC of Hochtor (Raggio et al. 2017; Büdel et al., 2014). Light regime could be a responsible factor, because all four sites share times where their BSC is protected from radiation by snow cover and times during high light exposure and desiccation due to receding snow. Appearance of photoautotrophic organisms up to these depths may be possible due to a diverse community composition with organisms and therefore is composed of organisms with different adaptions regarding light regime. Species such as *Nostoc* have been found to occupy mainly soil surface positions (Fig. 23, white triangles) where it must invest in UV- and desiccation protection, for example through pigment and EPS production. Crusts dominated by these highly pigmented organisms were classified therefore as dark crust (Belnap et al., 2004). A different strategy is avoidance, which requires vertical migration down from the soil surface (Garcia-Pichel and Pringault 2001). This is therefore only available to relatively large, mobile organisms such as the large filamentous cyanobacteria *Microcoleus*. In deeper layers of the soil, light is attenuated due to high densities of mineral and biogenic particles, shorter wavelengths penetrate less deeply than longer wavelengths, which provides a redundancy for pigment synthesis as UV protection (Belnap et al., 2004). Crusts that are dominated by these species are therefore called light crusts (Belnap et al 2004). Light regime and drying times define

~~the activity times of these cyanobacterial dominated BSCs, which are expected to follow radiation levels: *Nostoc* dominated (dark) BSCs would have the least amount of time active and *Microcoleus* dominated (light) BSCs the most activity time (Belnap et al., 2004).~~ The establishment of a highly diverse cyanobacterial community composition with representatives of both strategies would therefore lead to an increase in activity, and consequently the thickness of PAL. Additionally, activity

5 recovery of BSCs after seasonal changes may be accelerated by a vivid crust. For example, this may be the case in Hochtor, where BSC is composed of various cyanobacterial species with different ecological niches. ~~Nevertheless~~However, the community composition of these habitats ~~and especially of Hochtor~~ needs to be addressed by further molecular and taxonomic studies in order to investigate ~~proof~~ these ideas.

The main functional BSC groups such as cyanobacterial crust, green algae crust, cyanolichen~~s~~, chlorolichen~~s~~ and bryophyte

10 crust~~s~~s of Ny-Ålesund, Geopol and Livingston Island have already been shown to be distinct, which is for the most part linked to their geographic distribution and the differences between deeper and more skeletal soils (Williams et al., 2017). Carbon contents along the depth of the BSC obtained by CLSM 3D biomaps represent a summary of microclimatic conditions of the different habitats, their successional stage and structural features, which~~and~~ therefor~~e~~ support~~s~~ these results~~.~~: Highest C contents of approximately 40 mg C cm$^{-3}$ are reached in depths between 0.5 and 2 mm, except for

15 Livingston, where bryophytes dominate the BSC at the soil surface, leading to C peaks placed directly at the top. As described previously (Williams et al., 2017), Livingston Island shows a different~~higher~~ developmental vegetation stage where cyanobacteria and green algae have been~~are~~ replaced by bryophyte cushions and especially chlorolichens, situated on the crust surface that represent the climax community. Also at Geopol the C peak is close to the surface and combined with the thickest PIL, probably linked to the disturbance regime of cryoturbation. During polygon formation and especially

20 freeze-thaw interaction gravel and particles of different sizes are moved, this~~which~~ affects the cyanobacteria and green algae that are glued to them by their EPS (Cannone and Guglielmin 2010). The BSC structure may not be destroyed but is probably shifted so that organisms from the top that are immobile are moved to deeper positions, losing their favoured photosynthetic light regime position and possibly dying off. Dead cells and EPS can remain to some extent (Tamaru et al., 2005) but their maintenance as well as the amount of new grown BSC on top of the old structures is limited by the ongoing

25 processes of cryoturbation, a common occurrence in polar and alpine sites worldwide.

Northern circumpolar soils are estimated to cover approximately 18,782 x 103 km$^2$ and contain about 191 Pg of organic carbon in the 0–30 cm depth stratum (Tarnocai et al., 2009). Organic carbon stored in permafrost regions is one of the least understood and potentially most significant carbon- climate feedbacks due to the size of the carbon pools and the intensity of climate forcing at high latitudes (Schuur et al., 2008). Around Ny-Ålesund, Spitzbergen approximately 90 % of the soil

30 surface is covered by BSCs (Williams et al., 2017), whereby the first cm$^3$ of soil contains almost 18 % organic C, highlighting the sink character of these cold ecosystems. This becomes even more impressive, when compared to soil organic C contents of other BSC dominated habitats like the Andes with 4–8 % (Pérez 1997) or 1 % of the Kalahari ~~sand~~ (Mager 2010).

Nevertheless, the contribution of C from active photosynthetic organisms (apC) to the total C seems to be in a low range (2–3 %), which is probably linked to the methodological drawback of CLSM in terms of C determination, neglecting the EPS which is made of carbohydrates. Through their photosynthetic activity cyanobacteria ~~can~~ increase the C content within the BSC in the form of carbohydrates, this acts as an energy source that can be readily utilized by other soil organisms (Bertocchi et al., 1990). Mager (2010) for example ~~could~~ demonstrated that carbohydrates from the EPS made up to 75 % of the total organic C within the BSC in the Kalahari, which in comparison ~~hereby this factor~~ is determined~~captured~~ here by the loss on ignition as total organic C. Despite the fact that CLSM ~~does~~ neglects the EPS of unstained cyanobacteria and green algae, to some extent, which lowers the proportion of C this technique can determine, it can give highly comparable insights into BSC structures that have not been addressed by any other technique known so far.

## 5 Conclusion

In conclusion, CLSM is a suitable and highly comparable method to obtain 2D biomaps, which show the intact and undisturbed photoautotrophic community of BSCs to examine structural and clade specific features. The newly defined terminology and determination of PAL and PIL of BSCs as visual entities or C depth profiles will be helpful to investigate and compare successional stages and changes in BSCs of different sites or treatments. In addition, it is likely that BSCs contribute significantly to the global carbon flux of soils at cold sites, highlighting their role as $CO_2$ sinks. For this reasons it is vital to address the cyanobacterial community composition of the Arctic, Antarctica and the Alps with further studies before climate change induced species alterations take place.

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**Figures**

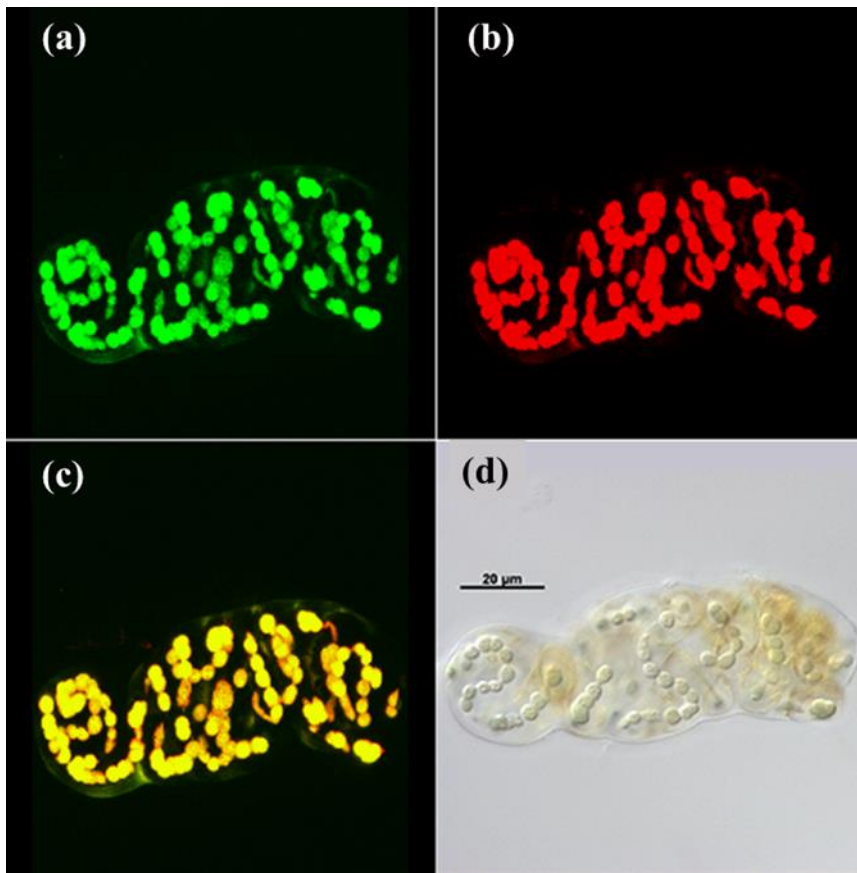
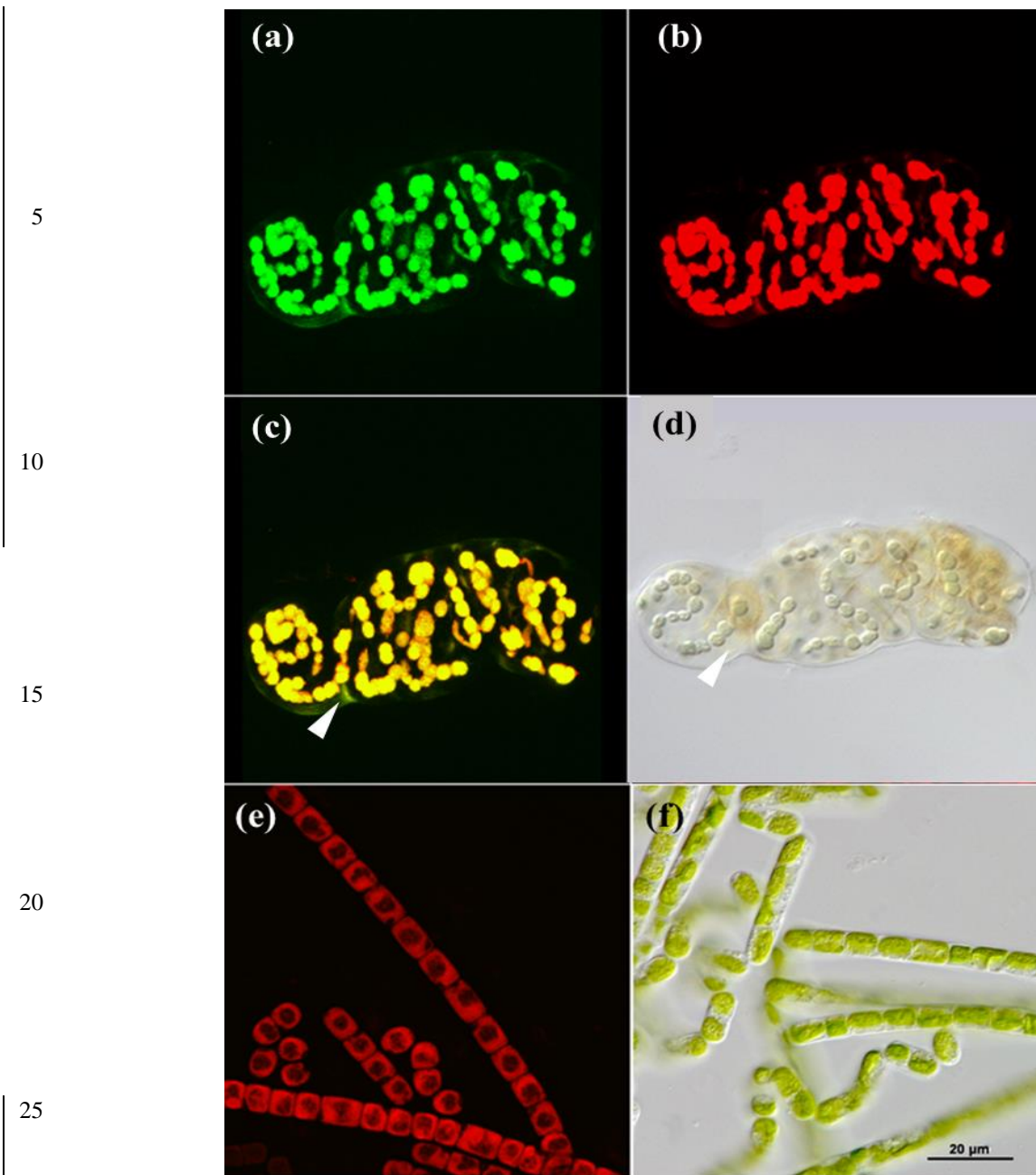



**Figure 1:** CLSM and light microscopy~~Cyanobacteria~~ micrographs of *Nostoc* sp. and *Klebsormidium* sp. Cyanobacterium (*Nostoc* sp.) visualized with CLSM (a–c) and light microscopy (d). White triangles indicate the EPS (c, d). Green algae (*Klebsormidium* sp.) visualized with CLSM (e) and light microscopy (f). Green channel (a) represents fluorescence from phycobillins at 639 nm, red channel (b, e) from chlorophyll-a at 555 nm and an overlap of both is shown in (c) for *Nostoc* sp.

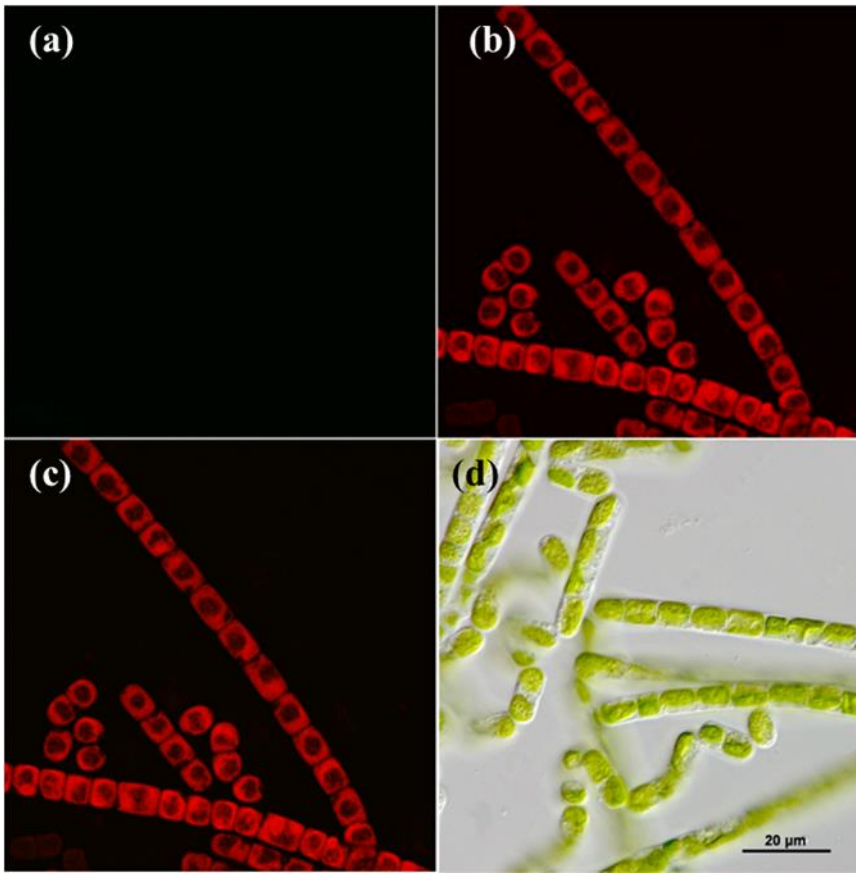



Figure 2: Green algae micrograph. *Klebsormidium flaccidum* visualiced with CLSM (a–c) and light microscopy (d). Green channel (a) represents fluorescence from phycobillins (not present in green algae), red channel (b) from chlorophyll a and an overlap of both is shown in (c).

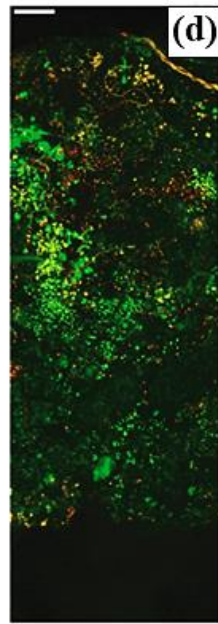
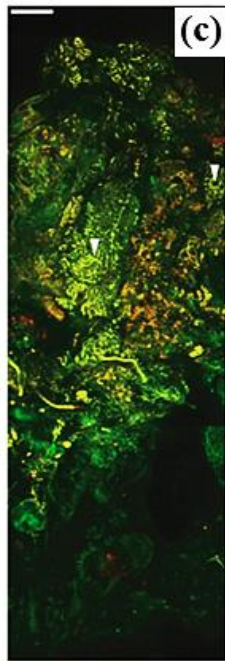
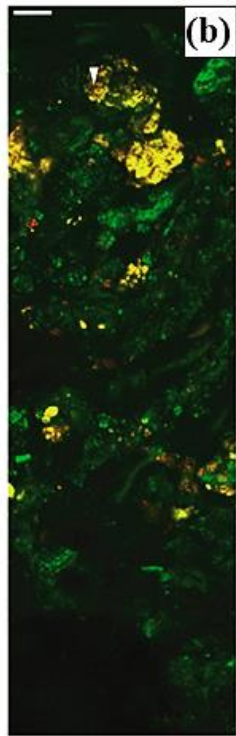
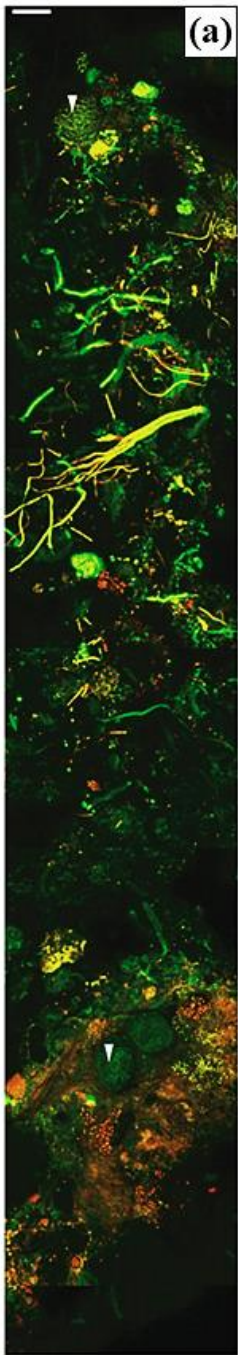
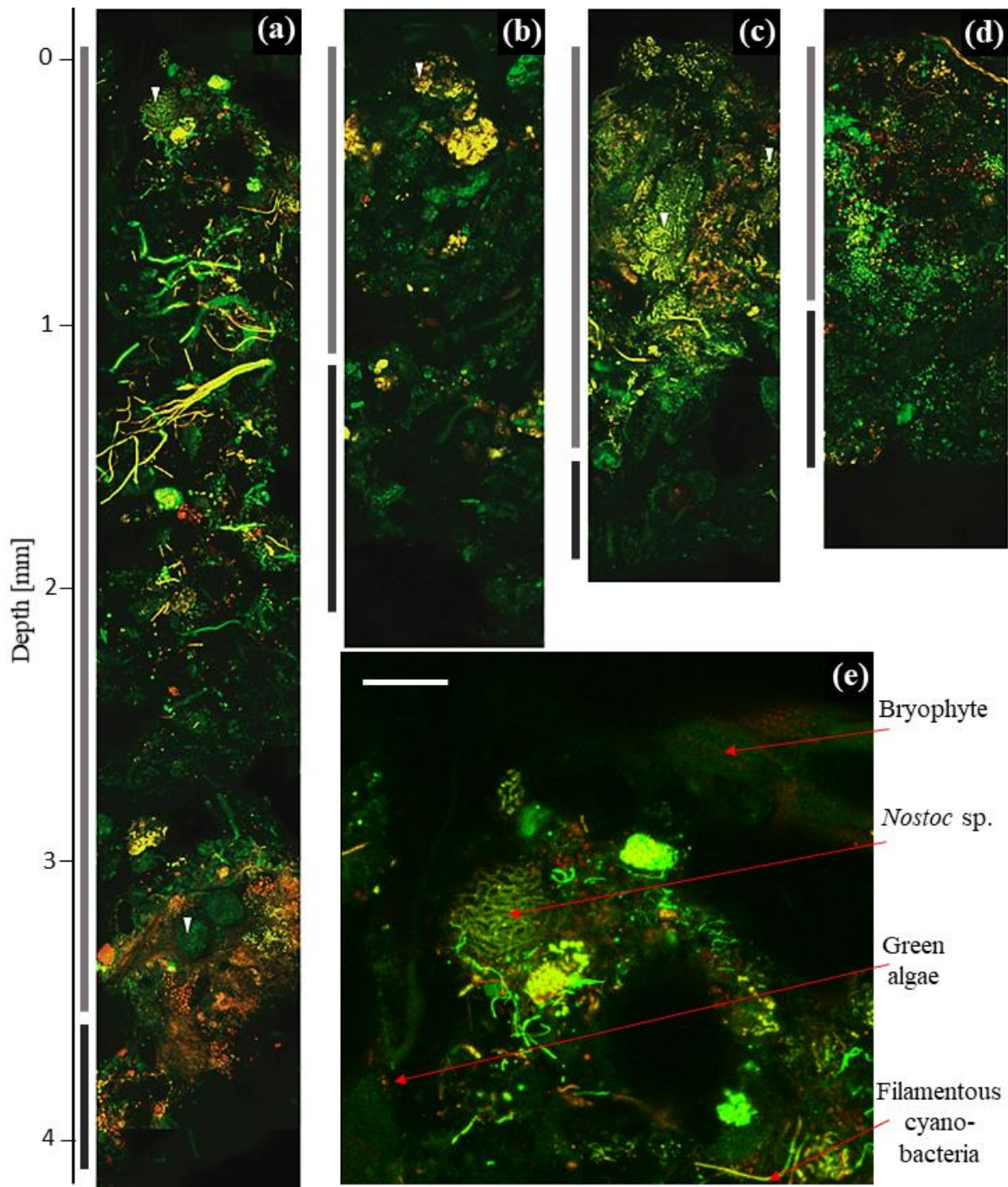



**Figure 23: 2D Biomap. Overlap of green and red channel as maximum projection of depth profile images showing Hochtor (a), Livingston (b), Ny-Ålesund (c) and Geopol (d). PAL (light grey) and PIL (dark grey) indicate different strata as bars in comparison to their depth. The beginning of the fluorescence signals indicates the BSC surface (0 mm). The dark black background above this is the agarose matrix. A zoom of the top layer of Hochtor demonstrates bryophytes, cyanobacteria and green algae (e). White triangles indicate different *Nostoc* species. White scale bar indicates 100 µm.**

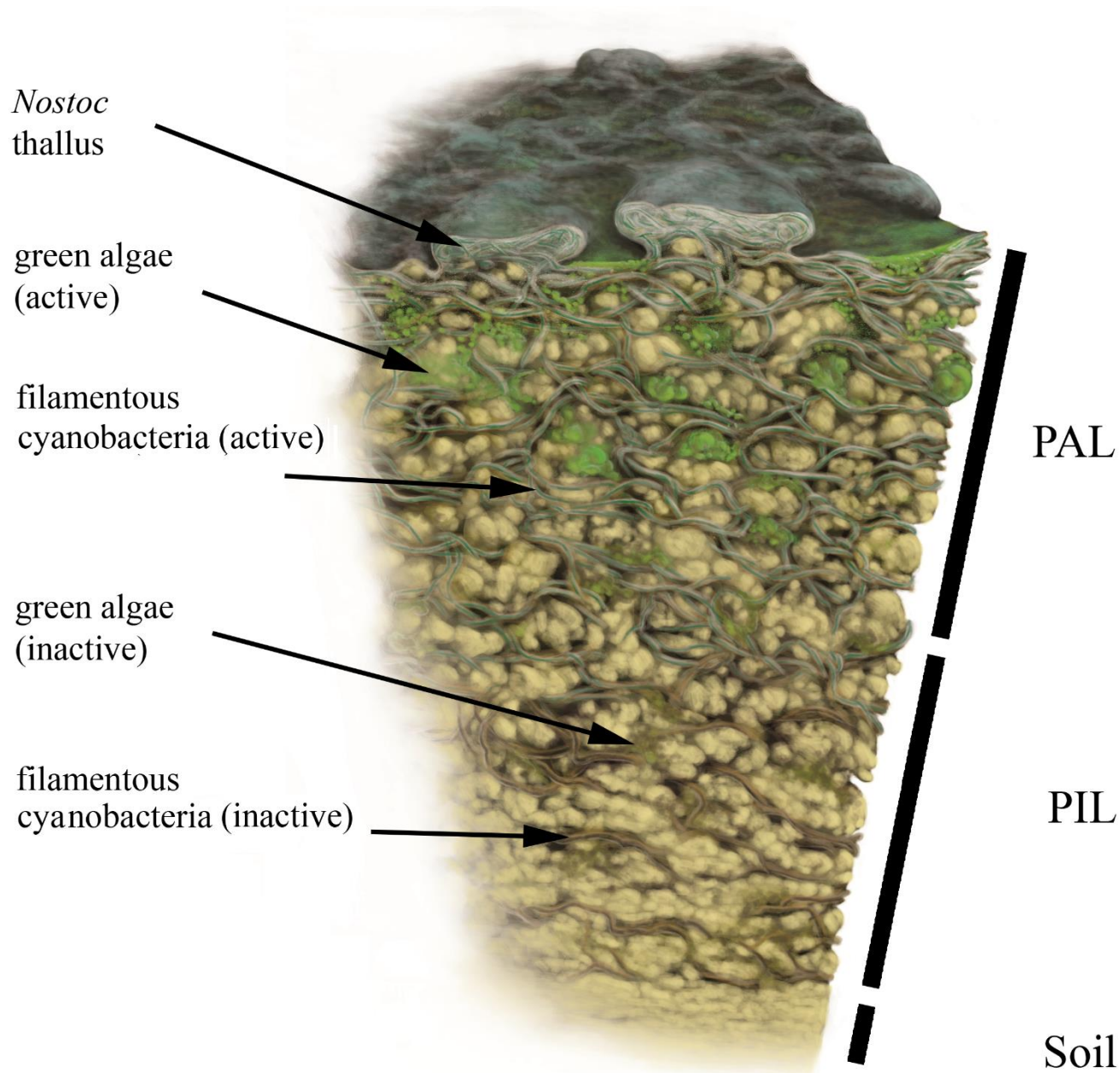



**Figure 34: Biomap Scheme.** Simplified illustration of a vertical BSC cross section with photosynthetic active cyanobacteria and green algae in the PAL (photosynthetic active layer) stratum as well as both fractions in their dead or inactive forms within the PIL (photosynthetic inactive layer) stratum. Illustrator: Frederik Spindler.

active photosynthetic Carbon [mg C*cm⁻³ soil]

PAL (photosynthetic active layer)

PIL (photosynthetic inactive layer)

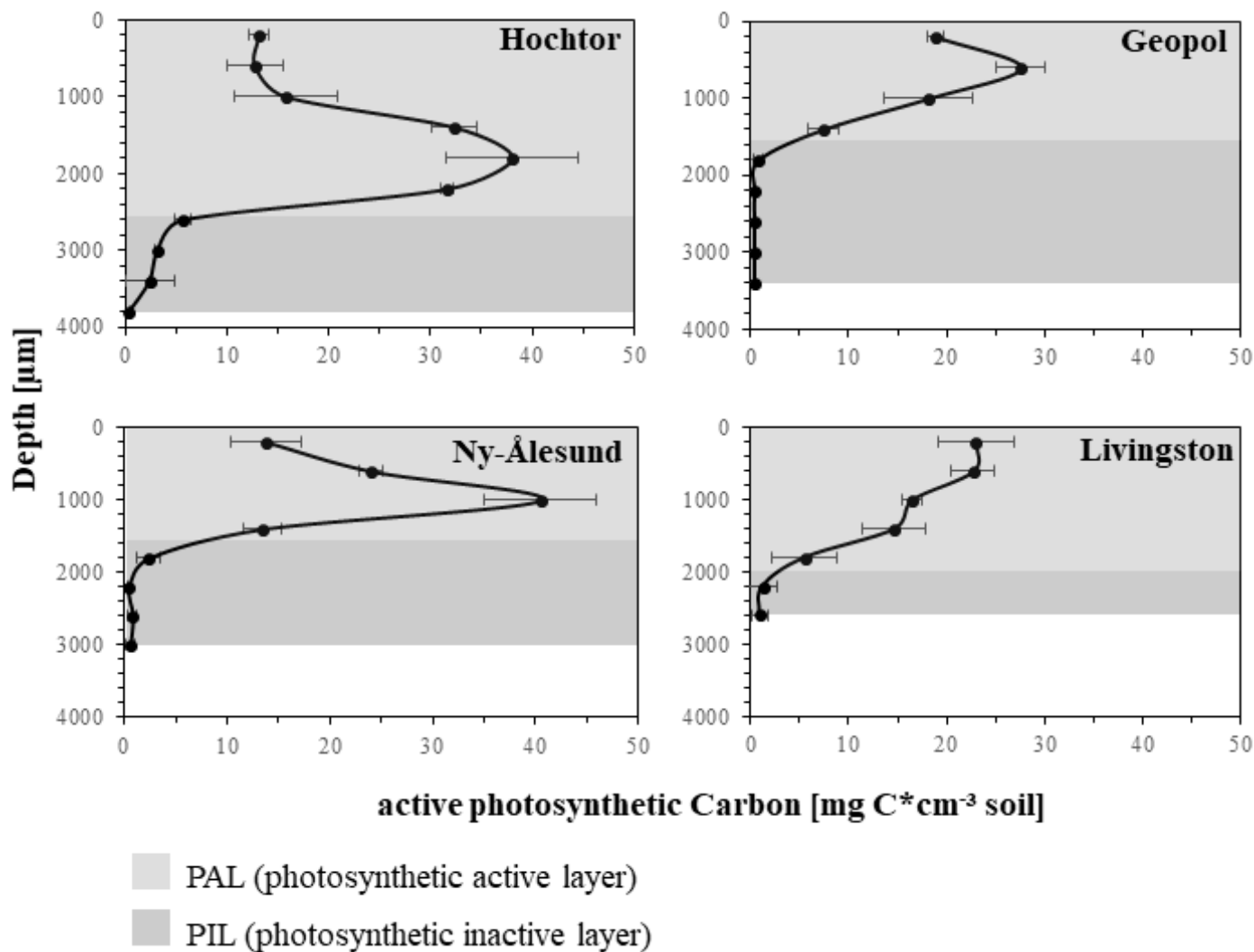
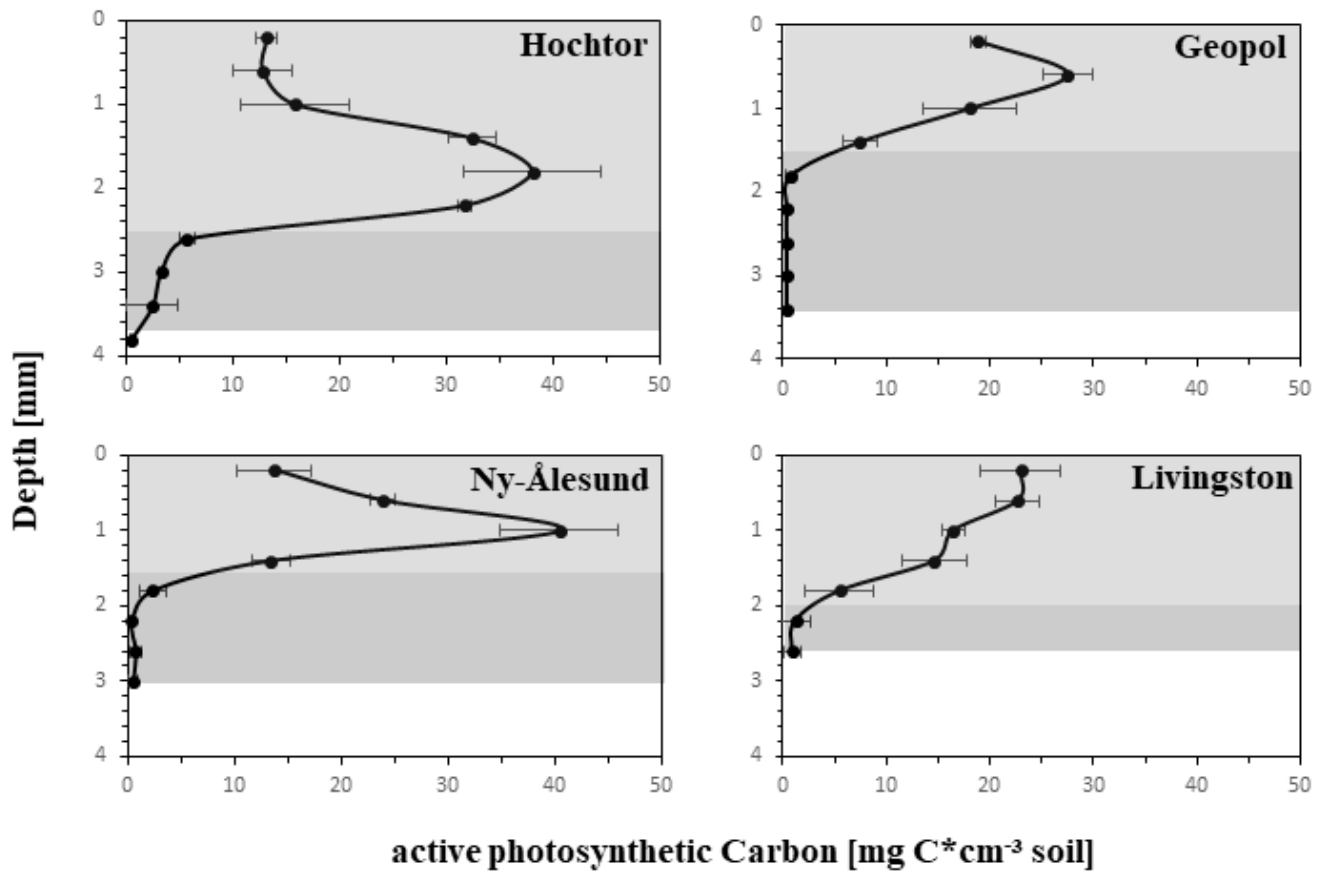



**Figure 45: Active photosynthetic carbon (apC) depth profiles. Carbon values calculated from 3D biomaps with CLSM–IA are plotted against soil depth from surface to where the crust ends with standard deviation. PAL (photosynthetic active layer) is indicated in light grey, describing the soil stratus that contains active photoautotrophic organisms. PIL (photosynthetic inactive layer) is shown in dark grey, marking the soil stratum with inactive photoautotrophic cyanobacteria and green algae. The value representing the deepest point describes the thickness of each biocrust.**

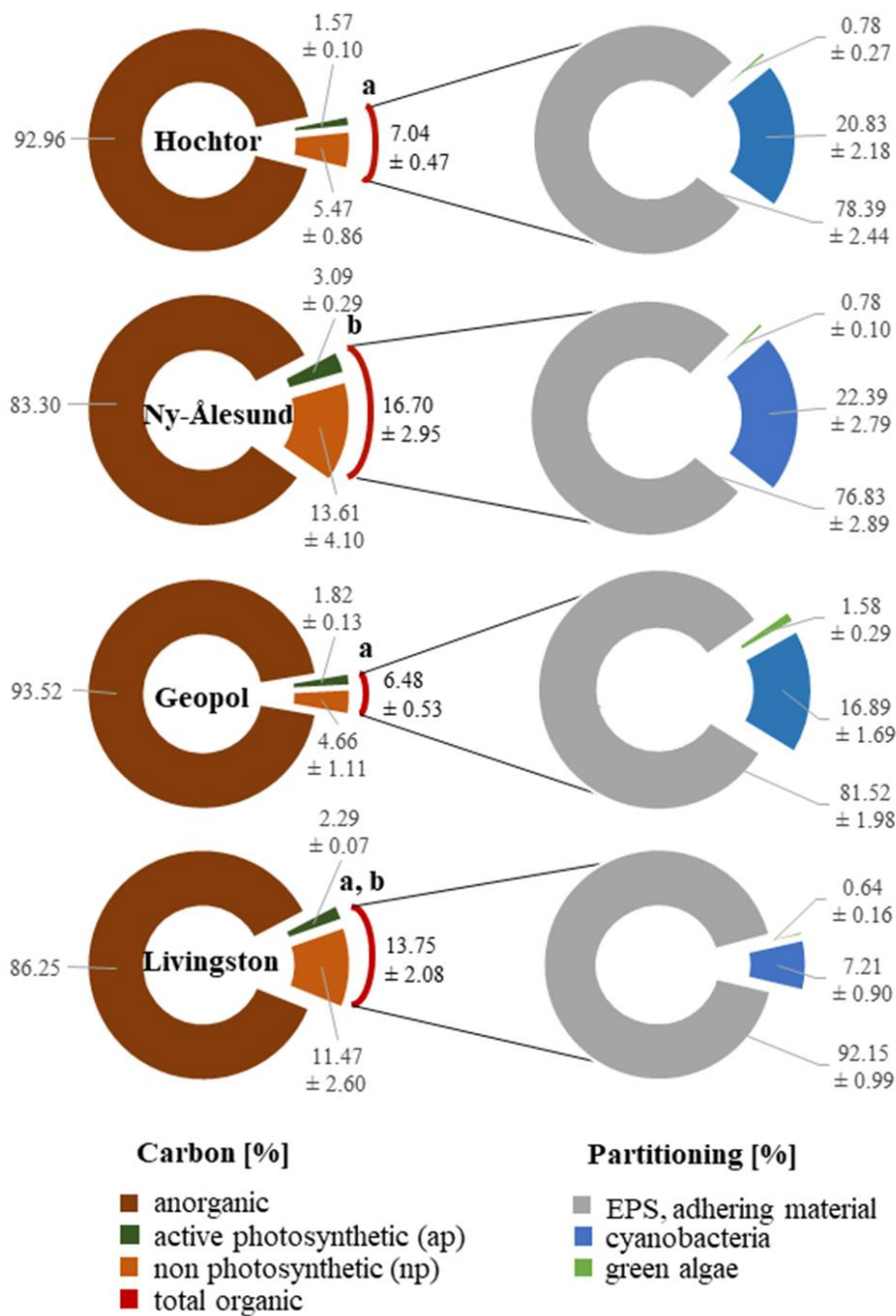
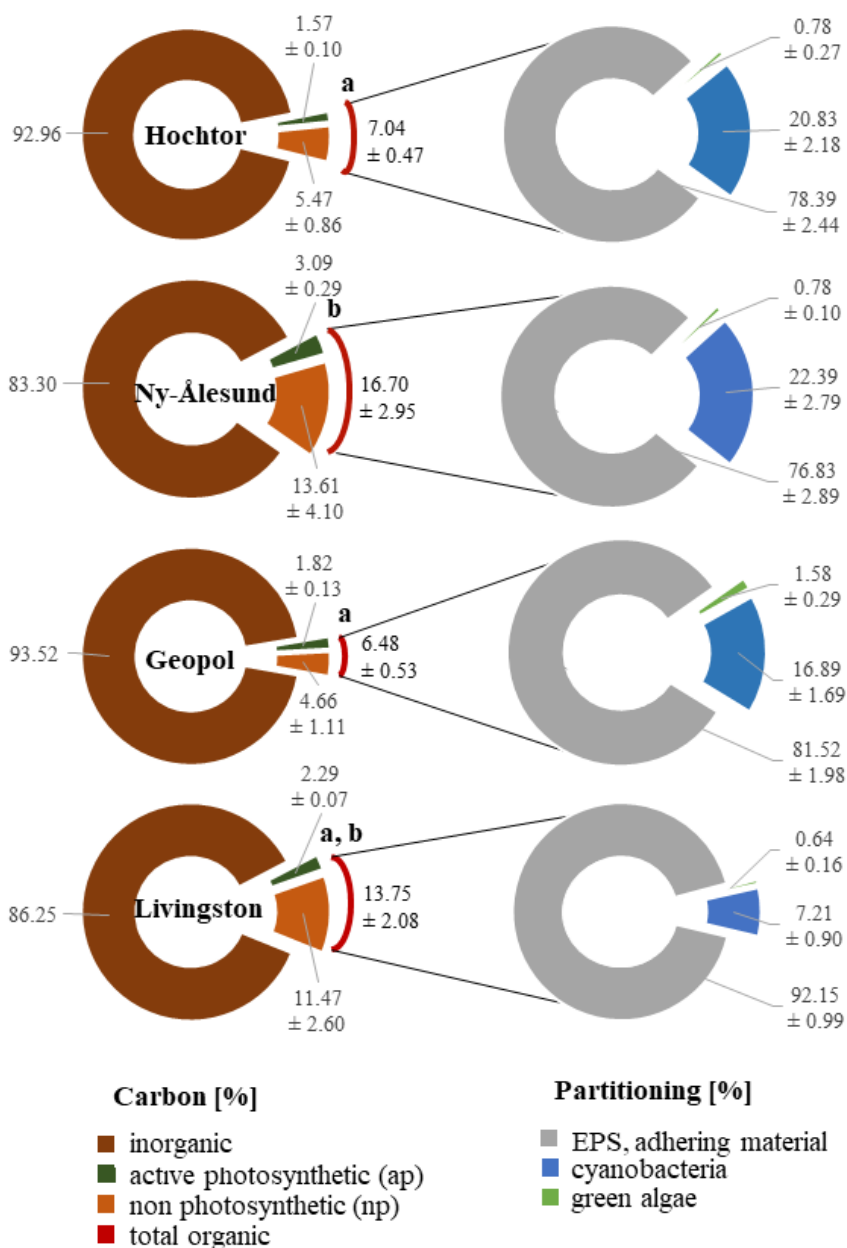
**Carbon [%]**

- ■ anorganic
- ■ active photosynthetic (ap)
- ■ non photosynthetic (np)
- ■ total organic

**Partitioning [%]**

- ■ EPS, adhering material
- ■ cyanobacteria
- ■ green algae

Carbon [%]
- ■ inorganic
- ■ active photosynthetic (ap)
- ■ non photosynthetic (np)
- ■ total organic

Partitioning [%]
- ■ EPS, adhering material
- ■ cyanobacteria
- ■ green algae

5 **Figure 56: Carbon and BSC group partitioning. Carbon content determined by loss on ignition and CLSM–IA of the 3D biomaps is combined in percent, applied to the volume of 1 cm³ (left circle). Total organic C obtained by loss on ignition from Hochtor and Geopol differ significantly from Ny-Ålesund (p≤0.05). Lower case letters are marking statistically significant differences. Area partitioning of cyanobacteria, green algae and adhering material within the BSC is expressed in percentage, applied to the total area of the 2D biomaps (right circle). Values are the mean ± standard deviation.**