Author Response to Reviews of

Measurement report: The ice-nucleating activity of lichen sampled in a northern European boreal forest

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ACP, doi:10.5194/egusphere-2023-2780

RC: ***Reviewer Comment***, AR: *Author Response*, ☐ Manuscript text

We thanks the referees for their positive comments and have addressed them below. In addition we have been made some minor changes throughout the paper and added a new reference to a new HyIce 2018 study in the introduction (Vogel et al., 2024).

1. Reviewer Comment # 3

RC: ***The authors have written detailed answers to the reports of referee #1 and #2, which I consider as being well argued and sufficient. However, it is really important to highlight the work of Eufemio et al. 2023, also when it was not measured at the same place, but the results are very similar. So I would expect that the authors show respect and make clear that their report is second. Nevertheless, the measurement report is valuable and must be published, since it adds interesting information and knowledge to the discussion for the scientific community focusing on heterogeneous ice nucleation triggered by biological materials from boreal forests. From my point of view, the boreal forests are key for the understanding of heterogeneous ice nucleation in the troposphere.***

AR: *Thank you for your feedback. In addition to our frequent mention of the Eufemio et al. (2023) paper we have added new statements throughout the introduction to highlight that our work has come second. However, we do stress that our study is unique, with different objectives and a different methodology.*

> Adding to evidence for lichenous INPs, this study shows that lichen from a European Boreal forest in Hyytiälä harbour INPs. This novel finding may be especially important in this snow covered habitat where few, if any, other biological INP sources are available.

> In a recent study, Eufemio et al. (2023) tested lichens collected across Alaska for their ice nucleating ability, pointing to their possible impact on cloud glaciation in a warming Arctic.

> In this paper we tackle the first part of the hypothesis, namely the question of if the lichens in the forest during the winter of HyICE-2018 contained ice-nucleating entities. Eufemio et al. (2023) have made this possibility obvious since they showed that lichen from across Alaska harbour INPs. It remains to us to confirm this for the Hyytiälä boreal forest. A positive outcome would provide motivation to address the question of whether sufficient quantities of INPs are released into the atmosphere to influence the INP population and subsequent cloud formation.

2. Reviewer Comment # 4

RC: ***Proske et al. have revised a manuscript based on INP measurements from several lichen species collected in Finland. The study presents results including size-resolved analysis via filtration and thermal treatments to assess the INP composition of the lichen samples. Although significant effort has been made in the revision process, several major issues remain that need to be addressed before the manuscript can be considered for publication.***

AR: *Thank you for your substantial feedback, which we have incorporated as detailed in the following.*

2.1. General comments

RC: ***Abstract: I understand the concern of the previous reviewers regarding the novelty of the work. The interesting aspect is that these lichens are present and consist of warm temperature INPs during winter when other more prominent/abundant biological sources are covered in snow, thus, could be important for influencing the wintertime airborne INP population. This concept should be highlighted more clearly, especially in the abstract, but additionally throughout.***

AR: *We have rephrased the sentence in the abstract that contained this thought, to make it more explicit:*

> This novel finding may be especially important in this snow covered habitat where few, if any, other biological INP sources are available.

In addition to the introduction, where we state:

> However, the source of these INPs was unclear since the surface was snow covered, which rules out leaf litter or bare soil as sources of INPs. We hypothesize that the trees which bore lichen, that were exposed even in the winter when the canopy and the ground were snow covered (see Fig. 1), might have been a source of INPs.

we have added a reiteration of this point to the beginning of the Results section:

> Our hypothesis is that these biological INPs originate from the lichen that is abundant in the boreal forest ecosystem even when there is snow cover.

RC: ***Hypothesis: Shouldn't this hypothesis be more specific, based on what was actually tested? The study only tests winter samples. I would guess there is some level of dormancy in the winter and the samples might behave differently in the summer. Also, the study does not actually test the hypothesis in that the presence of lichen is not investigated in the airborne INPs from HyICE-2018. Rather, the study tests the potential of local sources to contain INPs that could become airborne during the winter. The hypothesis should be rewritten to reflect what is actually verified/denied in this study and should be revisited in the discussion section.***

AR: *In our effort to explain a) the hypothesis that lichen could provide a local source of INP in the snow-covered winter and b) our approach of testing the first part of it, namely whether local lichen contain INP, we have*

apparently lost the reader. We have reformulated the corresponding section to make the distinction between the overall hypothesis and what we are able to contribute to addressing this hypothesis in this paper clearer:

> We hypothesize that the trees which bore lichen, that were exposed even in the winter when the canopy and the ground were snow covered (see Fig. 1), might have been a source of INPs. In this paper we tackle the first part of the hypothesis, namely the question of if the lichens in the forest during the winter of HyICE-2018 contained ice-nucleating entities. Eufemio et al. (2023) have made this possibility obvious since they showed that lichen from across Alaska harbour INPs. It remains to us to confirm this for the Hyytiälän boreal forest. A positive outcome would provide motivation to address the question of whether sufficient quantities of INPs are released into the atmosphere to influence the INP population and subsequent cloud formation.

RC: ***"Steps": It is very difficult to visually discern the steps in the current figures. Also, if trying to discuss different INP populations, it is best practice to use differential spectra. A couple of suggestions here: 1) make the current spectra bigger/wider so the small differences are easier to see and 2) make a figure with differential spectra, so that the two populations at -16C and -18C are evident.***

AR: *Thank you for the great suggestion! We have followed it by adding Fig. C1 that shows the differential spectra and included references to it in the text. Unfortunately not all samples had dilutions that lined up well enough to allow for a sensible interpolation, so we have only translated some of our data into differential spectra. However, the different INP species (peaks) corresponding to our 'steps' are clearly distinguishable.*

RC: ***Filtrations: Why were the same filtrations not executed on all the samples? Specifically, sample B was not subject to the 0.02 µm as sample C was. For the individual species, only 2 µm filtration was done. It is difficult to intercompare the samples when the same filtrations were not done. The authors could either omit the 0.02 µm results or very clearly describe why it was only done on one sample. Also, why were the same filtrations not done on the individual species? That may have helped determine which are the most dominant INPs in the sample mixtures, by being able to compare similarly-manipulated samples.***

AR: *We agree that it would have been best and regret to not have results for all filter sizes and samples, but have added a description as you suggested:*

> Only for sample C all filter sizes were used, as it was realized after the processing of sample B that further size differentiation would be desirable. For the species specific tests the samples were partly too small to use all filters so only the 2 µm filter was used.

RC: ***Proposed emission mechanisms: First, suspending lichen in a bunch of water would not replicate possible aerosolization methods in the real environment (lines 132-135). Suspension in water and atmospheric emission mechanisms could be completely different. This sentence should be omitted; the authors should instead state that there could be differences in the suspension solution versus what might actually become airborne. Second, the authors discuss how the smaller INPs could become airborne by adhering to large particles. Has this process been evaluated for lichen? What larger particles are present in lichen that these smaller INPs would stick to, that are subject to detaching from the lichen surface? I can see how this emission process is possible for soil surfaces, but it is not clear for lichen. Additional justification for these conclusions should be provided as the link between the results and possible emission processes is currently not clearly drawn.***

AR: *Regarding your first point, the lines you quote make the point that our sampling procedure improves upon previous studies because we are not washing the lichen prior to probing nor grinding it up. It continues to state that also our practice is clearly different from wind dispersal, which we have amended to be more clear:*

> *While this approach is clearly different from bioaerosol production from lichen via wind, it does bias the analysis towards the entities associated with lichen that are likely to become aerosolised.*

Your second point is covered in the Discussion section in l.334 to 340 (in the previous manuscript version). We could imagine the smaller particles to be fragments of dispersal particles. They could attach to whole spores, soredia or isidia, which have been shown to become aerosolised. The particular mechanism of smaller INP entities sticking to these larger entities has not been studied for lichen as far as we know (see l. 364ff, which have now been moved to the introduction).

RC: ***Reordering text: The results section was hard to follow - it did not flow naturally. I suggest reordering, so that the current section 3.1 is first (describing the individual species) followed by the start of the results up to line 278 (more complex investigation of potentially realistic ambient external mixtures). The text on lines 278-285 and the corresponding figure (comparing the extraction techniques) is more of a methodology testing. I suggest moving this to the methods as it does not test the hypothesis or apply to any sort of emission process that would realistically happen.***

AR: *Thank you for the suggestion, which we were happy to follow. Note that we have moved Fig. 6 and its discussion to the Appendix instead of to the Methods section where we felt it could overwhelm the reader with the results contained in the figure not discussed yet.*

RC: ***Along these lines, I found it unclear that B and C are called "samples" when in reality, they are roughly the same mixture just tested under different suspension techniques. (Side question: Why was one master sample not mixed and then aliquoted for the different extraction methods?) Perhaps just call them the same sample mixture, but in the methods, describe that they were tested differently. You could even call the spectra "hand shaken" and "rotary mixed" or something along those lines.***

AR: *We understand that this is a little difficult to follow, but the alternatives wouldn't necessarily be better. We want to do justice to the fact that these were two different 'samples', with different lichen samples (albeit collected at the same time and stored in the same bag) and extraction techniques. The fact that we have two samples is due to our finding that one could not simply draw more sample from the same suspension that still contained the lichen, as discussed in l. 247ff. Thus we needed to set up a fresh suspension where for sample C we took care to draw enough solution from the suspension for all filter tests we desired to do.*

RC: ***In general, there are many qualitative comparisons drawn between the results presented here and previous studies, and for inter-sample comparisons (i.e., the use of "steps" or indicating spectral features). It would be a stronger paper if actual concentration values were compared and differential spectra were used for the study sample intercomparisons. And for the filtrations, it would be helpful to present the % of the total INPs that were*** $10\,\mu\mathrm{m}$***,*** $2\,\mu\mathrm{m}$***,*** $0.1\,\mu\mathrm{m}$***, and*** $0.02\,\mu\mathrm{m}$***.***

AR: *We included differential spectra in C1 (discussed above). We attempted to produce percentage remaining plots (see below), but we feel these plots do not add anything that the fraction frozen plots already show. They also introduce issues such as fractions above unity, that are related to poor counting statistics in some cases.*

AR: *Also, we have created a new plot to compare with literature data (see response to specific comment below).*

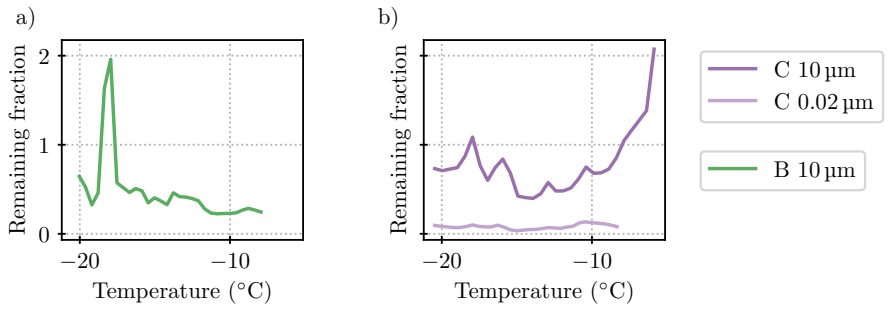

Figure 1: Relative contributions of the different size fractions to n_m for sample B in **a)**, and sample C in **b)**, i.e. $\frac{n_{\mathrm{m,10\,\mu m}}}{n_{\mathrm{m,unfiltered}}}$. To make the data comparable and allow for a division, the spline fit from de Almeida Ribeiro et al. (2023) was employed and the results were interpolated linearly. For the $10\,\mu\mathrm{m}$ size fraction of sample B, the point at $-14\,^{\circ}\mathrm{C}$ and $8.4 \times 10^4\,\mathrm{g}^{-1}$ needed to be excluded to allow for a smooth fit.

2.2. Discussion section

RC: ***A few things to point out here.***

RC: ***1. Some of this section belongs in the intro (lines 343-356), as it does not actually help describe or explain the study results.***

AR: *We have moved the section to the introduction as suggested.*

RC: ***2. This section would be much more impactful if there was a comparison figure shown, since there are a handful of previous studies evaluating INPs in lichen (something like spectral "ranges" as in Fig 1-10 of Kanji et al. (2017) or a bar plot of spectral ranges as in Fig 4 in Creamean et al. (2021)). It is difficult to put the current results into context without some sort of summarizing visual.***

AR: *We welcome this suggestion and have added a new figure to the discussion section. We took the time to digitise the old data from the Kieft papers and contacted Eufermio for their data rather than plotting ranges. The caveat with this comparison is that the preparation methods are different (which is why we refrained from doing it in the past), but we have included this caveat and discussed the figure appropriately. The discussion has been revised accordingly, but not reproduced here to be keep the response succinct. The fact that our activity is at the low end of the reported activities may well be related to the sample preparation method.*

RC: ***3. Intercomparing onset freezing temperatures with previous studies using different instruments is not recommended. Instrumental limitations and detection limits can yield different ranges of freezing onsets. Try to refrain from onset comparisons unless doing so with just the samples in the current study.***

AR: *We agree and have followed the referee's advice and removed that comparison. Onset comparisons can be logical if the activity is very steeply temperature dependent, but this is not a given here, so justification of the comparison would need to be given (which would distract from the main points we are making).*

RC: ***4. The main idea behind this study is to test what local sources might have been observed in the air. Thus, putting these findings into the context of those from the air during HyICE-2018 should be included here. This text basically exists on lines 369-377 in the conclusion section, but I suggest moving it to the discussion.***

AR: *We have followed your suggestion and moved that paragraph to the discussion section.*

RC: ***5. Along these lines, what other sources could be possible during the winter? What about anything on the needle surfaces or resuspension from the snowpack? I realize these were not tested here, but other realistic possibilities should be mentioned and cited.***

AR: *Yes, these other potential processes were not tested here so we do not want to discuss them in detail. We have inserted a brief mention of them in the discussion section.*

> Alternatively, the biological INP observed during HyIce-2018 might have come from a different source. Possibilities include release of INP from the needles or other surfaces of pine trees (Seifried et al., 2023) or perhaps from blowing snow that might release aerosol if snow particles sublime (Frey et al., 2020).

2.3. Specific comments

RC: ***Line 3: Specify that these were ambient/airborne INPs, not to be confused with the current work.***

AR: *We have specified this in the abstract as follows:*

> During the HyICE-2018 campaign, which took place in the boreal forest of Hyytiälä, substantial concentrations of airborne, heat sensitive biological INPs were observed despite many potential biological sources of INPs being snow covered. A potential source of INPs that were not covered in snow were lichens that grow on trees, hence we investigated these lichens as a potential source of biological INPs in this boreal forest environment.

RC: ***Lines 7-8: The abstract does not entirely reflect the actual findings. The authors report on the 0.02 µm results being the most substantial, but filtration was only done on one of the samples. In the paper, several times (e.g., lines 256-257, the discussion, and the conclusions), it is indicated that most of the INPs were in the 0.1-10 µm range. That should be reported here instead.***

AR: *We remain with our point that activity remains at small sizes, but have agreed to highlight the* $0.1\,\mu\mathrm{m}$ *filter size instead as it is tested more in our study.*

RC: ***Lines 44-46: Why is one study reported in onset freezing range and one in median? It is best to compare apples to apples and report median or another common value for both. (Intercomparing onset temperatures between different techniques is not advised; see comment above.)***

AR: *That's because the two studies only give us onsets and medians and no common value, which we agree is regrettable.*

RC: ***Lines 120-121: Why stored at room temperature and not at least close to the temperature in which the samples were collected? The authors should provide a statement about the caveats in possible changes to the samples while stored at room temperature. Often, vegetation samples are stored in a refrigerator (something like 4C) or even frozen. The authors cite Stopelli et al. (2014) which describe that certain conditions are known to activate the ice-nucleating activity of bacterial cells. While they are referring to samples in snow water, bacterial activity can change when the temperature of the air increases (i.e., what can happen in the summer compared to winter samples).***

AR: *We have added a sentence to make this caveat explicit. However, we also note that storing in a sealed bag at*

room temperature meant the samples were stored at low relative humidity, which tends to inhibit biological activity. Nevertheless, storage is a problem that we generally face and is unsatisfactorily resolved. Freezing is known to degrade samples, as is storing in a refrigerator, so these standard approaches are not necessarily suitable.

> By storing at room temperature, the samples were preserved at low relative humidity, conditions under which biological activity is inhibited. Nevertheless, we note that the storage at room temperature was pragmatic, and it is possible that the activity of the samples might be somewhat dependent upon the storage conditions.

RC: ***Table 1: Why were these particular percentages of species chosen for B and C? There needs to be some justification provided in the text.***

AR: *We have added a sentence clarifying that the percentages were chosen to mimic the perceived concentrations in the bag.*

RC: ***Line 320: Why is a quantifiable comparison not possible?***

AR: *As you state above, that is because onset freezing temperatures cannot be compared between setups. We have followed your advice above and removed that section now.*

RC: ***Line 322: Saying they "also" found high variability does not align with the current study. The species presented in Fig 7a look pretty similar. But this is also admittedly subjective. Making this statement more quantifiable, for example by using X orders of magnitude spread at select temperatures, and comparing the same to Eufemio et al. would get the point across, if indeed there was high variability found in both that study and the current one. If not found, why might this study not have "high variability"?***

AR: *We have made that statement more precise.*

> They also found high variability in ice nucleating activity between species of lichen (T_{50} of -8 and $-15\,^{\circ}\mathrm{C}$ for the boreal samples; compare to Fig. B1) as well as sensitivity to heat.

RC: ***Lines 335-336: What are the typical sizes of whole spore soredia or isidia? Some spores can have sizes down to*** $2\,\mu\mathrm{m}$ ***but I am not certain about these two specifically.***

AR: *According to Bowler and Rundel (1975), soredia are* 25 *to* $100\,\mu\mathrm{m}$ *in diameter; and isidia are between* 10 *and* $300\,\mu\mathrm{m}$ *in diameter and* 500 *and* $3000\,\mu\mathrm{m}$ *in height.*

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