Subpollen particles (SPP) of birch as carriers of ice nucleating macromolecules

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Abstract. Within the last years pollen grains have gained increasing attention due to their cloud forming potential. Especially the discovery that ice nucleating macromolecules (INM) or subpollen particles (SPP) obtained from pollen grains are able to initiate freezing has stirred up interest in pollen. INM or SPP are much smaller and potentially more numerous than pollen grains and could significantly affect cloud formation in the atmosphere. However, INM and SPP are not clearly distinguished and explanations on how these materials could distribute in the atmosphere are missing. In this study we focus on birch pollen and investigate the relationship between pollen grains, INM and SPP. According to the usage of the term SPP in the medical fields we define SPP as the starch granules contained in pollen grains. We develop an extraction method to generate large quantities of SPP and show that INM are loosely attached to SPP. Further, we find that purified SPP are not ice nucleation active: after several times of washing SPP with ultrapure water the ice nucleation activity completely disappears. To our knowledge this is the first study to investigate the ice nucleation activity of isolated SPP. To study the chemical nature of the INM we use fluorescence spectroscopy. Fluorescence excitation-emission maps indicate a strong signal in the protein range (maximum around λ_{ex} = 280 nm and λ_{em} = 330 nm) that correlates with the ice nucleation activity. In contrast, with purified SPP this signal is lost. We also quantify the protein concentration with the Bradford assay. The protein concentration ranges from 77.4 μg mL^{-1} (Highly concentrated INM) to below 2.5 μg mL^{-1} (purified SPP). The results indicate a linkage between ice nucleation activity and protein concentration. Even though purified SPP are not ice nucleation active they could act as carriers of INM and distribute those in the atmosphere.

1 Introduction

Aerosol particles in the atmosphere are climatically relevant if they affect cloud microphysics and formation of precipitation or directly scatter solar radiation. The aerosol effect on clouds is still one of the largest uncertainties in our climate system (Boucher, 2013) and the role of bioaerosols including pollen is far from being understood (Fröhlich-Nowoisky et al., 2016). Pollen are a subset of bioaerosols such as bacteria, viruses, algae, fungi and plant debris found abundantly in the atmosphere during the blooming season of plants. Pollen is the male gametophyte that contains vegetative and generative cells essential for fertilization and reproduction of a plant. In particular wind pollinated plants such as early flowering trees (e.g. birch) and grasses produce pollen in large quantities and rely on wind for dispersal and transportation to the female plant where fertilization occurs. Pollen are thus distributed in the lower atmosphere and can also travel long distances when meteorological conditions are favourable (Damialis et al., 2017; Mikhail Sofiev, 2013; Skjøth et al., 2007). Typical concentrations of pollen grains of a specific type range from 10 m^{-3} to a few hundred or even thousands per m^3 on a daily average. Peak concentrations in the vicinity of plants can assume a few 100 000 m^{-3} (Jochner et al., 2015; Šikoparija et al., 2018). Forests of the Northern Hemisphere are estimated to be large contributors to atmospheric pollen concentrations e.g. birch pollen concentrations outside the canopy of birch forests are in the range of 10 000 m^{-3} during spring (Williams & Després, 2017). The ability of pollen to induce liquid droplet and ice formation has been documented in several studies already years ago. Entire pollen grains were shown to act as giant cloud condensation nuclei (CCN) (Pope, 2010) and also as ice nucleating particles (INP) (Diehl et al.,

2002; Diehl et al., 2001; von Blohn et al., 2005). A summary of the role of pollen and other biological particles in cloud physics is given by Möhler and colleagues (Möhler et al., 2007). However, due to their large sizes (10-100 µm) leading to short residence times in the atmosphere their impact on cloud microphysics was estimated to be negligible on a global scale (Corinna Hoose et al., 2010; C. Hoose et al., 2010).

45 Only discoveries of recent years have demonstrated that not only the entire pollen grain has the ability to initiate ice formation but the presence of macromolecules originating from pollen grains is sufficient to induce heterogeneous ice nucleation (Pummer et al., 2012; Pummer et al., 2015). In their study, Pummer and co-workers (Pummer et al., 2012) suspended entire birch pollen grains in water for several hours. The solution is then decanted and filtrated yielding what is called pollen washing water. The washing water is shown to induce ice formation at similar temperatures as the entire pollen grains. The ice activity

50 of pollen grains is thus attributed to the existence of ice nucleating macromolecules (INM) that can be separated from pollen grains (Pummer et al., 2015). Such INM can also be obtained from other parts of a plant e.g. branches, leaves, and barks of a birch (Felgitsch et al., 2018). Aqueous extracts of pollen grains obtained in a similar manner were used in several other studies to investigate the cloud forming potential of many different types of pollen grains (Gute & Abbatt, 2020; Gute et al., 2020; Mikhailov et al., 2019; O'Sullivan et al., 2015; Steiner et al., 2015). In these studies, such aqueous extracts are often referred

55 to as subpollen particles (SPP). The term SPP originates in the medical sciences and is commonly used to describe starch granules with allergenic potential that are contained in pollen grains (Bacsi et al., 2006; Schäppi et al., 1999; Sénéchal et al., 2015). The term starch granule refers to the main component starch which is a polysaccharide and functions as an energy storage unit in plant cells (Baker & Baker, 1979; Buléon et al., 1998; Hancock & Tarbet, 2000). In studies related to atmospheric sciences the term is used in a less defined and sometimes confusing way. Aqueous extracts of pollen grains are

60 simply referred to as SPP even though they might not contain any particulate material or particles created artificially by atomizing solutions of aqueous pollen extracts are denoted SPP.

A recent model simulation estimates that the presence of SPP in the atmosphere could suppress precipitation in clean continental clouds by about 30% (Wozniak et al., 2018). However, estimations of the amount of such material in the atmosphere is based on rough assumptions, since data is missing. One reason for the lack of data is that little is known about

65 the nature and composition of INM and, as a consequence, direct measurement methods that can identify INM in the atmosphere are not available. Up to date only a few studies investigated the chemical nature of INM and found that INM contained in birch pollen washing water are water soluble and are likely composed of polysaccharides (Dreischmeier et al., 2017; Pummer et al., 2012; Pummer et al., 2015). However, there is also evidence that the ice nucleating activity stems from proteinaceous substances, e.g. Tong and colleagues show that the ice activity of birch washing water diminishes if

70 proteinaceous components are extracted (Tong et al., 2015).

One major difficulty in identifying INM is related to the complexity of a pollen grain. In fact, a pollen grain is a highly complex particle composed of different parts and materials (Fægri & Iversen, 1992). Figure 1 illustrates a birch pollen grain and its main parts. The actual cell is protected by a robust outer shell made of two layers: the exine (outer layer) and the intine (inner layer). The exine is composed of the mechanically resistant and chemically inert biopolymer sporopollenin, while the intine,

75 composed of cellulose and pectin, is a more fragile membrane. The cell is filled with cytoplasmic material including proteins, lipids and polysaccharides such as starch. Many pollen types, such as birch pollen grains, also contain pores. Pores are spots where the exine is missing and thus give access to the cell membrane (intine). Under humid conditions most fresh pollen grains are likely to expel cytoplasmic material, a process often referred to as pollen rupture. In literature two mechanisms are documented by which pollen grains can release cytoplasmic content including starch granules from their interior. First, entire

80 pollen grains can rupture by osmotic shock during hydration (D'Amato et al., 2007; Suphioglu et al., 1992; Taylor & Jonsson, 2004). Second, pollen grains can be stimulated to germinate and grow pollen tubes (Grote et al., 2003; Schäppi et al., 1997).

The pollen tubes rupture at their tips just as they would during fertilization on a female stigma and expel genetic and cytoplasmic material. This processed is referred to as abortive germination (Grote et al., 2003). In a more recent laboratory study Sénéchal and co-workers showed that SPP are also released from birch and cypress pollen after the grains were exposed

85 to humidity or to NO_2 (Sénéchal et al., 2015). Mechanical rupture caused by wind induced impaction might also generate SPP (Visez et al., 2015).

These processes have been examined since in several studies from the medical fields e.g. (Grote et al., 2003; Schäppi et al., 1997; Staff et al., 1999; Suphioglu et al., 1992; Taylor et al., 2004). Pollen rupture is hypothesized to occur in the atmosphere and offers an explanation of the presence of allergens in the fine aerosol fraction (< 5μm) i.e. the presence of allergens detached

90 from pollen grains. For example, birch pollen grains were shown to germinate on leaves after light rain and release starch granules. Simultaneously a rise in the allergen concentration in the air was measured without the presence of pollen grains (Schäppi et al., 1999; Schäppi et al., 1997). SPP are also hypothesized to cause the phenomenon of thunderstorm asthma (Taylor et al., 2007; Taylor & Jonsson, 2004). Thunderstorm asthma is the observed coincidence of severe asthma epidemics and thunderstorms (e.g. (Thien, 2018)). Due to their large size pollen grains are efficiently trapped in the upper airways when

95 inhaled. In contrast, SPP can penetrate deep into the lung and trigger asthma. Cases of thunderstorm asthma have been documented worldwide (D'Amato et al., 2019). An increase of submicron particles of biological origin possibly connected to pollen grains during rain events or thunderstorms has also been observed in a few recent studies not related to allergens that use chemical tracers and measurements of fluorescence particles as an indication for biological particles possibly originating from pollen grains (Huffman et al., 2013; Hughes et al., 2020; Rathnayake et al., 2017).

100 In this study we investigate SPP of birch pollen grains. The aim of our study is to shed light on the differences between INM and SPP. As discussed above, in atmospheric literature the term SPP is usually used in a rather unspecific sense to simply describe the remaining materials when aqueous extracts of pollen grains are generated. For the purposes of our study, we follow the usage of SPP as done in medical literature. Accordingly, we define SPP as the starch granules contained in the cytoplasm. With the extraction methods commonly used it is unclear whether materials are only washed off the pollen grains'

105 surfaces or also obtained from the cytoplasm. We therefore develop a method to extract SPP and separate those from other cytoplasmic materials. We thereby aim to answer the question whether SPP are ice nucleation active and if INM are linked to SPP. To or knowledge this is the first study to investigate the ice nucleating activity of SPP thoroughly extracted from pollen grains. Additionally, we use fluorescence spectroscopy to gain chemical information about the ice active substances and the Bradford assay to quantify protein concentrations.

2 Experimental Section

2.1 Pollen samples

We used commercially purchased birch pollen samples. The samples were purchased from ThermoFisher Scientific Allergon (www.allergon.com) and specified by the company as pollen from *Betula pendula* (common name: silver birch). Pollen are collected from trees in Southern Sweden and are carefully purified from other plant materials after sampling. The pollen sample

115 is reported to contain less than 2% of plant parts other than pollen grains. At the time of purchase the birch pollen grains were already 1 year old. Freshly harvested pollen samples were collected from birch trees at the Danube Island in Vienna. Collection took place when birch trees were reported to be ready to bloom by the Austrian pollen forecast service (www.pollenwarndienst.at). Additionally, phenological observations at the sampling location were performed (i.e. check for clearly visible anthers on the catkins) to confirm the maturity of the pollen grains.

2.2 Extraction of SPP

In order to extract SPP of birch pollen grains we developed an extraction process that starts with cracking the pollen grains so that the cytoplasmic content is available even without the process of pollen rupture. Fresh birch pollen grains naturally rupture when soaked with water and release their cytoplasmic content including starch granules. With commercially purchased pollen this ability is almost lost. Therefore, we use a mixer mill (Retsch MM400) to crush the grains. The extraction process is illustrated in figure 2. Crushing the pollen grains with the mixer mill is the first step of the extraction method (figure 2a, step 1). In order to crush the grains with the mixer mill we blend 0.5 g pollen grains with 2 mL ultrapure water (MilliQ, 18.2 MΩ) and pour the water pollen suspension into the grinding jar including one single ball as a grinder. The mixer mill is operated for 1 minute at 25 Hz (step 1). As a result, 30-40 % of the pollen grains are cracked and cytoplasmic material and SPP are gained (see figure 2b). It should be noted that the pollen wall cracks but does not fragment into very small pieces (below ~ 10 μm). We found very few wall fragments after this filtration process (analysed in electron microscopic pictures). Apart from the SPP, the cytoplasmic content appears soluble forming amorphous structures when dried.

In a second step, we use filter paper with 10 μm pore size to filter pollen wall fragments and remaining undamaged pollen grains. Particulate material larger than 10 μm is retained by the filter, while all other materials pass through. The resulting extraction product is referred to as sample A. Sample A contains SPP, other cytoplasmic materials (mostly soluble) and few small wall fragments.

In step 3, sample A is filtrated with a syringe filter (0.2 μm pore size, Nylon membrane, sterile) i.e. particles larger than 0.2 μm are retained by the filter (sample B). In step 4, the filter with the remaining material is rinsed with ultrapure water until most of the soluble material is washed off. The rinsing is done stepwise with 1 to 70 mL ultrapure water. After passing through the filter the filtrate is called sample C. The ice nucleation activity of sample C was tested for each rinsing step. As the purpose of this extraction was to test if INM can be separated from SPP, rinsing was continued as long as sample C was ice nucleation active. Rinsing was stopped after 70 mL when the ice nucleation activity of sample C had disappeared.

After rinsing, the filter is reversed and flushed with ultrapure water (step 5). A suspension of SPP in ultrapure water is obtained. This sample is referred to as sample D (figure 2c) and used to test the ice nucleation activity of SPP.

2.3 Determination of the geometric size distribution

The geometric size of the SPP was determined from images taken with a field emission gun scanning electron microscope (FEG-SEM; Zeiss Supra 55 VP). The shape of the SPP was approximated by a cylinder capped with hemispheres at each side with the diameters of the hemispheres being equal to the width of the cylinder. The width and length of the cylinder was measured manually with the help of an image analysis software (SmartTIFF V1.0.1.2) and the volume was calculated. This was done for 326 particles in total. The volume was then used to determine the volume equivalent diameter of the SPP i.e. the diameter of a sphere having roughly the same volume as the irregular shaped SPP. Additionally, the aspect ratio was used to describe the sphericity of the particle. The aspect ratio is defined as width/length and is 1 for spherical particles and smaller than 1 for elongated particles.

2.4 Ice nucleation measurements

INM content from birch pollen was quantified in immersion freezing mode by using the Vienna Optical Droplets Crystallization Analyzer (VODCA) setup (Felgitsch et al., 2018; Pummer et al., 2012): An emulsion of 2 μl sample solution and 4 μl inert oil-mixture (lanolin, paraffin) is prepared on a microscopic glass slide and transferred into a cryo-cell. Sample emulsions are cooled with a rate of 10°C min^{-1}. The freezing process was monitored by videos at four different spots of each sample glass slide via a microscope camera (MDC320, Hengtech, GER). The fraction of frozen droplets (n_{frozen}/n_{total}) were

plotted as a function of the temperature. Furthermore, the cumulative nucleus concentration (CNC) was determined using
160 equation 1 (Vali, 1971):

$$CNC(T_i) = -\frac{ln\left(1-\frac{n_{frozen}}{n_{total}}\right)}{V} \cdot d \qquad (1)$$

where n_{frozen} is the number of frozen droplets at the given temperature, n_{total} the total number of droplets, V the droplet volume
and d the dilution factor. CNC (T_i) indicates the number of INM per unit volume actively present above the temperature T_i.
To compare the values of INM from different samples we chose T_i at -25°C since most biogenic INM are active above that
165 temperature (Kanji et al., 2017; Murray et al., 2012; Pummer et al., 2015) and -34°C since homogeneous ice nucleation starts
at -35°C with the VODCA setup. Thus, the CNC (-34°C) value includes all heterogeneous freezing events. High concentrated
samples were diluted in the experiment to prevent underestimation of INM contents. Only droplets with a diameter between
15 and 40 µm (droplet volume: 1.8-34 pL) are counted in the evaluation and the average volume of 8.2 pL was used to calculate
CNC values.

2.5 Fluorescence spectroscopy

Autofluorescence active materials such as SPP and respective extracts (see figure 2) were characterized by fluorescence
spectroscopy using a FSP920 spectrometer (Edinburgh Instruments, UK), equipped with a Xe900 xenon arc lamp (450 W) and
a S900 single photon photomultiplier. Sample solutions were measured in a quartz glass cuvette (500 µL, Hellma Quartz
(Suprasil®), GER) using a designated cuvette optic. The excitation light beam is arranged in a 90° angle relative to the detector.
175 The software F900 allowed recording excitation-emission maps (excitation from 220 to 400 nm, emission from 320 to 500
nm). The excited state was held for 0.25 s dwell time and step width of the monochromators was set to 2 nm. To avoid first
and second order excitation (figure 5, grey area), we used a 295 nm low-pass filter (Stablife Technology®, Newport, USA)
and an offset of 10 nm. Two samples (sample A and B, see figure 3) were highly fluorescent active and thus diluted during the
measurements, to minimize the influence of quenching agents. Obtained excitation-emission maps were normalized to $5.0*10^4$
180 counts.

2.6 Quantitative protein analysis

The protein concentration of the sample solutions was determined by the common protein assay firstly described by Marion
M. Bradford (Bradford, 1976). The method is based on a protein-dye binding reaction. The dye-reagent Coomassie Brilliant
Blue forms a complex with proteins in solutions and shifts the absorption maximum from 465 to 595 nm. A standard curve
185 ranging from 2.5 to 25 µg mL^{-1} was prepared by diluting an albumin standard (Thermo Scientific, GER) with ultrapure water.
The samples from step A and B (see section section 2.2) were diluted 1:5 since their concentration was out of test specifications.
We pipetted 150 µL of each sample, standard and blank (ultrapure water) directly into a microtiter microplate (MaxiSorp,
Nunc-Immuno, Thermo Scientific, GER). By using a stepper pipette, 150 µL of Coomassie reagent (Thermo Scientific, GER)
were added to each cavity. The plate was shaken with a microplate shaker (PMS-1000i, Grant Instruments, UK). After
190 incubating for 10 minutes at room temperature, we measured the absorbance at 600 nm with a photometer (Sunrise, Tecan,
CH). For evaluation, the average blank value was subtracted from the absorption values. For the standard curve a linear
regression was determined and the total protein content of the sample was calculated.

3 Results and Discussion

3.1 Extraction process and size distribution of SPP

The extraction process in this study differs from the usual approach in other studies (e.g. (Gute & Abbatt, 2020; Pummer et al., 2012; Steiner et al., 2015)) especially in one aspect: to ensure that material from inside the pollen grains is obtained we first crack the exine of the pollen grains. This is done with a mixer mill. As seen in figure 2b the exine cracks and gives access to the pollen grain's interior including the starch granules. This step was necessary as we found that the commercially purchased birch pollen grains do not rupture nor develop pollen tubes. In contrast, after fresh birch pollen grains had remained in water for ~ 1 day we find several grains with pollen tubes and SPP in the sample. We find that this ability is almost entirely lost when freshly harvested birch pollen grains were stored in the lab for a few days to weeks. The highest germination activity (i.e. most pollen grains germinated) was observed when fresh pollen grains were exposed to water on the very same day they were harvested. With commercially purchased pollen grains we did not find any germination activity and also no SPP. In addition, we also treated pollen grains mixed with water up to 1 hour in the ultrasonic bath to see if pollen rupture could be induced this way. However, even after 1-hour ultrasonic treatment we did not find any ruptured pollen grains nor SPP (Figure S1). We believe that the usually applied extraction method, where pollen grains are only left in water and are then filtrated, do not actually yield SPP unless very fresh pollen grains are used. In this sense our method is unique and offers the possibility to study isolated SPP and gain further insight about the location of the INM within the pollen grain.

We also analysed the geometric size of the SPP gained by our extraction method and calculated volume equivalent diameters. The volume equivalent diameters of SPP range from 0.2 to 2.5 μm and are roughly normally distributed (Figure 4a). The mean value of the distribution as well as the maximum of the gauss-fit is about 1.1 μm. To describe the sphericity of the SPP the aspect ratio was calculated. It ranges from 0.27 to 1 where 1 describes a perfect sphere. The aspect ratio (figure 3b) is roughly log-normal distributed with a maximum at about 0.5 confirming that most SPP have a rather elongated form as can be seen in figure 2c. In fact, the length of the largest SPP reaches up to 4 μm. Considering a density of 1.6 g m^{-3} for SPP (Dengate et al., 1978) and neglecting the non-spherical shape, the aerodynamic diameter would be a factor 1.26 larger than the volume equivalent diameter. The aerodynamic diameter is essential to describe the behaviour of particles in the atmosphere. With this rough estimation we can conclude that the aerodynamic diameter of the SPP ranges from 0.25 to 3.2 μm. It should be noted that we used a filter with 0.2 μm pore size and that smaller particles might have been lost in the extraction process.

3.2 Ice nucleation activity

We analysed the ice nucleation activity of all samples obtained during the SPP extraction process (sample A, B, C and D, see figure 2). The goals of these measurements were: (1) to gain information about the location of the INM within the pollen grain and (2) to investigate whether SPP are ice nucleation active.

In step 4, the syringe filter was rinsed with up to 70 mL of ultrapure water. Rinsing was continued until the ice nucleation activity of the rinsed material (sample C) was completely lost i.e. only homogeneous freezing occurred at temperatures below -35°C. Here, the rinsing steps are labelled C01 to C70. The number indicates the water volume in mL used to rinse the sample up to this step i.e. C01 and C70 mean that the sample had been rinsed in total with 1 mL and 70 mL of ultrapure water, respectively. We analysed the ice nucleation activity of all C01 to C70 samples obtained during the washing process. Freezing curves of all samples are shown in the SI (figure S2). Figure 4 shows selected samples only to avoid an overcrowded figure.

The freezing curves follow the typical pattern (inclining curves and formation of a plateau) that results from diluting an ice active substance (Felgitsch et al., 2018). Sample A, B as well as C01 clearly exhibit heterogeneous ice nucleation. The concentration of INM active above -34°C was 13.2 pL^{-1} for sample A, 13.1 pL^{-1} for sample B and 8.7 pL^{-1} for sample C01 (figure 4a). After sample C10 the ice nucleation activity rapidly diminishes but only after 70 mL of washing the ice nucleation

activity is entirely lost and only homogeneous freezing takes place. After the last washing step, purified SPP (sample D) were

235   obtained by reversing the syringe filter and flushing the filter with ultrapure water. Sample D did not show heterogeneous ice nucleation activity. As losses of SPP in the syringe filter are expected during the washing process we also conducted a control experiment, where we generated a sample with highly concentrated SPP. In the control experiment we stopped the washing process after 30 mL and further separated SPP from solutes via centrifugation (1000 rcf, 3 min). A picture of the concentrated SPP precipitation is shown in the SI (figure S3). After several steps of centrifugation and cleaning the precipitate with ultrapure

240   water, even the accumulated SPP did not exhibit ice nucleation activity (see SI, Figure S4). The result indicates that purified SPP are not ice nucleation active. The results thereby show that INM can be separated from SPP and are not tightly attached to the SPP. Purified SPP are composed of starch and are contained in the pollen grains as energy storage units. Starch is a polysaccharide made of amylose and amylopectin (Hancock & Bryon 2000)(Buléon et al., 1998) inside the amyloplast membrane (Matsushima et al., 2016).

3.3 Chemical Analytics

3.3.1 Fluorescence spectroscopy

In order to gain information about the chemical nature of the INM we analysed the extracted samples using fluorescence spectroscopy. Since we found that the starch contained in pollen grains is not ice active, we focused this analysis on the emission/excitation wavelength range where proteins are expected. Biological ice nucleation is often linked to the presence of

250   proteins, acting as INP (Maki et al., 1974; Wilson et al., 2006). The amino acids tryptophan, tyrosine and phenylalanine, which are present in natural proteins, contain excitable π-electrons. Thus, proteins show auto-fluorescence when excited at around 280 nm with a Stokes shift of about 50 nm (Pöhlker et al., 2012). In figure 5a-c fluorescence excitation-emission maps of our samples show high intensity at λ_{ex} 280 nm and λ_{em} 330 nm. This maximum clearly indicates the presence of proteins. The signal correlates with heterogeneous ice nucleation of sample A, B and C01. Furthermore, we did not see any signal in this

255   range for purified SPP (figure 5d), leading us to the conclusion, that no proteins are detectable in that final fraction anymore. The thin line still visible in figure 5d is the Raman signal of water. We conclude that the ice nucleation of our samples might be linked to proteins, whereas the SPP mainly composed of starch are not ice nucleation active.

3.3.2 Quantification of the protein content

Even though proteins can show strong fluorescence signals, light absorbing substances in the extract might lead to quenching

260   effects (Papadopoulou et al., 2005) that decrease the protein signal. More specific measurements of protein content can be carried out using UV-Vis spectroscopy after staining the proteins with Coomassie Brilliant Blue (Bradford assay (Bradford, 1976)). Quantification via Bradford assay gave protein concentration of 77.4 µg mL^{-1} for sample A, 25.8 µg mL^{-1} for sample B and 5.3 µg mL^{-1} for C01. Values for D were lower than the limit of quantification (2.5 µg mL^{-1}). A summary of the determined values for samples analysed is given in Table 1. Comparing the protein concentration to CNC(-25°C), representing the

265   biological region (Kanji et al., 2017; Murray et al., 2012; Pummer et al., 2015), shows a general trend: a higher protein concentration coincides with higher CNC(-25°C) values. For lower temperatures (i.e. CNC (-34°C)) the dependency on the protein concentration is less pronounced since non-proteinaceous materials become ice nucleation active in the temperature window between -25°C and -34°C.

4. Conclusions

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In this study we develop an extraction method that gives access to the cytoplasmic material of pollen grains, even after the grains have lost the ability to germinate and rupture. We emphasise that birch pollen contain soluble and insoluble cytoplasmic materials. The insoluble material is mostly starch granules that we refer to as SPP in accordance with the usage of the term SPP in the medical sciences (e.g. (Bacsi et al., 2006)). INM are exclusively found within the aqueous solution but it took

275   several times of washing and dilution for a sample to lose the ice nucleation activity suggesting that INM are present in high concentrations. In contrast, we find that purified SPP are not ice nucleation active. Fluorescence spectroscopy reveals a strong protein signal in the remaining ice active solution that is not found with the highly concentrated SPP. Generally, we see a quantitative link between the ice nucleation activity (CNC (-25°C)) and the protein concentration during the washing procedure. Therefore, the INM as well as the proteins of the suspension must be soluble and extractable during the filtration

280   process in the same manner. We highlight the possibility that the ice nucleation activity of Betula pendula pollen is linked not only to polysaccharides (Pummer et al., 2015) but also to proteinaceous INM. INM and SPP are both contained in the cytoplasm. The abundance of INM suggests that INM and SPP might not naturally separate in the atmosphere. SPP could act as carriers of INM. Similarly, starch granules are known to act as carriers of allergens of different pollen grains (Bacsi et al., 2006; Schäppi et al., 1999; Staff et al., 1999). Pollen rupture generating airborne SPP could therefore be a possible mechanism

285   of how INM from pollen grains could disperse in the atmosphere without the presence of the grains.

Author contribution

JB wrote the manuscript with contributions from all co-authors. JB and HG developed the concept of the study. JB and HG acquired financial support for the project. JB worked out the details and guided experiments. JG and JB developed the extraction method. JG analysed the subpollen particles and created the graphical representation. TS and PB conducted the ice

290   nucleation measurements and respective data analysis including graphical representation. TS and PB performed the Bradford Assay, the fluorescence spectroscopy and the control experiment.

Data availability: All data are available from the corresponding author upon request

295   **Competing interest:** The authors declare that they have no conflict of interest.

Acknowledgement

The authors gratefully acknowledge the Hochschuljubiläumsstiftung der Stadt Wien (grant: H-318313/2018 and H-2075/ 2010) and the Austrian Science Fund (FWF; grant P 26040) for financial support. The authors would also like to thank Robin Hoheneder at the University of Natural Resources and Life Sciences, Vienna, for his help with the Bradford assay.

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Table 1. Protein concentration and cumulative nuclei concentration (CNC). LOQ = limit of quantification.

Sample	Protein Concentration [µg ml⁻¹]	CNC (-25°C) [pl⁻¹]	CNC (-34°C) [pl⁻¹]
A	77.4	7.6	13.2
B	25.8	4.5	13.1
C01	5.3	4.0	8.7
D	< 2.5 (LOQ)	0	0

515
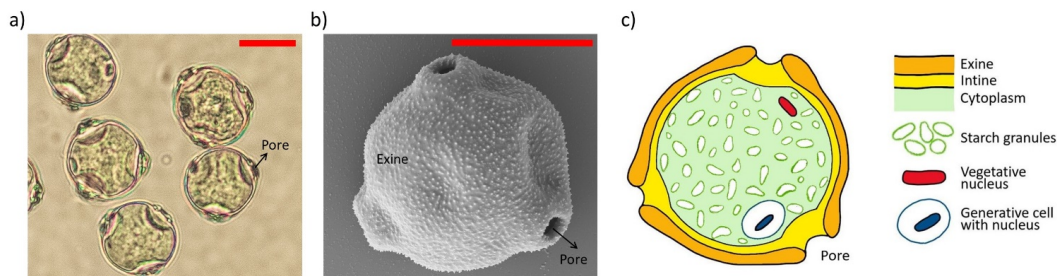
Figure 1: Different views of birch pollen grains. a) Light microscope image of pollen grains in water. b) Electron microscope image of a single pollen grain. c) Schematic sectional view with the main components of a birch pollen grain (Information for the drawing is taken from (Fægri & Iversen, 1992) and from www.paldat.org (*PalDat - Palynological*

520 ***Database.*). Red scale bar is 10 μm.**
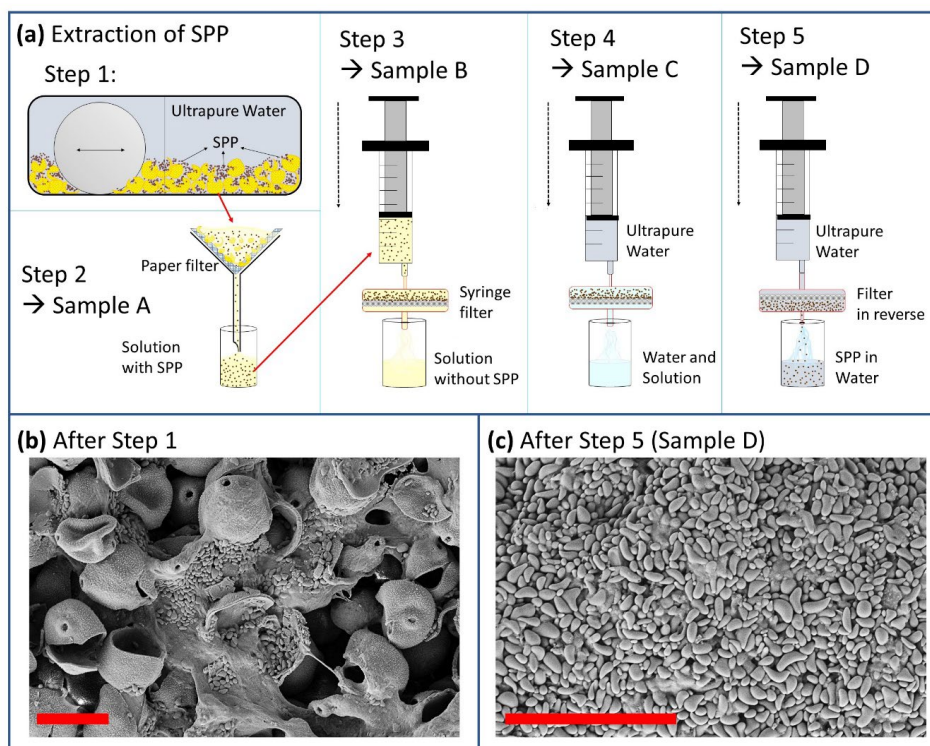
Figure 2: a) Extraction process of SPP. Step 1: Entire pollen grains mixed with ultrapure water are crushed in a mixer

525 **mill. Step 2: The sample is filtered so that entire pollen grains and large outer shells are excluded. Step 3: Filtration with a 0.2 μm pore sized filter. Step 4: The filter including the SPP is rinsed with ultrapure water to wash off soluble material. Step 5: The filter is reversed and the SPP are extracted with ultrapure water. b) Crushed pollen grains, cytoplasmic material and SPP (starch granules) after step 1. c) Extracted SPP after step 5. Red scale bar = 20 μm.**

530
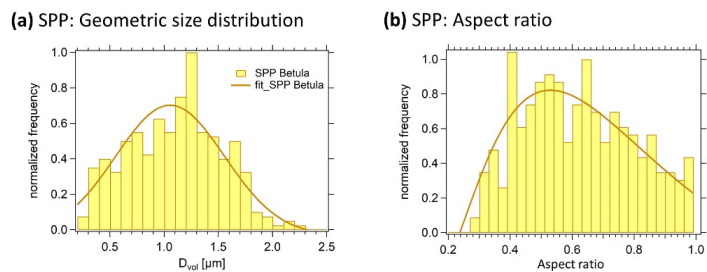
Figure 3: a) Size distribution of SPP. b) Aspect ratio of SPP.

535
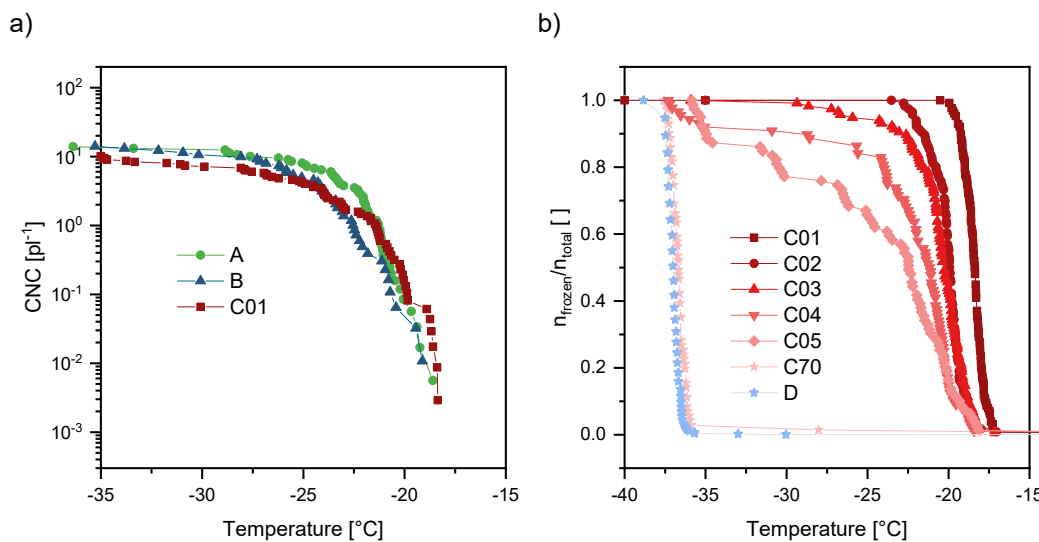
Figure 4: Ice nucleation spectra of extracted samples. a) Cumulative nucleus spectra of sample A, B and C01. b)
540 **Freezing spectra of SPP washing solutions (C01, 1 mL water used to wash SPP, C02, 2 mL water used to wash SPP,**
etc.) and of the purified SPP (D).
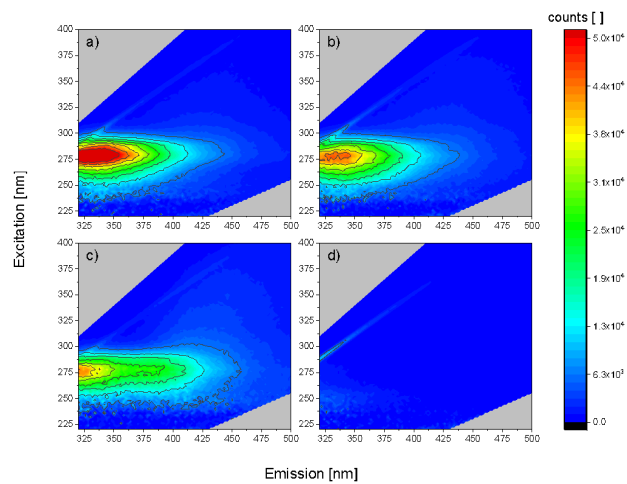
Figure 5. Fluorescence excitation-emission maps of obtained SPP samples. a) Sample A, 1:10 diluted with ultrapure water. b) Sample B, 1:10 diluted with ultrapure water. c) Sample C01. d) Sample D (washed SPP).

545