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

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Abstract. Ice nucleating particles (INPs) facilitate heterogeneous freezing of cloud droplets and thus modify cloud properties. Hence, it is important to understand the sources of INPs. During the HyICE-2018 campaign, which took place in the boreal forest of Hyytiälä, substantial concentrations of biological INPs were observed despite many potential biological sources of INPs being snow covered. A potential source of INP that were not covered in snow were lichens that grow on trees, hence we

5 investigated these lichens as a potential source of biological INPs in this boreal forest environment. INPs derived from lichen sampled during HyICE-2018 are shown to nucleate ice at temperatures as warm as $-5\,°C$ in aqueous solutions extracted from $0.03\,\mathrm{g\,ml^{-1}}$ lichen. In particular, we identify two species of INPs active at -16 and $-18\,°C$ and smaller than $2\,\mu m$ in size, which makes them good candidates for atmospheric INPs. These particles are smaller than whole lichen spores. In species specific tests we show that the four investigated lichen species harbour differently sized INPs, which might suggest that some

10 species of lichen are more important as a source of INPs than others. This study shows that lichen from a European Boreal forest harbour INPs, which may be especially important in habitats where few other biological INP sources are present, such as in a snow covered environment. The great terrestrial abundance of lichen INPs 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.

1 Introduction

15 Clouds are a crucial part of the hydrological cycle and strongly affect Earth's radiative balance (Boucher et al., 2013). Clouds properties are affected by a range of dynamical and microphysical processes, and it is becoming increasingly apparent that the formation of ice in clouds is amongst one of the least well understood of these processes. Heterogeneous freezing of clouds droplets on ice-nucleating particles (INPs) influences precipitation, cloud lifetime and the radiative effect of tropospheric

20 clouds (DeMott et al., 2010). However, the identity, sources and transport and therefore global distribution of INPs are poorly

constrained (Murray et al., 2021). This is especially so for biological INPs, which are known to be active at relatively high temperatures, but are highly variable in concentration (O'Sullivan et al., 2018).

Organic particles from different primary biological origins have been shown to exhibit ice-nucleation ability: biological particles in soil (Conen et al., 2011; O'Sullivan et al., 2014; Hill et al., 2016), on plants (Hill et al., 2014), in sea spray aerosol (DeMott et al., 2016), and in the sea surface microlayer (Irish et al., 2017; Wilson et al., 2015). Primary biological particles including bacteria, pollen, fungal spores, plankton and diatoms have been shown to nucleate ice (Alpert et al., 2011; Schnell and Vali, 1976; Lindow, 1989; Pouleur et al., 1992; Pummer et al., 2012). Subcomponents of living matter have also been shown to nucleate ice, including cellulose and lignins (Hiranuma et al., 2019; Bogler and Borduas-Dedekind, 2020) . Furthermore, it has been shown that nanometer scale entities washed off fungus and pollen can be potent INPs O'Sullivan et al. (2016); Pummer et al. (2012); O'Sullivan et al. (2015). In bioaerosols, ice-nucleating ability is a selective property and only few bacterial strains and fungal species have been found to nucleate ice at high temperatures (Hoose and Möhler, 2012; Murray et al., 2012). Globally, more abundant INPs such as desert dust particles dominate the INP distribution at temperatures below about $-15\,°C$ (Vergara-Temprado et al., 2017). However, biological INPs are thought to have an influence on the hydrological cycle and climate at least on regional scales (Prenni et al., 2009; Spracklen and Heald, 2014; Vergara-Temprado et al., 2017).

The HyICE-2018 campaign, which this study is a part of, focused on measuring INPs in a boreal forest environment in Southern Finland, between February and June 2018 (Brasseur et al., 2022). Both Schneider et al. (2021) and Brasseur et al. (2022) report measured biological INPs during the campaign. The snow-covered ground rules out leaf litter or bare soil as sources of INP, but even in winter snow-free lichens attached to the branches and trunks of trees are ubiquitous in Hyytiälä (see Fig. 1). Lichens of various types have been shown to nucleate ice in the past (Kieft, 1988; Kieft and Ahmadjian, 1989; Ashworth and Kieft, 1992; Moffett et al., 2015), hence in this study we explore the ice nucleating ability of lichens that are exposed in the forest understory and canopy when other potential sources of biological INP, such as leaf mould, leaf surfaces or fertile soil dusts in the region, are largely covered in snow (see Fig. 1).

Lichen are symbiotic organisms, composed of a fungal partner, the mycobiont, and a photobiont partner. In most lichen species, the photobiont is green algae, and in only 10% it is a cyanobacterium (Nash, 2008). The symbiosis might be seen as a successful one, as lichen are found worldwide, from the tropics to the polar regions (Nash, 2008). Three growth forms of lichens are traditionally distinguished (Hale, 1974): Crustose lichen are in intimate contact with their substrate and cannot readily be separated from it. Foliose lichen have a leafy plant body, up to $0.3\,m$ in diameter. Fructiose lichens appear hair-like. The four lichen species examined in this study were *Hypogymnia physodes* (foliose), *Evernia prunastri* (foliose), *Bryoria sp.* (fruticose) and *Platismatia glauca* (foliose). Pictures of them can be seen in Fig. 2, and microscope images in Fig. 3. Lichen reproduce either sexually or asexually, producing spermatia (1 to $5\,\mu m$), spores (1 to $510\,\mu m$) or vegetative diaspores (10 to $3000\,\mu m$, e.g. soredia, which are clumps of algae cells enveloped by elongated cellular fungal threads and are 25 to $100\,\mu m$ in diameter, or isida, see Fig. 3), which all become aerosolised (Hale, 1974; Bowler and Rundel, 1975). While spores are forcibly ejected, vegetative diaspores rely on external forces to be removed from the thallus (Bowler and Rundel, 1975). The vegetative strategy allows the invasion of new habitats, and species using this strategy often have a greater world distribution than their sexual counterparts (Hale, 1974; Bowler and Rundel, 1975).

Figure 1. Lichens attached to trees are ubiquitous in Hyytiälä and remain exposed even when the ground is covered in snow (first photograph taken on 07.04.2018 and second on 22.03.2018).

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. 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. Moreover, sonication and heat treatment at $70\,°C$ did not remove

60   ice nucleation activity, but heat treatment at warmer temperatures did. Heat treatment has been widely employed as a test for the proteinacous nature of INPs (Pouleur et al., 1992; Christner et al., 2008; Conen et al., 2011; O'Sullivan et al., 2014; Moffett et al., 2015; Daily et al., 2022), since heat denatures proteins, which are thought to be essential for many biologicals INPs' ice-nucleating activity (Lundheim, 2002). Kieft (1988) concluded that the INPs from the lichen are nonbacterial in origin and suspected them to be either membrane-bound proteins similar to those in bacteria or secondary metabolites. Kieft

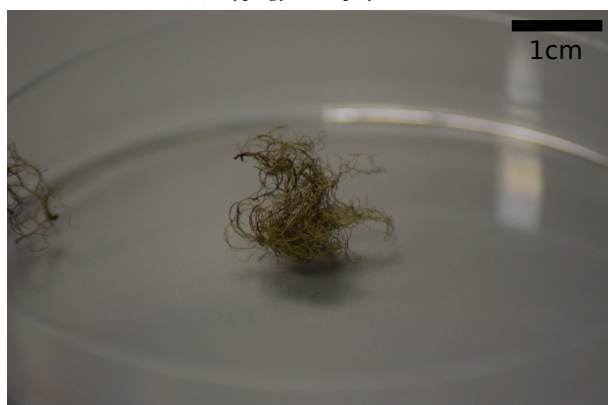
65   and Ahmadjian (1989) concluded that lichenous INPs are produced primarily by the mycobiont rather than the photobiont as the former showed ice nucleation activity at warmer temperatures. Kieft and Ruscetti (1990) argued that the sensitivity to proteases, guanidine hydrocholoride and urea could be taken as evidence for a proteinaceous nature of the INPs. 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). Ashworth and Kieft (1992) demonstrated ice nucleation activity in whole lichen thalli,

70   whereas in the studies before, the lichen had been ground and brought into suspension. Finally, Moffett et al. (2015) tested ice nucleation activity in 57 lichen and found that 74% of the samples nucleated ice above $-10\,°C$ in their particular assay, concluding that ice-nucleating activity is ubiquitous in lichen.

There are two hypotheses as to why ice-nucleating activity might have evolved in lichens (Kieft, 1988): Firstly, as proposed for ice nucleation active bacteria, lichen might benefit from nucleation of ice at relatively modest supercooling and the more

75   gradual formation of ice as it is less stressful to an organism than rapid crystallisation experience in greater supercoolings. During rapid freezing at great supercooling intracellular ice formation becomes more likely and this is usually lethal to cells

(a) *Hypogymnia physodes*

(b) *Evernia prunastri*

(c) *Bryoria sp.*

(d) *Platismatia glauca*

Figure 2. Pictures of the sampled lichen species that were tested for ice nucleation activity in this study.

(a)

(b)

Figure 3. Images obtained by stereomicroscopy of the sampled lichen that were immersed in water for the species specific testing. **a)** Soralia (see arrow) of *Platismatia glauca* that produce soredia, **b)** isidia (see arrow) of *Evernia prunastri*.

(Clarke et al., 2013; Daily et al., 2020, 2023). Secondly, ice nucleation might be a water-harvesting mechanism. Once a small amount of water is frozen on the thallus, more water may preferentially deposit on it. As most lichen species are extremely resistant to desiccation, the ice nucleation capacity could potentially serve as a means to rapidly modify their physiological activity with changing environmental conditions. In particular, as lichen lack stomata they are not able to actively control water loss (Kappen and Valladares, 2007), which points out the importance of physical processes in acclimation to varying moisture conditions.

In order to assess the potential effect that lichenous INPs have on cloud glaciation we need knowledge of both the concentration of lichenous particles in the atmosphere and their ability to nucleate ice. As a first step towards this goal we have tested the ice nucleating ability of lichens that are not covered in snow during late winter and spring in a boreal forest where biological INP were observed in the air.

2 Methods

In order to quantify the ice nucleating ability of lichens that are potentially relevant as sources of atmospheric INPs we collected samples and analysed their ice nucleating activity in the immersion mode using a droplet freezing assay. Samples were collected and as discussed below were sampled as mixtures of multiple lichen species. Our experimental approach was to subsample from these mixtures of lichen and test the activity of the mixtures of species, but also in a separate set of experiments to attempt to separate the various species.

2.1 Sample collection

The lichen was taken from Scots pine trees in a boreal forest environment in Hyytiälä with clean tweezers and placed in resealable plastic bags. One plastic bag contained specimen of *Evernia prunastri*, *Bryoria sp.* and *Platismatia glauca*, and two other plastic bags were filled with *Hypogymnia physodes*. The species were not collected seperately because they tended to grow in the same locations and clear separation in the field by eye was challenging. These bags were sealed and transported back to the University of Leeds on a passenger flight, at room temperature. They were stored at room temperature and experiments were conducted a month later. Lichen samples were imaged using a stereomicroscope (Stemi 508, Zeiss) and characterised with these pictures (see Fig. 3).

2.2 Sample preparation

$50 \, \mathrm{ml}$ of nanopure water was filtered through a $0.2 \, \mu\mathrm{m}$ filter (Minisart®, sartorius) and deposited into a $50 \, \mathrm{ml}$ polypropylene test tube. From this water, a blank was run on the Microlitre Nucleation by Immersed Particle Instrument (µl-NIPI, see section 2.5, Whale et al. (2015)) each morning to establish the baseline in the lab. In this way, the background signal could be evaluated, which determines the limit of detection for the instrument. The INPs examined in this study had freezing temperatures in between -5 and $-28 \, ^\circ\mathrm{C}$ (except for few dilutions where the freezing temperature was somewhat lower), with T_{50} approximately in between -15 and $-20 \, ^\circ\mathrm{C}$. Therefore, a blank was regarded as acceptable if the first droplet froze at below $-22 \, ^\circ\mathrm{C}$,

Sample name	Species present	Concentration $(\mathrm{g\,ml^{-1}})$	Dates of µl-NIPI runs
B	*Evernia prunastri* ($\sim 40\%$), *Bryoria sp.* ($\sim 10\%$), *Platismatia glauca* ($\sim 40\%$)	0.030	24.04., 25.04.18
C	*Evernia prunastri* ($\sim 40\%$), *Bryoria sp.* ($\sim 10\%$), *Platismatia glauca* ($\sim 40\%$)	0.033	02.05., 03.05., 04.05.18
Species specific tests	Every species separately		09.05., 10.05.18

Table 1. Overview of the lichen samples that were tested during this study.

T_{50} was at below $-26\,°C$ and the last droplet froze at below $-30\,°C$. The water from which the blank had been taken was then used further for immersing the lichen or for dilutions of suspensions. Samples that froze below $-25\,°C$ and thereby went into the range of considerable blank freezing (see Fig. A1), were excluded from the analysis.

Lichen was taken from its plastic bag with tweezers and placed in $50\,ml$ polypropylene centrifuge tubes (Falcon tubes, Fisher Scientific). The lichen's mass was noted as well as the amount of water which was then added to the tube. Lichen samples were taken from a mixture of lichen species stored in a single sealed plastic bag. While for sample B the composition was simply an accessible mix of the species, for sample C we tried to mimic the composition of sample B. The composition of the mixtures and the dates on which the samples were immersed in water and run on the µl-NIPI are given in table 1. For the species specific runs, samples of only one species of lichen were put into each centrifuge tube. Sample B was rotated carefully by hand for $10\,min$, while sample C and the species specific samples were left on a rotation mixer for $30\,min$, set at about 30 rotations per minute. In all procedures, care was taken to use relatively gentle approaches so as to minimise the break up of any structures in the lichen, since the atmospherically relevant INPs should be on its surface and readily removable.

2.3 Filtration

In order to learn more about the size of the ice-nucleating entities, samples were filtered prior to testing. $10\,µm$ (NY10, Merck Millipore), $2\,µm$ (TTP, Isopore™, Merck Millipore), $0.1\,µm$ (6809-6002, Anodisc, Whatman®) and $0.02\,µm$ (6809-6012, Anodisc, Whatman®) filters were used, placed within a $45\,mm$ metal housing. The filters were employed in front of a syringe filled with sample. It should be noted that the size of the particles immersed in water may be different from the size of the dry particles that might become aerosolised.

2.4 Heat Test

To test whether the ice nucleating particles are heat-labile, a polypropylene test tube with $1\,ml$ of sample solution was placed in a boiling water bath for $30\,min$. Different temperature heat treatments have been shown to have a different effect on biological INPs, and more deactivation happens with higher temperatures (Hara et al., 2016). In most studies, heat treatments that are meant to test for biological INPs involve heating at about $90\,°C$ for $10\,min$ (Hara et al., 2016; Christner et al., 2008; Moffett

et al., 2015; O'Sullivan et al., 2014). Longer periods of 20 and 45 min have been used as well (Garcia et al., 2012; O'Sullivan et al., 2015). In this study, the sample containers were placed in a bath of boiling water, hence the sample was warmed to above 90 °C, for 30 min as recommended by Daily et al. (2022). The sample was left to cool for a few minutes before being run on the μl-NIPI. Daily et al. (2022) have shown that not only biological INPs but also some minerals are affected by the wet heat

135 tests, but since our samples are clearly biological in nature we regard it as a valid method for the qualitative detection of protein based biogenic INPs.

2.5 Ice nucleation measurements with the Microlitre Nucleation by Immersed Particle Instrument

Droplet freezing techniques are widely used to study immersion mode freezing (Vali, 1971; Murray et al., 2012). In these techniques, droplets of a suspension are cooled and freezing events are recorded as a function of temperature. The volume of

140 the droplets determines the temperature range that can be investigated. In a bigger volume of the same concentration there are more INPs and therefore it is more likely that rarer INPs are present in the droplet, which are active at higher temperatures and dominate the freezing. Hence, multiple instruments with different droplets sizes and dilutions are needed to investigate ice-nucleating particles and their range of freezing temperatures.

  The Microlitre Nucleation by Immersed Particle Instrument (μl-NIPI) is used for droplet freezing experiments with 1 μl-

145 droplets. As such it was first described by Atkinson et al. (2013) and has since been employed in a range of studies (O'Sullivan et al., 2014, 2015). It has been part of an intercomparison between 17 ice nucleation measurement techniques in Hiranuma et al. (2015) and DeMott et al. (2018). In Whale et al. (2015), one can find a detailed instrument description.

  For the freezing experiments, a 22 mm diameter silanised glass slide (Hampton Research, HR3-231) is put on the aluminium plate after being rinsed thoroughly with methanol and nanopure water. If the samples had been prepared hours before being

150 tested, they were vortexed before being run on the μl-NIPI in order to homogenize the sample and stir up any particles that might have sedimented. Samples that were run in Leeds were vortexed for at least 10 s before each run. 30 to 50 1 μl-droplets were then pipetted directly onto the slide using a multi dispense pipette (Sartorius eLINE®). Suboptimal mixing before pipetting is visible in the results where e.g. dilutions do not line up, as discussed in Sec. 3. The aluminium plate was covered with a Perspex chamber. The chamber has openings for a camera (Microsoft LifeCam HD) and two pipes for flooding with dry nitrogen. The

155 nitrogen flow prevents frost growth and freezing due to contact with frost. Another benefit is that the nitrogen flow reduces potential contamination with aerosol particles from laboratory air.

  The cold stage was directed to cool with a temperature ramp of $1\,°C\,min^{-1}$. The starting temperature was between $10\,°C$ and room temperature and the run was stopped as soon as the last droplet was observed to be frozen. When the EF600 was set to cool, the data logger software was started. The temperature of the aluminium plate as a function of time was logged, and

160 images of the droplets on the glass slide were recorded with the digital camera at a rate of $1\ frame\ s^{-1}$. The frames of the video were manually looked through, noting for each droplet the frame in which it showed the first signs of freezing so the fraction of droplets frozen, f, could be calculated. The ice active site density per mass of lichen, n_m, was calculated following Murray

et al. (2012) (who reference Vali (1971)):

$$n_m = -\frac{\ln(1-\mathrm{f})}{V_d} \cdot d \cdot \frac{V_w}{m_{\mathrm{lichen}}} \tag{1}$$

where the factor d is the dilution of the droplets, V_d is the droplet volume, V_w is the wash volume, and m_{lichen} is the mass of lichen per sample. Dilutions of samples were made in order to reach lower temperature ranges in the measurements. The errors of the INP concentration measurements were calculated following the procedure described in Harrison et al. (2016), which in turn is based on Wright and Petters (2013), and the temperature uncertainty is $\pm 0.4\,^\circ$C.

3 Results

Previous studies have shown that a substantial fraction of the INP observed during the HyICE-2018 campaign was of biological origin, based on a heat test (Schneider et al., 2021). Our hypothesis is that these biological INPs originate from the lichen that is abundant in the boreal forest ecosystem. However, because INP typically make up a small portion of aerosol particles, their identification in aerosol samples is challenging. Thus, samples of lichen were taken in Hyytiälä and tested for their ice nucleation activity. Figure 4 shows all measurements that were made on lichen sample B, a mixture of *Evernia prunastri* and *Plasmatia glauca* with only a small fraction of *Bryoria sp.* (see table 1).

In Figure 4a), the ice nucleation measurements that were conducted on different size fractions of sample B1, are plotted together for comparison. The spectra for the unfiltered sample and the sample that had been filtered through a $10\,\mu$m filter look similar, qualitatively and quantitatively. This indicates that the ice-nucleating entities can become independent of the lichen and that these entities are smaller than $10\,\mu$m. The ice-nucleation activity drops by 1 to 2 orders of magnitude at respective temperatures for the sample that had been filtered through a $0.1\,\mu$m filter. This suggests that the size of the entities that nucleate ice falls in between $0.1\,\mu$m and $10\,\mu$m. In order to better constrain this size, a $2\,\mu$m filter sample was included in the next set of experiments (sample C, see below).

A striking feature that is present in all three size fractions of sample B1 are the 'steps' in the spectra (sections that are almost vertical), visible at $-16\,^\circ$C and $-18\,^\circ$C. A sharp rise in INP concentrations at a specific temperature suggests that there is a single INP species with a specific temperature of freezing onset present in the sample. As there is no data for the concentration of the INP species active at or above $-16\,^\circ$C for the $0.1\,\mu$m size fraction, it can be concluded that this entity is between $0.1\,\mu$m and $10\,\mu$m in size. The species active at $-18\,^\circ$C, however, is also detected in the $0.1\,\mu$m size fraction.

All heat tests conducted on these samples show a striking loss of activity, with a decrease in the temperatures where ice nucleation was observed. The onset freezing temperature that could be observed with $1\,\mu$l droplets dropped by $6\,^\circ$C to $8\,^\circ$C, depending on the size fraction. In fact, the heat test decreased the concentration of INPs active at temperatures greater than -14 $^\circ$C so much that they could not be detected with the μl-NIPI technique. The loss of activity is greatest for INPs active at temperatures above about$-16\,^\circ$C; being more than an order of magnitude. INPs that induce freezing at lower temperatures seem to be less heat-labile. In the unfiltered and $10\,\mu$m size fraction, after the heat test, activity is centred around $-18\,^\circ$C, just below the step at $-18\,^\circ$C (see Fig. 4 b) and 4c)). However, in the $0.1\,\mu$m size fraction (see Fig. 4d)), the heat test diminishes

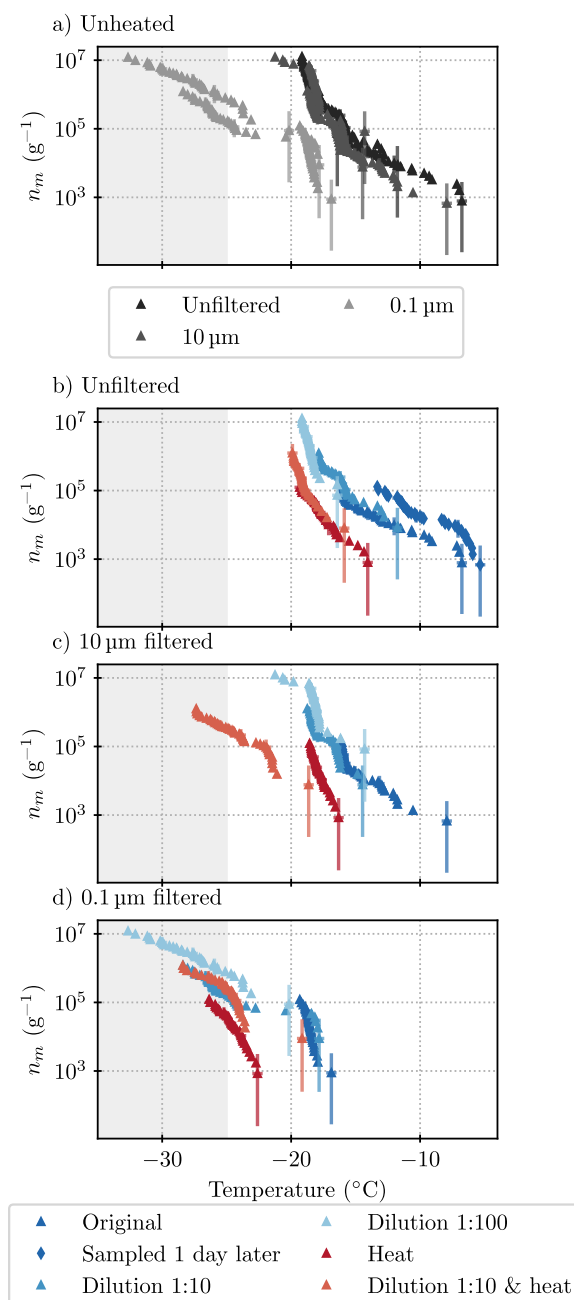



Figure 4. Results acquired from all ice nucleation measurements on lichen sample B (INP per gram of lichen sample): **a)** all unheated samples (summarizes all blue points from the plots below), **b)** unfiltered samples, **c)** samples filtered through a 10 µm filter, **d)** samples filtered through a 0.1 µm filter. Higher dilutions are depicted as lighter shades of blue, heat tested samples in red. The grey shading indicates the temperature at which the blanks started to freeze and thus results are deemed unreliable. The majority of results are for sample B1, but there is one run with B2 in panel b. Uncertainties are included for every 10th data point and deduced as described in Sec. 2.5.

195 activity at $-18\,°C$. Therefore, the INPs that are active around $-18\,°C$ and dominate the heated unfiltered and $10\,\mu m$ samples are concluded to be larger than $0.1\,\mu m$. The spectrum of the heat test in the smallest size fraction, $0.1\,\mu m$, is very close to the unheated sample spectrum below $-24\,°C$, indicating that the INP that are smaller than $0.1\,\mu m$ and active below $-24\,°C$ are heat stable (although these results are very close to our background). Overall, the heat tests show that the INPs that are active at temperatures warmer than $-18\,°C$, including those species responsible for the steps at $-16\,°C$ in the unheated spectra, are

200 heat-labile. In the heated samples larger than $0.1\,\mu m$, INPs that are active at around $-18\,°C$, dominate. It seems likely that the species active at $-18\,°C$ is only partially heat-labile or in fact consists of two species: it dominates in the heated samples of size fractions larger than $0.1\,\mu m$, but its activity diminishes upon heating of the $0.1\,\mu m$ size fraction.

In order to test if the activity of sample B changed with time, we took a sample of aqueous solution (B2) from the original centrifuge tube that contained water and lichen. This sample had been rotated by hand prior to B1 being sampled, and then left

205 for 1 day in a fridge (at $4\,°C$) before sample B2 was taken. The activity in this sample was up to about an order of magnitude greater than in sample B1 (see Fig. 4b)). A potential explanation for this is that the lichen shed additional INP into solution while the lichen was sat in water. However, we note that precipitation samples have been seen to become more active with time, possibly related to the formation of ice-active protein aggregates (Stopelli et al., 2014). While this is in itself interesting and warrants future work, it also informed us that we could not take more samples from the original lichen-water mixture for

210 further tests (such as additional filter tests). Hence, it was necessary to make up fresh suspensions.

In order to investigate the size of these INPs present in the lichen samples further, a new sample of lichen (sample C) was taken out of the same bag as sample B. When sampling from the bag of lichen, we aimed to obtain the same mix of lichen species in this sample as in sample B (see Table 1). Figure 5 shows the results of the experiments conducted with sample C. Inspection of Figure 5a shows that while the freezing characteristics are qualitatively similar to sample B there are also some

215 important differences that we discuss here.

Again warm temperature INPs are present, and as in sample B, INP concentrations decreases substantially when the sample is filtered through a $0.1\,\mu m$ filter (Figure 5a)). Meanwhile, the $2\,\mu m$ size fraction gives about the same signal as the $10\,\mu m$ fraction. The bulk of the INPs present in the mixture of lichen species in lichen sample B and C are therefore concluded to be in between $0.1\,\mu m$ and $2\,\mu m$ in size. The steps that can be clearly distinguished in all three size fractions of sample B1 are

220 less prominent in sample C. The step at $-18\,°C$ can be seen in all size fractions, albeit naturally at lower concentrations in the $0.1\,\mu m$ and $0.02\,\mu m$ size fraction. The step at $-16\,°C$, however, can only be identified in the $0.1\,\mu m$ and $0.02\,\mu m$ size fraction (see Fig. 5e) and 5f)). Only when the larger INPs are removed can the activity of these INPs be seen in the spectrum, because their concentrations are lower than that of the INPs larger than $0.1\,\mu m$. That the step at $-16\,°C$ was visible in the spectra of sample B1 might be explained by the differing composition of the samples. The heat tests on sample C again show a clear

225 decrease in activity, by about 1 to 2 orders of magnitude. The higher temperature INPs are more heat-labile than the ones active at colder temperatures, just like in sample B1. Also, as before, in the $10\,\mu m$ size fraction in Fig. 5c), an INP species active at $-18\,°C$ is evident in the spectrum of the heated sample. However, heat tests of the smaller size fractions diminish activity at $-18\,°C$.

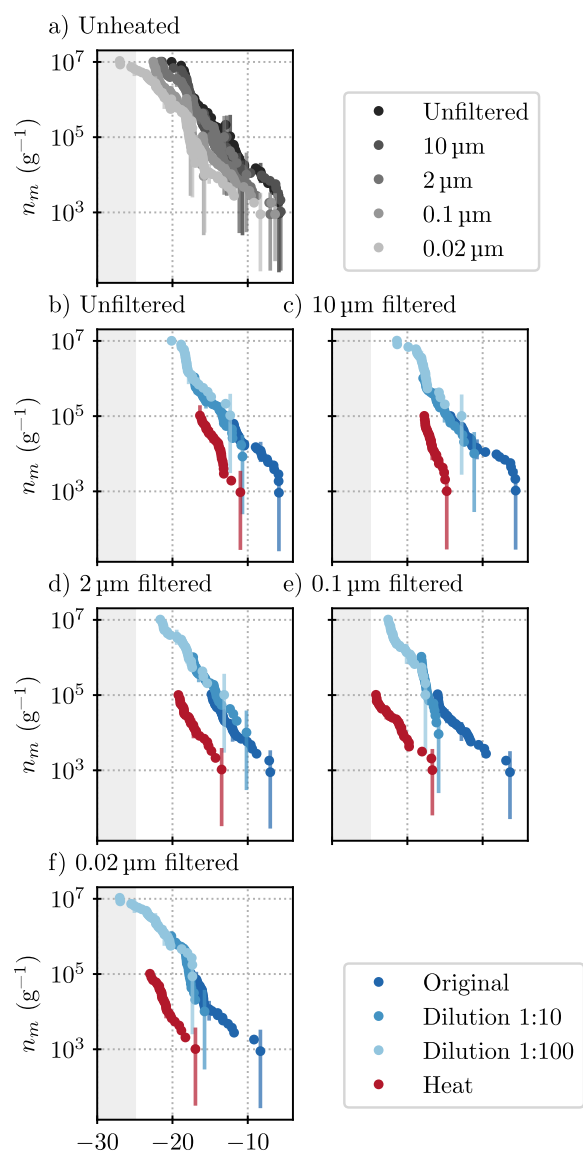



Figure 5. Results acquired from all ice nucleation measurements on lichen sample C: **a)** all unheated samples, **b)** unfiltered samples, and samples filtered through a **c)** 10 μm, **d)** 2 μm, **e)** 0.1 μm, and **f)** 0.02 μm filter. Higher dilutions are depicted as lighter shades of blue, heat tested samples in red. The grey shading indicates the temperature at which the blanks started to freeze and thus results are deemed unreliable.

Species	Mass of lichen	Washvolume	Concentration
	(g)	(ml)	$(\mathrm{g\,ml^{-1}})$
Hypogymnia physodes	0.8709	30	0.029
Evernia prunastri	0.9109	35	0.026
Bryoria sp.	0.0185	2	0.0093
Platismatia glauca	0.3515	20	0.018

Table 2. Lichen concentrations in the species specific tests

Taking the results from lichen sample B1 and C together, there is an INP species active at $-18\,°C$ present in all samples,
whose concentration decreases when filtered through $2\,\mu m$ pore size filters or smaller, but remains distinguishable in the spectra
of the $0.02\,\mu m$ size fraction. The species is partially heat-labile as a step at $-18\,°C$ is also visible in the heated samples, but
only in the size fractions larger than $2\,\mu m$. These different characteristics for different size fractions point to different states
of the INP species, for example as either attached to a larger particle or free in solution as it has been proposed for nano-
INP (O'Sullivan et al., 2015) or as different aggregates as proposed for Pseudomonas syringae (Turner et al., 1990). The INP
species active at around $-16\,°C$ is heat-labile and smaller than $0.02\,\mu m$ for sample C (see Fig. 5). The identification of two
INP species, active at certain temperatures and of a determined size, allow the relation of these findings to future studies that
might find similar species.

The comparison of sample B1 and C can be seen in Fig. 6. In between -18 and $-21\,°C$, the spectra of sample B1 and C lie
close to each other. At higher temperatures, the concentrations of the unfiltered original and the size fraction filtered through a
$10\,\mu m$ filter are both lower for sample B1 than for sample C, by about 1 order of magnitude. The $0.1\,\mu m$ size fractions show a
difference of up to 2 orders of magnitude. As outlined in section 2.2, lichen sample B and C were mixed on the rotary shaker
for $10\,min$ and $30\,min$, respectively. The different procedures might contribute to the discrepancy in the INP concentration
spectra, with a greater concentration of sub $2\,\mu m$ being released with the rotary shaker. 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.

3.1 Ice nucleating ability of the individual lichen species

In order to learn more about the different species, the ice nucleation activity of each lichen species was tested individually. The
results are plotted in Fig. 7. The species specific ice nucleation activities look very similar to each other. Only the spectrum
of *Hypogymnia physodes* has a shape distinct from the others, with a greater activity below $-7\,°C$ and a lower activity above
$-7\,°C$. One might conclude that the three species which were stored in one bag (*Evernia prunastri*, *Bryoria sp.* and *Platismatia
glauca*) simply show the same INP concentrations because these small particles were spread equally throughout the bag.
However, the $2\,\mu m$-filtered size fractions, shown in Fig. 7 is inconsistent with this idea. *Evernia prunastri* and *Platismatia

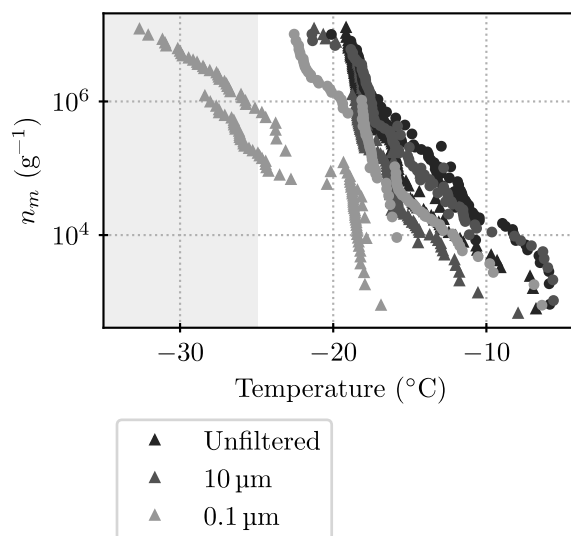



Figure 6. Comparison of the filtered size fractions of lichen sample B (triangles, compare Fig. 4) and C (circles, compare Fig. 5). The grey shading indicates the temperature at which the blanks started to freeze and thus results are deemed unreliable. For better readability no error bars are shown.

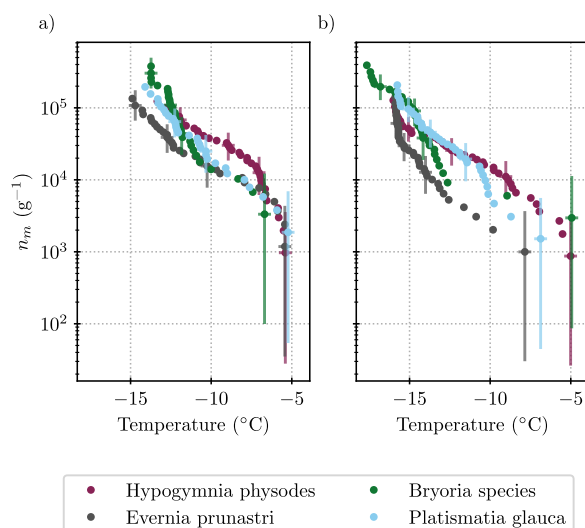



Figure 7. INP measurements that were conducted for each species of lichen separately: comparison of the a) unfiltered samples and of b) those that were filtered through a $2\,\mu m$ filter.

255 *glauca* are the species that were present the most in the bag as well as in the mixed samples (see table 2). The INP concentration of the *Platismatia glauca* sample in the 2 μm size fraction is half an order of magnitude higher than that of the *Evernia prunastri* sample at for example $-10\,°C$, while the spectra of the unfiltered samples lie within error of each other. So in fact, the different lichen species seem to harbour differently sized INPs.

4 Discussion

260 In other studies, lichen samples were ground into a powder and different instruments were used to detect ice nucleation (Kieft, 1988; Kieft and Ahmadjian, 1989; Kieft and Ruscetti, 1990; Ashworth and Kieft, 1992), because those researchers were interested in the benefit of INPs for their harboring plants rather than their atmospheric relevance. This makes a quantitative comparison of INP concentrations difficult.

The most direct comparison can be drawn between the experiments conducted on *Platismatia sp.* in Ashworth and Kieft 265 (1992) and the experiments on *Platismatia glauca* in this study, as these samples are from the same genus. In fact, both species were found to have an onset freezing temperature of about $-5\,°C$, with a concentration of 1×10^3 INPs per gram of lichen. However, the INP concentrations of *Platismatia sp.* rise much sharper in Ashworth and Kieft (1992), resulting in a concentration of 1×10^8 INPs per gram of lichen at $-12\,°C$, whereas in this study, the *Platismatia glauca* sample showed a concentration of 1×10^5 INPs per gram of lichen at $-12\,°C$. The other species investigated in this study can be compared 270 only to the whole variety of species examined in the literature, as there are no studies on the exact same species as sampled during HyIce. Consistently, Ashworth and Kieft (1992), Kieft and Ahmadjian (1989) and Kieft (1988) find a great spread of INP concentrations for their 7, 18 and 15 species tested, respectively. The results of this study lie within that spread. The most notable feature is that the concentrations reached in this study are more than three orders of magnitude lower than the highest reached consistently in the literature. While in their species tests, Ashworth and Kieft (1992); Kieft and Ahmadjian (1989); 275 Kieft (1988) reached concentrations of up to 1×10^8 INPs per gram of lichen at $-12\,°C$, the highest concentrations seen in this study are about 1×10^5 INPs per gram of lichen at $-12\,°C$. The reason for this difference might lie in the preparation of the samples, as with grinding more INP are expected to come into suspension/solution than with the relatively gentle agitation employed here.

It is noteworthy that these earlier studies quote their blanks as freezing in between $-10\,°C$ and $-15\,°C$, while blanks in this 280 study showed background freezing mostly occurred below about $-25\,°C$, allowing measurements at lower temperatures in or study. Moffett et al. (2015) reassumed ice nucleation experiments on lichen after a gap of roughly 20 years. They used whole lichen samples immersed in water and recorded the onset freezing temperature. Their study includes two *Evernia prunastri* samples from the UK. The freezing onset temperatures of $-5.6\,°C$ that was obtained for one sample ($-10\,°C$ for the other) agrees well with the one that was obtained in this study. Overall, the samples tested in the present study show a higher onset 285 freezing temperature than the median of onset freezing temperatures, $-7.2\,°C$, obtained in Moffett et al. (2015).

Kieft and Ruscetti (1990) is the only literature study that looks at the size of the INPs in droplet assays. Their samples were filtered through a $0.2\,μm$-pore-size filter. The samples had been centrifuged and only the supernatant was used for testing,

whereas in this study the whole suspension was filtered. Kieft and Ruscetti (1990) mention that their "extraction procedure did not remove all of the nuclei from the lichens" (Kieft and Ruscetti, 1990, p. 3521). This supports the results and the hypothesis brought forth in the present study: As the present study has found the bulk of lichenous INPs to be in between 0.1 and $2\,\mu m$ in size, it seems likely that these are not whole spores or diaspores. The very smallest recorded lichen spore is $1\,\mu m$ in size, and vegetative diaspores are even larger. Hence, as proposed earlier, the INPs might be smaller particles (nano-INPs) or fragments of dispersal particles. As pointed out in O'Sullivan et al. (2015), for example, pollen harbour nano-scale entities that are attached to the pollen grains and are more numerous than the whole pollen grains. Similarly, the INPs found in this study are smaller than whole spores or soredia. They could become airborne when attached to those larger propagules or wind might pick up these smaller particles by coincidence. However, when looking at their atmospheric relevance, it is not only important to know the INPs' size fraction, but the structure of the lichen that the particles stem from is bound to be important as well. For example, looking at Fig. 2, one can imagine that wind blowing over these lichens, as a possible way of dispersal for the INPs, interacts with each of them very differently. For example, *Bryoria sp.* is found to be hanging from trees, swaying in the wind. Hence, particles that stem from this lichen species seem likely to be dispersed and lifted into the atmosphere more easily than particles on species that are tightly bound to trunks and branches.

As of now, little is known about the atmospheric abundance of ice nucleation active particles that stem from lichen (Després et al., 2012). However, the wide distribution of lichen indicates that they have a successful mechanism for dispersal (Hale, 1974), which in turn suggests high concentrations of propagules. Wind removal of soredia has been successfully demonstrated by Bailey (1966). It was found that while more soredia were removed at higher moisture content of the thallus, the wind speed necessary for removal increased with higher moisture content as well. Armstrong (1993) conducted a wind tunnel experiment and found humidity to lead to a substantial decrease in soredia dispersal. Armstrong (1991) identified humidity to be the most significant variable for soredia dispersal, stating that more soredia dispersed at lower humidity. It has been suggested that high moisture content promotes soredia production, but that low moisture content facilitates release (Marshall, 1996). In a year-long aerobiological monitoring program over Antarctica, Marshall (1996) found lichen soredia to be more abundant than spores. Soredia were collected every month, suggesting that soredia are produced year-round. The release was found to be independent of any specific meteorological variable. In a field study in an old-growth forest in Finland, samples of the lichen Lobaria pulmonaria were genetically analysed (Ronnås et al., 2017). Symbiotic propagules had a maximum dispersal range of $100\,m$, while ascospores dispersed several kilometers. Tormo et al. (2001) placed traps $6\,m$ above ground level in Spain. The mean soredia concentration was $0.4\,m^{-3}$ with a daily maximum of $5\,m^{-3}$. The concentration was higher during the day. Positive correlations with temperature and again negative correlations with humidity were found. The lichen tested in the present study were sampled at subzero temperatures (see Fig. 5 in Schneider et al. (2021)). Correlating meteorological variables with the INP concentrations obtained from air filter measurements (Brasseur et al., 2022), could shed light onto the hypothesis that lichenous INPs could be a local source in Hyytiälä.

5   Conclusions

A possible local source of INPs in Hyytiälä was explored with the thorough examination of the ice-nucleating ability of lichen sampled during the HyICE-2018 campaign. In accordance with literature (Kieft, 1988; Ashworth and Kieft, 1992; Moffett et al., 2015), the mixtures of three lichen species sampled in Hyytiälä were shown to harbour INP active at temperatures as warm as $-5\,^{\circ}$C with concentrations of $1 \times 10^3\,\mathrm{g}^{-1}$ of lichen. These findings were expanded through size segregation and heat tests. Many of the lichenous INPs were found to be between 0.1 and $2\,\mu$m in size when immersed in water and those active at temperatures higher than $-18\,^{\circ}$C were heat labile. Out of the cumulative nucleus spectra, two species of INP could be identified: one species, active at $-16\,^{\circ}$C, was found to be heat labile and smaller than $0.02\,\mu$m; the other species was active at $-18\,^{\circ}$C. The latter's concentration decreased upon filtering through a $2\,\mu$m pore size filter, but it was still detectable in the $0.02\,\mu$m size fraction. In the smaller size fractions ($< 2\,\mu$m), this INP species was heat labile, but in the larger size fractions it was not. These differing characteristics in different size ranges suggest that the INP species was present in two different states, attached to a larger particle or free in solution or in different states of aggregation. In the species specific experiments, the four species of lichen showed similar ice-nucleating activity, ranging from 1×10^3 INP per gram of lichen at about $-5\,^{\circ}$C to 4×10^5 INP per gram of lichen at about $-14\,^{\circ}$C. However, the species harbour differently sized INPs, as the activity decrease seen upon filtration through a $2\,\mu$m pore sized filter varied by about one order of magnitude in between species. This implies that some species of lichen are more important as a source of INPs than others. Combined with knowledge about biological differences between these lichen species, these findings might contribute to the identification of lichenous INPs. The size of INPs found in this study suggests that whole spores or soredia are not required for ice nucleating activity, but rather smaller entities nucleate ice. This is analogous to pollen and fungal materials, where nanoscale ice-nucleating entities can become seperate from their host (O'Sullivan et al., 2014). This encourages the idea that these INPs could become airborne, either attached to (or part of) spores or soredia or simply carried aloft by wind by themselves. As the lichen species investigated here were picked in Hyytiälä without any knowledge about their ice nucleation activity and all were found to harbour INPs, these findings hold promise (in combination with literature data) that lichen in general represent a source of atmospheric INPs, as they populate many terrestrial environments in great abundance. This source may be especially relevant in winter when the ground and with it other sources of biological INPs are snow covered in boreal forests. Expanding upon lichen dispersal studies with emphasis on INPs, for example wind tunnel experiments could be employed, controlling environmental factors such as temperature, relative humidity and wind speed. Further it would need to be evaluated if and in which concentrations lichenaceous INPs reach atmospheric heights where they might nucleate ice in clouds.

Code and data availability.   Processed measurement data and plotting scripts are archived at https://doi.org/10.5281/zenodo.8355809.

Appendix A: Blanks

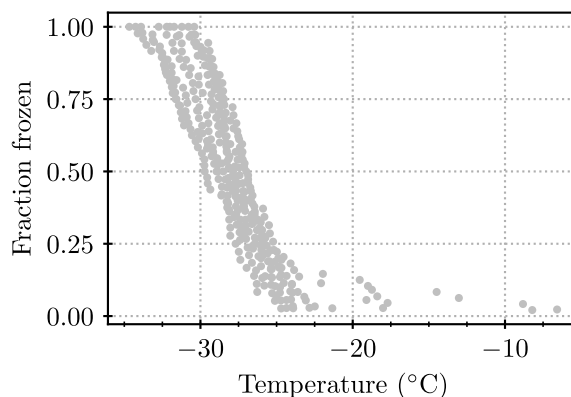



Figure A1. Blanks that were run on the morning of each day that experiments were conducted, as described in Sec. 2.2.

350 *Author contributions.* UP conducted the experiments and analysis and wrote the manuscript. MPA and BJM conceived the idea of the study. MPA, GEP, MH and BJM contributed to the design of the study and the analysis of the results. BJM sampled the lichen. MH and UP took the lichen images. JB identified the lichen species sampled. All authors provided comments and edits to the manuscript.

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