

Author Response to Reviews of

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

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

We thank the reviewers for taking the time to write constructive comments.

1. Reviewer Comment # 1

RC: *The work by Proske et al. investigates the role of lichen IN from a boreal forest as biological INPs. The authors investigate four different lichen species and state in their introduction that ...While considerable attention has been paid to INPs of bacterial origin, there has been comparably little interest in the ice-nucleating ability of particles that stem from lichen. However, a quick google scholar search revealed that this statement is incorrect. In fact, a study by Eufemio et al. (2023) investigated Lichen as potential INPs in great detail. It is puzzling how the authors have overlooked this study as the topic is identical to their study. In fact, of the four investigated lichen species in this manuscript, three were already reported by Eufemio et al. The samples were also derived from similar snow-covered ecosystems. Eufemio et al. had also already done tests for heat stability and freeze-thaw cycles. In light of that manuscript, the current study does not add any new information to the topic besides confirming the results by Eufemio et al.*

AR: *We thank the referee for bringing this important recent paper by Eufemio et al. to our attention. We agree that it should be cited in the introduction and our results should be discussed in light of this paper. However, we disagree with the statement that our study 'does not add any new information to the topic'. Eufemio et al. present a survey of 29 lichen species from Alaska and find that the T50 values range from -5.2°C to -14.5°C. They produced these results by taking bulk lichen samples and grinding them, thus releasing ice-nucleating entities from within the body of the lichen as well as those that might reside on the surface and might be more likely to become aerosolised. They also showed that the heat sensitivity is complex, with different species responding in different ways. They did not study the size of the ice-nucleating entities. Based on Eufemio et al. it is not clear at all what we would have expected in our study for lichens collected in southern Finland. Our study is the first to report the ice nucleating activity of lichens from S. Finland's Boreal forests. Furthermore, Hyttiala is an important atmospheric research site and it is therefore all the more important to characterise potential sources of INP there. We have added references and discussion of Eufemio throughout the paper. In doing so, we restructured the introduction. We explicitly discuss the Eufemio paper in the following:*

*Several studies have shown that lichens from a range of environments and across multiple lichen species nucleate ice (Kieft, 1988; Kieft and Ahmadjian, 1989; Ashworth and Kieft, 1992; Moffett et al., 2015; Eufemio et al., 2023). In an early study Kieft (1988) examined 15 lichen. Nearly all of them showed ice-nucleating activity at -8°C , with -2.3°C as the highest onset temperature. The bacteria that could be cultivated from the lichen showed no ice nucleation activity. Moffett et al. (2015) and Eufemio et al. (2023) between them surveyed the ice nucleation activity of 86 lichen samples and found that while ice nucleation was ubiquitous these lichens had remarkably varied ice nucleating abilities. Moffett et al. (2015) report onset freezing ranging from -5.1°C to -20°C , while Eufemio et al. (2023) report median freezing temperatures between -5.2°C to -14.5°C . In addition, there is substantial variability in ice nucleation between different samples of the same species of lichen. For example, one sample of *Evernia Prunastri* nucleated ice at -5.6°C while another nucleated ice at -10°C (Moffett et al., 2015). These studies show the ubiquity of ice nucleation in lichens, but given the observed variability in ice nucleating activity, we cannot simply infer that lichens in one environment possess the same ice nucleating activity as the same lichen species in other environments.*

And in the discussion we include:

Eufemio et al. (2023) recently presented a study of 29 lichen species from Alaska, some of which were sampled from boreal forests. They also found high variability in ice nucleating activity between species of lichen as well as sensitivity to heat. They performed detailed analysis on three lichen species demonstrating that there are two populations of ice active material, one active around -7°C and one at around -14°C . They also showed that, while the samples were generally sensitive to heat, these different populations of ice active material responded differently to their heat treatment. They interpreted this as evidence that there are different molecular compositions of ice nucleating materials in lichens.

We have also improved the conclusions section, including adding a short paragraph on how our measurements are consistent with measurements of ambient INP during HyICE-2018.

The size and heat sensitivity of ambient INPs during HyICE-2018 has some consistency with the properties of the lichenaceous INPs we studied here. Schneider et al. (2021) report that the ambient INPs were strongly heat sensitive with all activity above -13°C being removed on heating. The size of INP during HyICE-2018 is also reported by Porter et al. (2020) who revealed that the $0.25\ \mu\text{m}$ to $0.5\ \mu\text{m}$ fraction contained more INPs (above -22°C) than any of the larger size fractions in their tests. Porter et al. (2020) comment that the more normal dependency, based on literature data, is that larger aerosol particles contribute more INPs than smaller aerosol particles, hence their finding was unexpected. As mentioned in the introduction, during HyICE-2018 the forest floor was covered in snow, thus preventing emission of bioaerosol associated with leaf litter or soil, whereas copious quantities of lichen were exposed to the air. Thus, a viable explanation for the heat sensitivity and the size of ambient atmospheric INPs during HyICE-2018 is that they are derived from lichens.

RC: *In addition, the current work has additional flaws in their manuscript that should be addressed. The study puts emphasis on the potential size dependency of the lichen INP. Yet again an important study on this topic seem to have been overlooked as Kieft and Ruscetti (1992) used gamma radiation to determine size of the INs.*

AR: *We have added a sentence on this paper in the introduction:*

Using a relationship between molecular size and the likelihood to become deactivated on exposure to gamma radiation, Kieft and Ruscetti (1992) found a logarithmic relationship between freezing temperature and protein size.

RC: *How did the authors determined which lichen species they investigated? visual inspection? genetic testing?*

AR: *We used a visual inspection method, similar to Eufemio et al., in combination with the knowledge of what common lichen species are present in Southern Finland. While DNA analysis would provide robust data, in a 'normal' production forest in a boreal climate zone, the possible number of species is rather modest, so visual assessment is sufficient. The species listed here are very typical to this kind of forest.*

RC: *How did the authors ensure that the lichen were not contaminated with e.g. bacteria on top of the lichen. Any washing steps prior to analysis?*

AR: *We took the approach where we did not wash the samples, unlike in Eufemio et al. paper. Washing would likely remove ice nucleating material that is loosely bound to the surface of lichen. We think that it is this loosely bound material, such as the soredia or isidia (that can become aerosolised) that is likely to be important in the atmosphere. Furthermore, washing might remove bacteria that are now thought to be part of the symbiosis in lichen (Grimm et al., 2021). Hence, we chose not to wash the samples prior to use. We have added a new paragraph in the methods section to make our rationale clear.*

Examples of the structures that can become aerosolised are shown in Fig. 3. Panel a shows soredia on *Platismatia glauca*, while panel b shows isidia on *Evernia prunastri*. These vegetative diaspores can be broken off the thallus through the action of wind, rain droplets or even animals. The recognition that it is likely these fragile structures on the surface of the lichen that preferentially become airborne helped us to design a droplet freezing assay that is appropriate. In some previous ice nucleation studies the lichens were ground with some water to produce a pulp that was then suspended in water (Kieft, 1988; Eufemio et al., 2023). This approach might be appropriate for studying the water harvesting properties of lichens, but may be less relevant for understanding atmospheric implications. In addition, the practice of washing lichen samples to remove non-lichen ice-nucleating entities may inadvertently remove the soredia and isidia, the very entities of particular interest. Hence, we used an approach where lichen was exposed to water and gently agitated in order that fragile structures, like the soredia or isidia, might be removed (details in the next section). The large pieces of lichen were allowed to settle to the bottom of the vial and then the aqueous supernatant, which was clear to the eye, was sampled for the droplet freezing assay. While this approach does not replicate bioaerosol production from lichen, it does bias the analysis towards the entities associated with lichen that are likely to become aerosolised.

RC: *Lichen is a symbiosis of more than two partners, the idea that is only two is outdated and has been disproven.*

AR: *Thank you for pointing us to this relatively new knowledge. We had focused on the text-book understanding, which is obviously somewhat out of date. We have added the concept to the text as follows:*

For many years lichens were thought to be symbiotic organisms composed of a fungal partner, the mycobiont, and a photobiont partner (Nash, 2008). However, it is now recognised that in addition to the mycobiont and photobiont (algae/cyanobacteria), lichen species can accommodate several additional symbionts, including yeasts and bacteria, associated with the fungus or locally living in the microhabitats of lichen thalli (Aschenbrenner et al., 2016; Cernava et al., 2017; Grimm et al., 2021).

RC: *p.3 l. 76, the Reference Schwidetzky et al. (2023a) would be fitting as it is the first study that provides conclusive evidence that fungal IN are proteins*

AR: *We added this citation:*

We also know that some fungal materials produce proteins that nucleate ice effectively and these proteins can become separated from the mycelia (O’Sullivan et al., 2015; Schwidetzky et al., 2023b).

and

This size dependence is consistent with the idea that larger aggregates of proteins have the potential to nucleate ice at higher temperatures (Schwidetzky et al., 2023a).

2. Reviewer Comment # 2

RC: *The manuscript presents an analysis of the ice forming activity of lichen wash water. Lichen samples growing on Scots pines in Hyytiälä, Finland, were collected, washed, and the ice forming activity of the water was determined using a drop freezing experiment. In addition, the washing water was filtered and subjected to heat-treatment to study the size and nature of the ice nucleating particles.*

RC: *The manuscript is well written and gives an overview of many previous studies. However, the new experimental work does not appear to go beyond the screening a few samples and the interpretation of results appears speculative. In its current form the manuscript does not present substantial and convincing new data.*

AR: *This is a study that is explicitly focused on lichens in Hyytiälä in light of the measurements that show a substantial population of biological INP from an unknown source. It is not intended to go beyond the common lichens that were present in the forest during the HyICE campaign. Importantly, no one has studied the lichens present in S. Finland in the past and given the massive variability observed in other locations in ice nucleating activity (even within single species) it was not clear to what extent the lichens in Hyytiälä would nucleate ice. Furthermore, many previous studies have taken entire pieces of lichen or ground lichen samples to release ice nucleating entities into water. We have taken a more subtle approach of washing the lichens in an attempt to release only those entities that might become aerosolised. We have successfully addressed the question whether lichens in Hyytiälä contain ice nucleating material that could conceivably become aerosolised. This shows that it is worth the time and effort involved in performing future aerosolisation work where someone might attempt to quantify the production of INPs from lichen as a function of wind speed, RH etc. In order to make our objectives clearer, we have restructured the introduction and included the following at the end of the introduction:*

In this paper we report the ice nucleating ability of lichens that were present and exposed in the boreal forest at the Station for Measuring Ecosystem-Atmosphere Relations (SMEAR) II located in Hyytiälä, Finland during the HyICE-2018 campaign. The HyICE-2018 campaign was focused on measuring atmospheric INPs between February and June 2018 (Brasseur et al., 2022). Schneider et al. (2021) report the presence of heat sensitive biological INPs during the campaign. 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. In this paper we tackle the question of if the lichens in the forest during HyICE-2018 contained ice-nucleating entities. We see this as a first step to addressing our hypothesis and 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.

In order to address the referees concerns around speculation, we revisited our abstract and focused it. The modified section is below:

INPs derived from lichen sampled during HyICE-2018 are shown to nucleate ice at temperatures as warm as -5°C in aqueous suspensions extracted from 0.03 g mL^{-1} lichen. Successive filtration to smaller sizes removes some of the most active INPs in suspension, but substantial activity remains even when filtering to $0.02\text{ }\mu\text{m}$. The small size of the INPs from lichen means they have the potential to either be emitted directly into the atmosphere or be associated with larger particles, such as lichenous reproductive aerosol types (spores, or diaspores). We also show that the INPs from lichens from Hyytiälä are sensitive to heat, which is similar to the INP sampled from the atmosphere of Hyytiälä and consistent with the presence of ice-active proteins. This study shows that lichen from a European Boreal forest in Hyytiälä harbour INPs, which may be especially important in this snow covered habitat where few, if any, other biological INP sources are available. The great terrestrial abundance of lichens in Hyytiälä, and around the world, calls for further research to combine their ice nucleating ability with dispersal studies to evaluate the flux of lichenous INPs into the atmosphere as well as to what extent these particles reach heights and locations where they might influence cloud properties.

2.1. Introduction

RC: *Line 79-80: Explain how ice formation might help to change physiological activity and indicate in what way.*

AR: *We have edited this section:*

Once a small amount of water is frozen on the thallus, more water may preferentially deposit on it. Later, when the temperature increases, this ice may melt and the liquid water would become available to the lichen. This process is all the more important, since lichen lack stomata and are therefore not able to actively control water loss as many plants do (Kappen and Valladares, 2007).

2.2. Methods

RC: *Line 90 ff: Clarify why the approach was to use a mixture of lichen species for heat-treatment and detailed filtering experiments rather than a single species. The approach seems more suitable for an initial*

screening or a feasibility study.

AR: *We wanted to know if the lichens present in Hyttiäla contained ice nucleating material. Knowledge of the ice nucleating ability of the single species is clearly academically interesting, but the first order question is if the lichens in Hyttiäla contain ice nucleating material. We have made this clearer:*

We examined mixed samples of lichen for their ice nucleating ability, size of the ice-nucleating species and the heat sensitivity rather than solely focusing on single species in order to reveal if the lichens in Hyttiäla harboured ice nucleating entities and obtain an indication of their activity. This allowed us to address our stated objective of determining if there is a potential source of biological INPs associated with the prolific lichen population in Hyttiäla.

RC: **Line 110 ff. Was the lichen separated from the tree bark?**

AR: *Yes, it was. This is now stated in the methods.*

RC: **Line 114: Explain why an attempt was made to mimic sample B.**

AR: *We wanted roughly the same proportions of the lichen species to get a replica and to see how different the results would be if we changed the method of agitation in water. Sample B was carefully rotated in water by hand for 10 mins, while C was mixed on a rotary mixer for 30 mins at 30 rotations per min. These details have been added to the table as well as the text. In the text we have clarified this and now state:*

Both sample B and C had a similar proportion of the different lichen species, but the sensitivity to the mixing method was explored.

RC: **Line 118 ff., 123ff: Explain why the particle fraction resembling the windblown particles is suspended (or not) in a gentle, wet extraction. What difference can be expected between wind and water extraction? Smaller or larger particles, more or less numerous? With the aim of using this type of measurement to estimate the atmospheric ice formation potential of lichens from the lichen mass, the similarity of particles washed off to wind-blown needs to be clarified.**

AR: *At no point have we claimed that the wet extraction processes produces a ‘particle fraction resembling the windblown particles’. But, we do think our approach is more relevant to the atmosphere than simply grinding whole lichen samples. We have added the following text to the methods to explain our rationale:*

Examples of the structures that can become aerosolised are shown in Fig. 3. Panel a shows soredia on *Platismatia glauca*, while panel b shows isidia on *Evernia prunastri*. These vegetative diaspores can be broken off the thallus through the action of wind, rain droplets or even animals. The recognition that it is likely these fragile structures on the surface of the lichen that preferentially become airborne helped us to design a droplet freezing assay that is appropriate. In some previous ice nucleation studies the lichens were ground with some water to produce a pulp that was then suspended in water (Kieft, 1988; Eufemio et al., 2023). This approach might be appropriate for studying the water harvesting properties of lichens, but may be less relevant for understanding atmospheric implications. In addition, the practice of washing lichen samples to remove non-lichen ice-nucleating entities may inadvertently remove the soredia and isidia, the very entities of particular interest. Hence, we used an approach where lichen was exposed to water and gently agitated in order that fragile structures, like the soredia or isidia, might be removed (details in the next section). The large pieces of lichen were allowed to settle to the bottom of the vial and then the aqueous supernatant, which was clear to the eye, was sampled for the droplet freezing assay. While this approach does not replicate bioaerosol production from lichen, it does bias the analysis towards the entities associated with lichen that are likely to become aerosolised.

RC: *Does exposure of lichens to Milli-Q water cause lysis?*

AR: *This is an interesting question that we haven't been able to find a clear answer to.*

RC: *Section 2.4. Clarify whether samples are first filtered and then heated or vice versa.*

AR: *This became a little confusing because we did tests where we explored the effect of the order of treatments. But, in those experiments we also had issues with the reproducibility associated with poor mixing. We have decided to remove the dilution series of the heated samples. The undiluted heat tests demonstrate the heat sensitivity and by removing the dilutions we remove complexity and ambiguity (see below). We also only did the dilutions for a subset of the heated samples.*

RC: *Section 2.5.: For brevity, consider referring to previous descriptions and only mention steps specific to the present experiments.*

AR: *We feel that the description of the technique is already concise and specific to the present experiments. It isn't clear what we would remove.*

RC: *Line 162 ff.: If I understand correctly, the initially added lichen mass did not dissolve in the water. Why was the initially added lichen mass used to normalise the measurements if the lichen mass was not contained in the droplets? Also, filtering removes the lichen mass from the suspension. Justify the use of the initially added lichen mass to normalize data from filtered samples.*

AR: *We normalise to the initial mass of lichen because we need to be able to compare results from the various heat and filtration tests. By normalising to initial mass of lichen, changes in n_m reveal relative changes in activity. This is a common approach and understood by the community (e.g. Murray et al. (2012)). We have added:*

We normalise to the initial mass of lichen in order that we can quantify the relative changes in activity on dilution, heat tests and filtration.

2.3. Results

RC: *Line 171: Clarify how the hypothesis can be confirmed by the present study? Considering that the water samples may not be representative of aerosolized samples (line 124-125) and that both the concentration of lichen particles in the atmosphere and their ice forming capacity need to be known to make this assessment (line 83-84).*

AR: *We made the scope of our work much clearer in the final paragraph of the introduction. We agree that more work is needed, but our study shows that the next phase of this work is indeed justified and worthwhile.*

RC: *The measurement data can possibly be better interpreted if they are presented as fraction frozen instead of as site density normalized to lichen mass. Below is a comparison of the fraction frozen of non-heat-treated, non-diluted samples B and C (data taken from the provided Assets to the manuscript).*

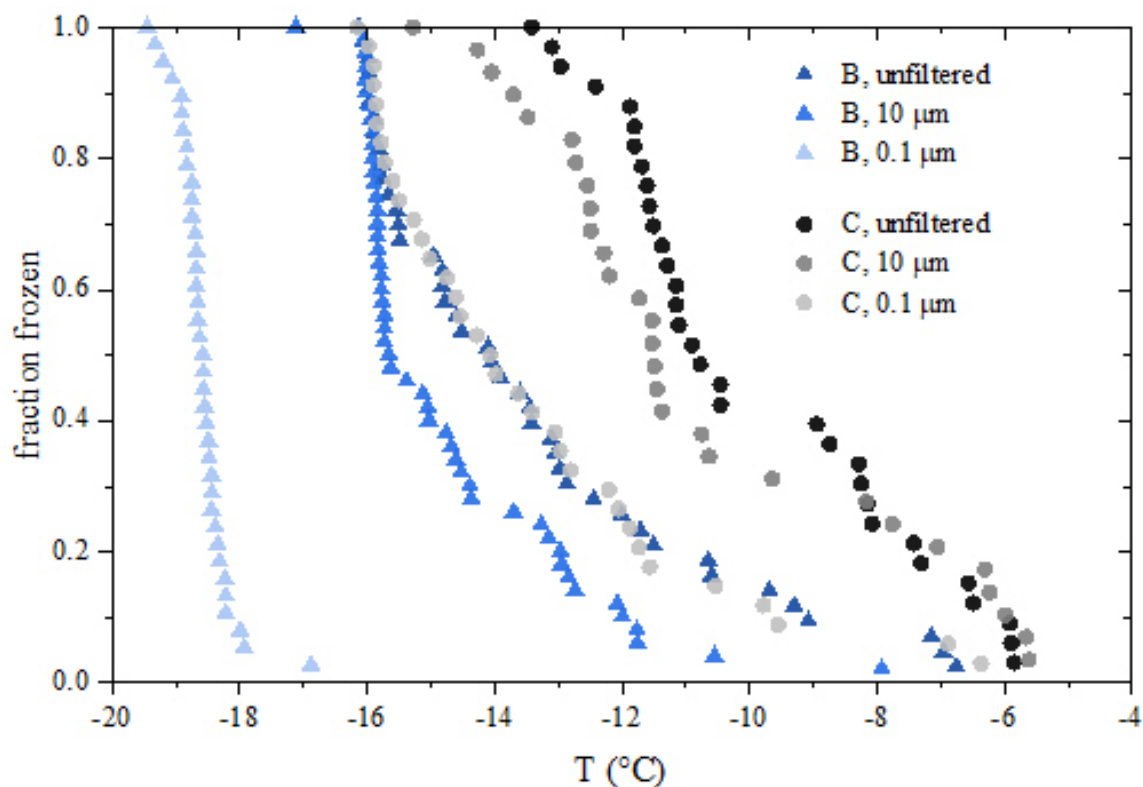


Figure 1

AR: *Fraction frozen curves are fine for comparing like-for-like experiments. But, when the samples are diluted or if different concentrations of lichen are used, then fraction frozen curves cannot be compared. The community has moved away from showing fraction frozen curves (e.g. Murray et al. (2012)). However, we do take on board the more general principle that Fig 6 was hard to understand as there was just too much data in it. We have produced a revised fig 6, with multiple panels.*

RC: *Line 177-178: As can be seen in the plot above, there is a clear offset in the fraction frozen (FF) between the unfiltered and the 10 μm filtered B sample (blue triangles). They cannot be considered similar.*

AR: We have qualified what we mean by similar by adding in 'within 2 °C'.

RC: **Line 193: The experimental detection limit makes the comparison of heat-lability at different temperatures questionable. There is no data above -15°C in the heat-treated data that can be compared.**

AR: On the contrary, the heat test is very clear. We direct the reader to Daily et al. (2022). But, there are clear shifts in the spectra on heating, with no observed freezing above -16°C after heating.

RC: **As shown in your Fig. 4 c) and d), there seems to be a large offset between original and dilutions of heat-treated samples. Can this be explained?**

AR: There are some offsets, yes. We had done a bit more work on this aspect, but removed it from the paper for the sake of simplicity. We found that there are sometimes issues around pipetting these samples, where if the suspension isn't well mixed you can get more or less material in the dilution than expected. We took these learnings to improve our approach. Given the ambiguity in the dilutions of the heat test we have removed these results from the figure. This does not change the conclusions, but removes ambiguity and complexity.

RC: **Line 220: Explain why lower concentrations can be expected for smaller size fractions.**

AR: If you remove the higher temperature INP, you naturally reduce the concentration across the full spectrum given n_m is a cumulative quantity. We have added:

(because higher temperature INP are removed by filtration and this reduces the concentration across the full spectrum since this is a cumulative quantity)

RC: **Line 224: The differences between sample B and C (see figure above) are surprisingly large considering all single lichen samples showed similar spectra.**

AR: Yes, we think this is attributed to the different extraction method. We have improved the pertinent paragraph which now reads:

The comparison of sample B1 and C can be seen in Fig. 6. Generally, greater concentrations of INP were present in sample C than in sample B1. As outlined in section 2.2, lichen samples B were mixed by hand for 10 min and C was mixed on a rotary mixer 30 min. The different procedures might contribute to the greater concentrations of INPs being released with the rotary mixer. This is consistent with the results for B1 and B2 where we saw more INP released with time, indicating sensitivity to the exact experimental procedure. It also should be noted that the experimental procedure for estimating the composition of the samples to be the same by eyesight was rather crude. Hence, different species of lichen may have different ice-nucleating characteristics. To explore this further we attempted to separate out the lichen species and test them individually.

RC: **Ideally, the single species experiments should reproduce the results of the mixed sample when their spectra are added together, weighted according to their proportion in the mixed sample. Can the results of the single species experiments be used to deduce what the difference between B and C might have been?**

AR: With standardised processes this might be possible, but to what end? The objective here was to test if the samples contained INPs.

RC: **Line 225 ff: Clarify how “more” and “less” heat-labile particles can be explained? Shouldn't “all” or “none” entities that are ice active at a certain temperature be affected?**

AR: *There are degrees of heat sensitivity. This is covered in detail by Daily et al. (2022), who test the heat test. Even with psuedomonas Syringae only a fraction of the ice-nucleating proteins are destroyed and the different classes of protein aggregates are affected to different extents.*

RC: *Fig. 5: Why were no dilutions measured for heat-treated samples C?*

AR: *This is now a mute point given we have removed the heat treated dilutions from B due to issues with reproducibility.*

RC: *Line 231-232: Unclear where this step can be seen. There is no data for the heat-treated samples C, unfiltered and 10µm at -18°C and a step can also be seen in Fig.5e) for 0.1µm.*

AR: *This is much clearer in the revised Fig 6.*

RC: *Line 232: Define which “characteristics for different sizes” are referred to and clarify how this can be used to infer the state of these INPs.*

AR: *We have made this clearer, by inserting '(activity and heat sensitivity)'.*

RC: *Line 238: The logarithmic scale used in Fig. 6 could be misleading here. Overlap can be checked more directly in FF plots. The figure above shows a comparison of the FF of sample B and C. It shows that the freezing signal of the two samples differs considerably.*

AR: *We have revised Fig. 6 to make it clearer.*

RC: *It is striking that sample B, unfiltered produced the same FF as sample C, filtered through 0.1 µm, raising overall doubt on the presented interpretation of size dependence. The data should be replotted as FF and features discussed based on such figures. The nm -plots obscure the data by scaling and plotting on a logarithmic ordinate.*

AR: *As mentioned above, this difference is attributed to the different extraction methods. It is not possible to plot the data in a meaningful manner as fraction frozen plots since the data contains dilutions.*

RC: *Line 240: What is considered 1 order of magnitude (small difference) in Fig. 6 corresponds to about 4°C (large difference) in the FF plot above.*

AR: *We have now qualified the shift in temperature as well as concentration in appropriate places in the text.*

RC: *Line 253: Indicate which small particles.*

AR: *We agree that this isn't clear. Revised to: 'simply show the same INP concentrations because entities such as the soredia and isidia may have been spread throughout the bag'.*

RC: *Line 257: Given the difficulty of reproducing measurements from sample B and C, it seems highly uncertain whether the result of a single measurement is reliable. All measurement should be repeated several times before they are compared and interpreted. Since droplet freezing experiments are neither time consuming nor expensive, several repetitions of experiments are desirable before conclusions are drawn.*

AR: *There is a discrepancy between sample B and C, because the preparation method was different. The discussion mentioned above is now clearer. In an ideal world we would repeat everything in triplicate, but in practice compromises need to be made due to limitations of resources and time. Rather than perform each measurement in triplicate we chose to perform dilutions, which also give a good idea of reproducibility in the region of overlap, but have the added benefit of extending the dataset to lower temperatures.*

2.4. Discussion

RC: *Line 262: Can recommendations be given on how to measure the atmospherically relevant ice formation activity of lichens in a comparable way?*

AR: *This is something we have been giving thought to and had planned to do. However, the pandemic prevented us from doing this work. At the end of the conclusions section we briefly mention the idea of making use of a wind tunnel and that we could use an online INP counter to quantify INP production as a function of RH, T etc. This would clearly require substantial resources and is beyond the scope of what we can do for this paper.*

RC: *Line 264-278: A comparison of “INPs per gram of lichen” between studies in which the lichen material remained in droplets for freezing experiments (ground powder) and experiments with washing water in which the lichen matter is not contained in the water droplets seems incorrect. Explain how this comparison of concentrations can be justified. Consider limiting the comparison to temperatures at which specific features are observed.*

AR: *We prefaced this section with a statement that comparison between different approaches is difficult for exactly this reason. We have emphasised this and now state ‘This makes a quantitative comparison of INP concentrations challenging and therefore needs to be done with some caution.’*

RC: *Line 290 ff: The reported size of the lichen INPs is questionable as the same freezing curve was measured from sample B, unfiltered and sample C, filtered through 0.1µm filter (see figure above).*

AR: *We dealt with this misunderstanding above.*

RC: *Line 304: What is the size of the propagules? Consider adding a definition.*

AR: *This refers to spores, soredia and isidia etc. We defined sizes in the introduction. We have added ‘(e.g. spores, soredia and isidia)’*

RC: *Line 318 f: A correlation to which meteorological variable would provide a strong indication of lichen INP?*

AR: *We have added in brackets the variables we are referring to.*

RC: *Line 327: The decrease in concentration with size indicates that a large fraction of these INPs are larger. Clarify what fraction of the -16°C species is smaller than 0.02µm.*

AR: *We would rather not quantify the fraction, this would serve no obvious purpose and be prone to uncertainty.*

RC: *Line 329: This conclusion does not appear to be supported by the data. Figure 4 shows a reduction in all sized after heat treatment.*

AR: *The point is these INP are removed only when also filtered to small sizes.*

RC: *Line 330: Clarify which characteristics are referred to and explain how they support the suggestion of these two different states.*

AR: *We have attempted to clarify our thinking here:*

These differing sensitivities to heat across different size ranges suggest that the INP species responsible for freezing at -18°C was present in two different states, attached to a larger particle or free in solution or in different states of aggregation. If it was attached to larger entities or in large aggregates it would be lost on filtration, but is also apparently heat stable. In contrast when it is attached to small particles or in free solution, it is more sensitive to heat.

RC: *Line 335-336: Clarify how it can be concluded whether a species is an important source of INP based on the current results without knowing the abundance of lichen particles in the atmosphere.*

AR: *We have replace 'are' with 'may be'.*

RC: *Line 337ff: Since there is no information on the abundance of such smaller lichen entities in the atmosphere, it is speculation whether they contribute INP. Furthermore, the fact that they may be dissolved in water does not necessarily indicate their presence in the atmosphere.*

AR: *Yes, that is correct. That is why we need wind tunnel measurements.*

RC: *Line 340ff: As the authors explain below (Line 343ff), the ice activity of lichens without atmospheric concentration data is not evidence that they are a source of atmospheric INPs, and other approaches are necessary to clarify the importance of lichens as INPs.*

AR: *This is correct and consistent with what we say.*

2.5. Technical corrections

RC: *Line 18: Provide a reference to “formation of ice in clouds is amongst one of the least well understood of these processes.”*

AR: *Done. (Murray et al., 2021; Tan et al., 2016)*

RC: *Line 29-30: References should be in brackets.*

AR: *This has been corrected.*

RC: *Line 47: should it be “Fruticose” instead of “Fructiose”?*

AR: *This is correct and this has been corrected.*

RC: *Line 123: Clarify what is meant by “metal housing”. The filter holder?*

AR: *Replaced with 'Advantec 301000 stainless steel filter holder'*

RC: *Line 158: Define “EF600”*

AR: *This is the machine's name. Added '(EF600 Stirling engine chiller, Grant-Asymptote)'*

RC: *Line 176: consider introducing sample B1 and B2 in Sect. 2.2*

AR: *Added 'Sample B is split into B1 and B2 in the manuscript, B2 was sampled from the same suspension one day later, so had had more time to release INP into suspension/solution.'*

RC: *Line 280: ... temperatures in our study.*

AR: *Corrected.*

RC: *Line 290: Provide a reference for the smallest lichen spore size of 1µm.*

AR: *This is defined in some detail in the introduction.*

References

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