

## 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

## 1. Reviewer Comment # 1

AR: *We thank the reviewer for taking the time to write constructive comments.*

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  $-8^{\circ}\text{C}$ , with  $-2.3^{\circ}\text{C}$  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  $-5.1^{\circ}\text{C}$  to  $-20^{\circ}\text{C}$ , while Eufemio et al. (2023) report median freezing temperatures between  $-5.2^{\circ}\text{C}$  to  $-14.5^{\circ}\text{C}$ . 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  $-5.6^{\circ}\text{C}$  while another nucleated ice at  $-10^{\circ}\text{C}$  (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  $-7^{\circ}\text{C}$  and one at around  $-14^{\circ}\text{C}$ . 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  $-13^{\circ}\text{C}$  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  $-22^{\circ}\text{C}$ ) 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).*

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