Interactive comment on "Lignin's ability to nucleate ice via immersion freezing and its stability towards physicochemical treatments and atmospheric processing" by Sophie Bogler and Nadine Borduas-Dedekind

Anonymous Referee #1 Received and published: 4 August 2020

The manuscript presented ice nucleation ability of a commercial lignin via immersion freezing. This study measured the frozen fraction for lignin at different carbon contents. It was shown there is a nonlinear relationship between freezing temperature (T50) and lignin concentration of 2-200 mg C/L. This study also investigated the effects of sonication, heating and reaction with H2O2 and O3 on ice nucleation ability of lignin. The filtration at 0.22 and 0.2 micrometer was used to exam the size dependence of lignin's ice nucleation ability. This study provides additional data sets for the better understanding in the ice nucleation potential of lignin-like aerosol particles. Part of the methods and conclusions need clarifications before it can be considered for publication.

We thank the reviewer for their feedback and address the individual comments in the section below.

Comments:

Line 100, 145, why the molecular biology reagent water was used as background water? The milli-Q water was also used during the filtration, what are the differences between these two in terms of ice nucleation measurements?

 \rightarrow Ice nucleation measurements are sensitive to contaminations which increase the water background's freezing temperature in FINC. Thus, we chose a very pure, commercially available water source, the molecular biology reagent water, for background measurements and as a solvent to minimize contaminations introduced by the water. The milli-Q water is less pure, results in higher background freezing temperatures in FINC and was therefore only used for a first rinsing step in line 145 to avoid wasting expensive molecular biology reagent water. The rinsing procedure was subsequently complemented by rinsing with molecular biology reagent water as well.

To add clarity, we modified the sentence which now reads, "Lignin solutions were prepared from powder kraft lignin (471003, Sigma Aldrich, average M_w 10 000 g mol⁻¹, Error! Reference source not found.) dissolved in molecular biology reagent water (hereafter termed *background water*, W4502, Sigma Aldrich, Germany) to minimize contamination from the solvent."

Line 180, how can ozonation approach used in this study represent the atmospheric aging by ozone? What are the potential differences or impacts?

 \rightarrow We thank the reviewer for this comment. Admittedly, the oxidation setup in this study is simplified compared to atmospheric oxidation by ozone. We think the most important difference is connected to the specific reaction kinetics in the cloud droplet compared to the experimental bulk phase. Particular to our setup, the ozone bubbling in solution may be enhancing the partitioning kinetics of gaseous ozone into the solution. Nevertherless, our approach illustrates an upper exposure limit to ozone and the lack of change observed in lignin's IN ability after exposure underlines the biopolymer's recalcitrance.

In section 3.3.2 of the manuscript, we revised our argument for clarity upon receiving this reviewer's comment as follows:

"We argue that our experimental setup with bulk solutions represent an upper limit for the reactivity of lignin towards O_3 in the atmosphere. The bubbling of a flow of O_3 within the solution is not directly comparable to atmospheric gas phase O_3 partitioning. Indeed, bubbling supplies a larger water-air interface thereby increasing the partitioning of gaseous O_3 into solution, leading to higher dissolved O_3 concentrations in our experimental setup. Nevertheless, the lack of change in freezing temperatures during these experiments further illustrates the recalcitrance of lignin's IN activity. Tropospheric O_3 concentrations average around 35 - 40 ppbv globally and rarely reach 100 ppbv, even in more polluted regions (Tiwari and Agrawal, 2018). Thus, atmospheric processing by O_3 likely does not influence lignin's IN activity. This stability in IN activity after <u>oxidation by O_3 up to 1 ppm</u> and 6 h duration was also observed by Kunert et al. (2019) and by Attard et al. (2012) who investigated fungal and bacterial ice nuclei, respectively. Notably, the oxidative gas mixtures in both these studies contained additionally nitrogen dioxide (NO₂) as a second oxidant in the same concentration as O_3 , and <u>still no significant</u> <u>effect was observed</u>. [...]"

Line 190, without detail description of FINC, it is not easy for readers to judge whether the method is appropriate. First, how the temperature uncertainty is determined? Secondly, what are the temperature differences across the whole PCR tray wells?

→ We thank the reviewer for addressing this caveat and agree that the description of our ice nucleation setup with FINC is brief and concise. However we are happy to refer the reader to the pre-print manuscript under review by Miller et al., 2020 in Atmospheric Measurement Techniques Discussions titled "Development of the drop Freezing Ice Nuclei Counter (FINC), intercomparison of droplet freezing techniques, and use of soluble lignin as an atmospheric ice nucleation standard" (Manuscript No.: amt-2020-361) which addresses the open questions about FINC in extensive detail.

Note to reviewer and editor: A link to the manuscript will be available shortly (it has passed pre-review and technical validation) and will be linked to this paper's discussion.

Third, is there any correction on the data with the background water? When looking at the 2mgC/L sample, there may be over 0.3 FF contributed by the background water to 0.5 FF at T50? For 2mgC/L sample, it is about 0.2 FF. For these lower concentration samples, the background water could contribute significantly to the ice nucleation events.

→ There is no correction with background water. We followed the recommendation by (Polen et al., 2018) and by our own FINC manuscript (pre-print Miller et al. 2020 in AMTD). Briefly, the frozen fraction represents a cumulative probability of freezing. The nature of a probability however prevents the use of a subtraction correction which has incorrectly been applied in the past to remove the contribution of background freezing. Thus, we show the whole frozen fraction curve for both background water and sample which does indeed include an overlap for the 2 mg C/L and 5 mg C/L with the one standard deviation uncertainty range of the background water. Here, we admit that measurements of these lower lignin concentrations are at the detection limit of our ice nucleation setup and the freezing contributions from the water background and sample solution cannot be fully disentangled. Therefore, we chose the higher concentration of 20 mg C/L for the subsequent experiment series. We also refer the reviewer to the next point for a detailed discussion of sources of error.

Line 204, what are the estimated uncertainties in TOC concentrations and the uncertainties in ice-active mass site density (nm)? For most of the data in Fig.4C, the values of nm are well within one order of magnitude. How does it look like if uncertainties are considered? Is it still significantly different? We thank the reviewer for this comment. We discuss in the supplementary information, Section S5, that the TOC analysis in our solutions was challenging with our available instrumentation. We therefore relied on the supplier's specification of carbon content (~ 50%). Since we submitted this manuscript, we have also been able to confirm this value independently with an acid digestion of lignin followed by TOC analyzer measurement. We can report TOC concentration uncertainties as a sum of (1) the uncertainty of the balance (+/- 0.01 mg) and (2) volumetric flask (+/- 0.06 mL). The variability in nm introduced by these sources is insignificant and does not affect our conclusions of the observed spread in the dilution series.

Additionally, to test the limits of the spread in n_m we observed, we added Figure S8 which considers the effect of a hypothetical maximum TOC variability of 50 % on lignin's n_m values in the dilution series. Even then, the spread in n_m remains significant compared to the error.

To further clarify these uncertainties, we have now added error bars to Fig.4c, we have added Figure S8 and we reworded the discussion in Section 2.5.2 as follows:

"[...] Without dividing by TOC, Eq. 1 results in the number of IN sites, also plotted in **Error! Reference source not found.** Note that the TOC content was calculated based on the 50 % carbon content by mass as specified from the vendor's elemental analysis (Sect. 2.1). Uncertainties in the TOC content were quantified based on sample preparation, and included the balance (\pm 0.01 mg) and the volumetric flask (\pm 0.06 mL) and illustrated as error bars in Figure 4c. An additional discussion of errors related to n_m can be found in the SI (Sect. S5). [...]"

Anonymous Referee #2 Received and published: 9 August 2020

This manuscript describes the ice nucleation activities of lignin after different physicochemical treatments such as sonication, heating and hydrogen peroxide digestion and simulated atmospheric processing such as photochemistry and ozone oxidation. The authors use custom-built freezing ice nuclei counter (FINC) to measure freezing temperatures in the immersion freezing mode. They also investigated effect of dilution of lignin and observe that dilution decreases frozen fraction but interestingly when the frozen fraction values were normalized by organic carbon content then active site sites per mg of carbon increases with decreasing lignin concentration. Overall, the authors found that physicochemical treatments don't not have much effect on the freezing temperatures of lignin. This manuscript can be published after appropriate revisions, mostly providing some discussion of results and elaborate some of reasoning behind the experimental design. Especially there are different treatments were performed, the authors need to justify why they did those, why they did and didn't observe any changes in freezing temperatures.

We thank the reviewer for their feedback and address the individual comments both directly below and by revising sections in the manuscript.

General comments: The authors selected a lignin compound that is robust against degradation and stable structure. Then several physicochemical treatments were performed. It is probably expected that there is not significant different in changes after degradation. Then why this particular lignin material was used?

→ We thank the reviewer for this attentive comment. Indeed, at the beginning of our study we expected to observe more degradation upon processing lignin. This expectation was based on the results from e.g. Borduas-Dedekind et al., 2019 and Gute and Abbatt, 2018 who processed dissolved organic matter (DOM) and showed how the complex material degraded after atmospheric processing. We knew lignin is part of DOM and wanted to follow-up these studies by processing this subcomponent in depth and observe its specific behavior. As the reviewer is pointing out, we now present results that underline how lignin is stable and robust against degradation by physicochemical and atmospheric processing.

Still, we see our findings as relevant because establishing the biopolymer's stability strengthens lignin's atmospheric relevance. If removal from the atmosphere other than by wet and dry deposition is hard to achieve, lignin will have a long atmospheric lifetime and contribute to the overall ice nucleating activity throughout that time.

In the text, we added a sentence as the end of the introduction to highlight how our initial hypothesis had to be revised. The text now reads,

"Although we expected lignin, as a subcomponent of organic matter, to be reactive towards these processing treatments, we found that it was particularly recalcitrant."

Overall, discussion of the result needs to be elaborated. For example, if you didn't observe any changes in freezing temperatures after some treatment- please explain what might may cause this.

 \rightarrow We thank the reviewer for this comment and hope to address it fully by specifying where we see the source of stability within the polymer. Indeed, the stability likely results from the lack of labile functional groups in lignin's structure which limits the polymer's reactivity (we added a figure of the polymer to figure 1). For example, the carbon backbone of the polymer is neither is carbon-based and is strong and the esters and ether groups would require harsh acid hydrolysis to be broken apart. On the other hand, the chromophoric, aromatic substructures are subject to a reaction in the photochemical processing we applied, as shown by a decrease in UV/Vis absorbance. However their decay did not result in changes in freezing temperatures. Evidently, these specific substructures are either not the source of lignin's ice nucleating ability or the products after photochemical processing are equally active in nucleating ice.

Further, upon receiving this reviewer's comment we have now strengthened our argument in section 4.3. as follows: "[...] Only harsh treatment conditions such as heating above 260 °C substantially reduced lignin's IN ability in immersion mode freezing to FINC's limit of detection. We link the source of lignin's stability to the lack of labile functional groups in its structure. For example, the carbon backbone of the polymer is strong, and the ester and ether groups would require harsh acid hydrolysis to be broken apart. We emphasize that due to this robustness, lignin can likely be part of heat-stable components that are observed after heating treatments of complex organic INP samples. [...]"

Additionally, as an illustration, we have revised Figure 1 in the manuscript which now includes a polymeric exemplary structure of lignin based on the structure provided by our supplier Sigma Aldrich. This figure is mentioned in lines 63, 65, 112 and 614.

Typically, heating treatment is used to understand the effect of biological material not just to remove the contributions of organic matter. Please discuss.

 \rightarrow We think this question can be clarified by our definition of organic matter as a generic term that includes biological material. So any treatment that removes organic matter in general would also target biological material.

To further add clarity, we have revised the introduction, section 3.2 and section 3.2.2 in the text: "In this study, we focused on the ice nucleation (IN) abilities of organic matter. We define organic matter as a generic term for material that is made of mostly C, H, O, N and S covalently bonded which includes biological material as a specific subset. [...]", "[...] In particular, heating and the reaction with hydrogen peroxide have been used frequently to remove organic material including biological material from IN samples [...]", "[...] Heating procedures are commonly used to remove the contributions of organic matter including biological material to IN activity in complex samples containing mixtures of heat-labile and heat-stable material. [...]"

Uncertainty analysis of freezing temperatures and IN active sites need to be incorporated. Please provide details about the freezing experiment set up. I was bit surprised with the detection limit of the instrument. Maybe authors should discuss limitation of this set-up.

→ We thank the reviewer for addressing this caveat and agree that the description of our ice nucleation setup with FINC is very brief and concise. However we are happy to refer to the newly submitted manuscript by Miller et al., 2020 in Atmospheric Measurement Techniques titled "Development of the drop Freezing Ice Nuclei Counter (FINC), intercomparison of droplet freezing techniques, and use of soluble lignin as an atmospheric ice nucleation standard" (Manuscript No.: amt-2020-361) which addresses the open questions about FINC in extensive detail. The manuscript has now gone through the pre-review and should be posted momentarily. We will post a link to the manuscript describing the FINC validations and detection limit as soon as possible.

It raises concern because some of the lower concentration lignin (e.g., 2-5 mg C/L) are very close to the detection limit below -20dgree C or so. Please also discuss about normalization of carbon concentration.

→ There is no correction with background water. Instead, we decided to show the whole frozen fraction curve for both background water and sample which does indeed include an overlap for the 2 mg C/L and 5 mg C/L with the one standard deviation uncertainty range of the background water. Here, we admit that measurements of these lower lignin concentrations are at the detection limit of our ice nucleation setup and the freezing contributions from the water background and sample solution cannot be disentangled fully at the lower frozen fractions. Therefore, we chose the higher concentration of 20 mg C/L for the subsequent experiment series.

→ We thank the reviewer for addressing the discussion on normalization of carbon content. Upon receiving this comment we have revised our analysis as follows. First, we have modified the nm calculation to include all 288 recorded freezing data points which optimizes the representation of ice nucleating activity. Then, we have added a discussion of uncertainty in TOC content. As discussed in the supplementary information, Section S5, the TOC analysis in our solutions was very challenging with our available instrumentation. We therefore relied on the supplier's specification of carbon content (~ 50%). This limits our ability to report uncertainty in the TOC concentrations to sources of uncertainty during the solution preparation, i.e. the uncertainty of the balance (+/- 0.01 mg) and volumetric flask (+/- 0.06 mL) used. The variability in nm introduced by these sources proved not to be large enough to significantly alter the discussion of the observed spread throughout the dilution series.

Nevertheless we have now added the detailed uncertainty values to Fig.4c and introduced the calculation in Section 2.5.2 as follows:

"[...] Note that the TOC content was calculated based on the 50 % carbon content as specified from the vendor's elemental analysis which we assume to be constant (Sect. 2.1). <u>Uncertainties in the TOC content were quantified</u> based on sample preparation, and included the balance (\pm 0.01 mg) and the volumetric flask (\pm 0.06 mL) and illustrated as error bars in Figure 4c. An additional discussion of errors related to n_m can be found in the SI (Sect. <u>S5).</u> [...]"

Lastly, to test the limits of the spread in n_m we observed, we have added Figure S8 that considers the effect of a hypothetical maximum TOC variability of 50 % on lignin's n_m values in the dilution series. Even then, the spread in n_m remains significant compared to the error.

Please provide some explanation, why did you use sonication? If sonication is mostly used to extract material from filter but for your experiments you have lignin in powder form. Then why didn't you see any changes in freezing temperatures?

 \rightarrow We thank the reviewer for these questions and hope to answer them fully as follows: in our physicochemical sections we included some of the most common tools for pre-treating atmospheric samples, among which is sonication. Sonication is a widely used tool for lab work and in the atmospheric community specifically for extracting filter material. We agree that our sample preparation did not include the extraction of lignin from filters. However, our experiment in solution established in general that lignin is not reactive towards the radical pathway with hydroxy radicals that can be formed upon sonication (Miljevic et al, 2014) and the lack of reactivity results in a lack of change in freezing temperatures.

Refer to section 3.2.1. where we clarified our statement as:

"Based on these observations, the effect of pre-treating or extracting organic aerosol samples using sonication is predicted to have no impact on lignin's IN activity."

Similarly, why the authors expected to see changes in ice nucleation activity due to decay of chromophores during simulated atmospheric processing experiments.

 \rightarrow We thank the reviewer for this comment. A decrease in ice nucleation after photochemical processing has previously been linked to the structural decay of chromophores in dissolved organic matter (DOM) e.g. by Borduas-Dedekind et al., 2019. For our study, we have extended the investigation to include

oxidation by ozone as alternative atmospheric processing pathway that may affect ice nucleation activity. As lignin is a subcomponent of DOM that contains chromophores, we expected it to behave similarly to the complex DOM. Additionally, the chromophores are a central structural component, so their loss may have an impact on the overall structure. These structural properties may be the source of ice nucleation activity.

To further clarify this question, we have added to the following lines in the manuscript, section 3.3.: "[...] Atmospheric processing causes aging in the aerosols, altering its physical and chemical properties. For example, photochemical processing causes degrades chromophores. These changes may subsequently have an impact on their IN activity (Attard et al., 2012; Borduas-Dedekind et al., 2019; Gute and Abbatt, 2018; Kunert et al., 2019). [...]"

Can you provide little bit more information about lignin content in soils, plant debris and other sources (any quantification?) that can be aerosolize to atmosphere, this information maybe help to strengthen the atmospheric implications part.

→ We thank the reviewer for this question which has identified an open point of research. There is a lack of detailed quantifications of lignin fractions in atmospheric aerosols on a global scale. Still, there is sufficient evidence for lignin's presence and relevance in the atmosphere. In particular, biomass burning events are important point sources where concentrations up to 150 ng lignin m⁻³ have been measured (Myers-Pigg et al., 2016). The current wildfire events in e.g. North America (09/2020) and the long-range transport of this smoke throughout the continent underline the growing importance of this source.

Furthermore, estimates for atmospheric plant debris exist based on measurements of atmospheric cellulose as a tracer. For example, Sánchez-Ochoa et al. (2007) reported annual average concentrations of plant debris between 33.4 and 363 ng m⁻³ depending on the sampling location. Puxbaum and Tenze-Kunit (2003) reported an average of 750 ng m⁻³ over a time series of 9 months at an urban sampling location. As lignin and cellulose are two highly related biopolymers in terms of their sources, we think these quantifications are a first, valid upper estimate for concentration ranges of lignin.

We have added this information to the section 4.3. as follows: "[...] However, lignin concentrations in the atmosphere have been estimated to be up to 150 ng m⁻³ after biomass burning related events (Myers-Pigg et al., 2016). Lignin is therefore likely more abundant in the atmosphere <u>at certain time-points</u> than other plant extracts and bioaerosols, despite being less ice active. <u>Another quantitative estimate for lignin's relevance in the atmosphere is based on quantification of atmospheric plant debris. For example, Sánchez-Ochoa et al., 2007 reported annual average concentrations of plant debris between 33.4 and 363 ng m⁻³ depending on the sampling location. (Puxbaum and Tenze-Kunit, 2003) reported an average of 750 ng m⁻³ of plant debris and of 374 ng m⁻³ of cellulose over a time series of 9 months at an urban sampling location. As lignin and cellulose are related biopolymers, these values may provide an upper limit for a concentration range of atmospheric lignin. [...]"</u>

Minor comments: Probably it is more appropriate to place Fig 3 before Fig.2

 \rightarrow We thank the reviewer for this suggestion and have adapted the order of Figure 2 and 3 accordingly.

Please provide error bars in frozen fraction and active sites plot.

→ We thank the reviewer for this comment. The error bars in the frozen fraction figures report the uncertainty in freezing temperature of each well, that results from the uncertainty in the bath and the spread over 3 trays, which is constantly +/- 0.5 °C for both sources specific for FINC (Miller et al., 2020). To avoid cluttering in the figures we have opted to report this uncertainty only in the written text (Section 2.5) and have now further added the value to the caption in Figure 4a. To illustrate this decision, here is the frozen fraction plot with added uncertainty at each 1/288 step of freezing:



Regarding the active sites plot, we refer the reviewer to our answer to the comment: "Please also discuss about normalization of carbon concentration."

Figure 6: There is a decrease in freezing temperature after 260 degree C. At 260 degree C it reached already close to the background water. Then what causes a decrease in freezing temperature at 300 degree C? Probably most of the material is depolymerized at this temperature. Please explain.

→ We thank the reviewer for addressing this caveat in our discussion. As the freezing temperatures continuously decrease with increasing heating temperatures higher 180 °C we expected this trend to include the highest heating temperature of 300 °C as likely more and more material is depolymerized. Instead however we observed a turning point of this decreasing trend at 300 °C and admittedly, we have no explanation for this observation at this point. Still, we decided to be transparent and show the dataset of the whole measurement range to create room for further discussion and interpretation ideas that can be followed up by further experiments.

To address this specific caveat more openly in the manuscript, we have added the following sentences to section 3.2.2.: "[...] Therefore, to completely remove the contributions from lignin to IN activity in ambient samples, a temperature above 260 °C is necessary. It is likely that when heat-stable organic fractions have been observed in complex samples after a heat-treatment < 260 °C, lignin fragments were contributing to the remaining IN activity (e.g. in Hill et al., 2016; Suski et al., 2018). Of note, we observed an unexpected increase of the T_{50} value to -21.8 °C for a heating temperature of 300 °C, challenging the decreasing trend in IN activity. However, the reason for this increase at 300 °C remains unclear. [...]"

Note to editor:

Since the original manuscript was submitted, the method development in quantifying the carbon content in the lignin solution with the available instrumentation has improved (SI, Sect. S5). With information on the carbon content newly available, we saw a potential impact on the interpretation of our results specifically for the photochemical processing section. This is why we re-ran those experiments and adapted our discussion in Section 3.3.3 accordingly: "[...] Interestingly, the observed decrease in absorbance matches closely in both experiments, indicating that the same functional groups were affected by photochemistry. The preliminary analysis of the TOC content (SI, Sect. S5) showed a potential average decrease of 25% by weight after 25 h of UVB irradiation. However, we know that this decrease is not large enough to substantially influence our interpretation of ice nucleating activity after normalization to carbon content (SI, Fig. S8). Furthermore, we tracked the production of photoproducts formed from the chromophoric decay of the polymer, including formic acid, acetic acid, and oxalic acid. [...]"

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Lignin's ability to nucleate ice via immersion freezing and its stability towards physicochemical treatments and atmospheric processing

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Abstract. Aerosol-cloud interactions dominate the uncertainties in current predictions of the atmosphere's radiative balance. Specifically, the ice phase remains difficult to predict in mixed-phase clouds, where liquid water and ice coexist. The formation of ice in these clouds originates from heterogeneous ice nucleation processes, of which immersion freezing is a dominant

- 10 pathway. Among atmospheric surfaces capable of templating ice, mineral dust, biological material, and more recently organic matter are known to initiate freezing. To further our understanding of the role of organic matter in ice nucleation, we chose to investigate the ice nucleation (IN) ability of a specific sub-component of atmospheric organic matter, the biopolymer lignin. Ice nucleation experiments were conducted in our home-built Freezing Ice Nuclei Counter (FINC) to measure freezing temperatures in the immersion freezing mode. We find that lignin acts as an ice active macromolecule at temperatures relevant
- 15 for mixed-phase cloud processes (e.g. 50% activated fraction up to 18.8 °C at 200 mg C L⁻¹). Within a dilution series of lignin solutions, we observed a non-linear effect in freezing temperatures; the number of IN sites per mg carbon increased with decreasing lignin concentration. We attribute this change to a concentration-dependant aggregation of lignin in solution. We further investigated the effect of physicochemical treatments on lignin's IN activity, including experiments with sonication, heating and reaction with hydrogen peroxide. Indeed, harsh conditions such as heating to 260 °C and addition of 1:750 g of
- 20 lignin to mL of hydrogen peroxide were needed to decrease lignin's IN activity to the instrument's background level. Next, photochemical and ozone bubbling experiments were conducted to test the effect of atmospheric processing on lignin's IN activity. We showed that this activity was not susceptible to changes under atmospherically relevant conditions, despite chemical changes observed by UV/Vis absorbance. Our results present lignin as a recalcitrant IN active subcomponent of organic matter within for example biomass burning aerosols and brown carbon. They further contribute to the understanding

25 of how soluble organic material in the atmosphere can nucleate ice.

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1 Introduction

To reduce modelling uncertainties of radiative forcing from aerosol-cloud interactions, an improved understanding of ice formation in the atmosphere is necessary (Stocker et al., 2013). Atmospheric ice crystals influence cloud properties by altering their microphysical and radiative properties, thereby affecting precipitation patterns and cloud lifetime (Storelvmo et al., 2011).
35 In fact, the majority of precipitation originates from the ice phase (Müllmenstädt et al., 2015). In mixed-phase clouds where water and ice co-exist, ice crystals grow at the expense of supercooled water droplets through the Wegener-Bergeron-Findeisen process. As the saturation water vapour pressure over ice is lower than over water, ice crystals grow and consequently dominate the phase distribution of water in the cloud (Korolev and Field, 2008). Ice nucleating particles (INPs) are necessary to induce freezing at warmer temperatures via a heterogenous freezing pathway since homogenous freezing of nanoliters of cloud water droplets becomes instantaneous only at temperatures below –38 °C. In mixed-phase clouds, the immersion freezing pathway dominates heterogenous freezing and occurs when solid or dissolved INPs initiate freezing from within a supercooled cloud droplet (Hoose and Möhler, 2012; Knopf et al., 2018). It remains crucial to study the pathways of ice crystal formation in these clouds to advance our quantitative understanding of warming and cooling factors contributing to the aerosol-cloud radiative effects (Storelvmo et al., 2011).

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In this study, we focused on the ice nucleation (IN) abilities of organic matter. We define organic matter as a generic term for material that is made of covalently bonded C, H, O, N and S, and includes biological material as a subset of organic matter. Organic aerosols are ubiquitous in the environment (Jimenez et al., 2009) and their ice nucleating ability is highly variable and depends on their chemical composition (Knopf et al., 2018). Recently, dissolved organic matter from lakes and rivers have

50 been identified as efficient soluble INPs (Borduas-Dedekind et al., 2019; Knackstedt et al., 2018; Moffett et al., 2018). The analytical challenge, however, of resolving the chemical composition of complex organic matter hinders our ability to identify the specific functional group, chemical moieties or conformations acting as a surface to template ice. Thus, detailed elucidations of the IN active component of organic matter, including organic aerosols, can help improve ice crystal concentration predictions in mixed-phase clouds.

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We chose to reduce sample complexity by investigating the IN activity of a specific commercially available sub-component of organic aerosols, the biopolymer lignin. Indeed, lignin is the second most abundant organic polymer on earth after cellulose. An estimated 30% of the organic carbon present in our biosphere is part of this polymer (Boerjan et al., 2003). Lignin functions as an essential structural component in the cell walls of plants where it builds a complex matrix with cellulose and

- 60 hemicellulose (Faraji et al., 2018). As such, lignin provides stability, enables the efficient transport of water and solutes through plant stems and protects the plant against external pathogens (Boerjan et al., 2003). The biopolymer is best described as a complex class of aromatic heteropolymers built from three main precursor monomers of hydroxy-cinnamyl alcohols: namely p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 1) (Vanholme et al., 2019). The monolignols are most commonly linked through a stepwise and entirely chemically-controlled polymerization in irregular patterns via ether bonds.
- 65 (β-O-4, α-O-4) or carbon-bonds (Chandra and Madakka, 2019; Ralph et al., 2019) <u>Gigure 12</u>. Evidently, a variety of formation pathways exists, which likely increases the polymer's structural heterogeneity and its robustness against degradation and enables its protective role in cell wall structure.

Lignin is emitted into the atmosphere through three main pathways: (1) sea and lake spray aerosols, (2) soil dust and (3) biomass burning plumes_γ(1) Lignin is part of the organic matter in lakes, rivers, and oceans as plant debris. This material enters the water body either via runoff from the watershed or through deposition, for example from overhanging plants. The production of lake, river or sea spray aerosol transfers this biological material including lignin and other biogenic macromolecules into the atmosphere (Axson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2016; Knackstedt et al., 2018; Meyers-Schulte and Hedges, 1986; Olson et al., 2018; Meyers

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al., 2019; Slade et al., 2010; Zark and Dittmar, 2018). The aerosols containing this complex organic matter have been shown to nucleate ice at temperatures relevant for mixed-phase clouds (Borduas-Dedekind et al., 2019; Knackstedt et al., 2018;

- 85 Moffett et al., 2018). (2) Soils can be a source of lignin-containing organic matter to the atmosphere. Wind erosion can transport soil dust and plant fragments into the atmosphere, for example during agricultural harvesting (Suski et al., 2018; Tobo et al., 2014). Studies from e.g. Conen et al. (2011), Hill et al. (2016), Pratt et al. (2009), Suski et al. (2018), and Tobo et al. (2014) have demonstrated the potential of this material to act as INPs before. Further, lignin could be part of organic coatings on soil minerals known to be INPs and thereby influence the mineral's IN activity. Indeed, examinations of organic coatings on
- 90 minerals have revealed the importance of the organic component (Augustin-Bauditz et al., 2016; Birkel et al., 2002; Perkins et al., 2020). (3) Lignin also reaches the atmosphere through biomass burning processes, where its monomeric pyrolysis products are common tracers of organic matter (Shakya et al., 2011; Simoneit, 2002). In addition, large fractions of polymeric forms of lignin remain present in biomass burning organic aerosols even after pyrolysis (Myers-Pigg et al., 2016; Shakya et al., 2011; Stefenelli et al., 2019). In a study by Myers-Pigg et al. (2016), up to 73-91% of the lignin material in a wildfire
- 95 smoke plume was in polymeric form. We therefore expect lignin to be present and relevant for aerosol-cloud interactions. The IN potential of these biomass burning aerosols has also been investigated before (e.g. McCluskey et al., 2014; Prenni et al., 2012), but results from field and laboratory studies remain contradictory and further elucidations require more details about particle composition and morphology (Bond et al., 2013; Kanji et al., 2017).
- 100 An assessment of the IN activity of plant-derived material including lignin and complex ambient samples has recently been initiated by Steinke et al. (2019). This study concluded that the individual plant-derived organic compounds show lower IN activity than complex ambient plant-derived samples. In fact, lignin was one of the least IN efficient components in plant-derived material with IN activity surface density values between 10⁸ and 10⁹ m⁻², i.e. 2 orders of magnitude lower than the complex samples. With our study, we aim to continue the discussion of IN activity in plant-derived material and
- 105 macromolecules in immersion freezing (Pummer et al., 2015) and contribute IN results for lignin in more detail. We aim to improve our understanding of organic matter's IN abilities on a molecular level by investigating lignin as a model organic aerosol component. To determine lignin's possible impact on aerosol-cloud interactions, we characterized its IN activity in our drop-Freezing Ice Nuclei Counter (FINC, Miller et al., 2020). Additionally, we extended the investigation to the effects of physicochemical treatments such as sonication, heating, reaction with hydrogen peroxide, and atmospheric processing such as
- 110 photochemistry and oxidation by ozone, on lignin's IN activity (Figure 2: Overview of conducted experiments to characterize lignin as an IN macromolecule (INM), Although we expected lignin, as a subcomponent of organic matter, to be reactive towards these processing treatments, we found that it was particularly recalcitrant.

2 Materials and Methods

2.1 Lignin sample preparation

- 115 Lignin solutions were prepared from powder kraft lignin (471003, Sigma Aldrich, average M_w 10 000 g mol⁻¹, Figure 1) dissolved in molecular biology reagent water (hereafter termed *background water*, W4502, Sigma Aldrich, Germany) to minimize contamination from the solvent. Targeted carbon concentrations were based on a carbon content of 50% resulting from the producer's elemental analysis data (47-51%). As specified by the producer, this technical kraft lignin has a remaining sulphur content ≤ 3.6 %. This sulphur is introduced into lignin as an impurity during the delignifying kraft process in wood pulping by addition to double bonds in its aliphatic carbon chain (Lin and Dence, 1992). Sample solutions were stored in amber
- 120 pulping by addition to double bonds in its aliphatic carbon chain (Lin and Dence, 1992). Sample solutions were stored in amber glass vials at 4 °C in between processing and measurements. Sterile conditions are important to minimize background freezing and thus all solutions were prepared in a calibrated laminar flowhood (Labculture® Class II, Type A2 Biological Safety

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Cabinets, ESCO). In addition, all glassware was cleaned before use by rinsing 3 times with distilled water and 3 times with acetone followed by drying in the oven at 120 °C for minimum 1 hour.

2.2 Analytical chemistry instruments

2.2.1 UV/Vis spectrometry

130 To characterize the chromophores within lignin, we measured the absorbance of aqueous sample solutions with UV/Vis spectrometry (Varian Cary 100 Bio, Agilent) (see Fig. S15). A baseline correction with background water was applied to all sample measurements.

2.2.2 Ion chromatography

Acetic acid, formic acid, oxalic acid and pyruvic acid were quantified via ion chromatography (DX-320, Thermo Scientific,
 135 USA) using the method from Borduas-Dedekind et al. (2019). Briefly, we used the instrument with an EG40 eluent gradient generator, a Dionex Ion-Pac AG11-HC RFIC 4 mm column and a guard column, a Dionex AERS 500 4 mm electric suppressor and an electrical conductivity detector. The KOH gradient was set as follows: 0 to 11 min, 1 mmol L⁻¹; 11 to37 min, 1 mmol L⁻¹ to 40 mmol L⁻¹; 37 to 38 min, 40 mmol L⁻¹; 38 to 41 min, 1 mmol L⁻¹. The retention times of the acids were 7.9 min, 10.6 min, 13.5 min and 26.2 min for acetic acid, formic acid, pyruvic acid and oxalic acid, respectively. The calibration curves for

140 each acid are in the supplementary information (SI, Fig. S1). Note that none of our samples had detectable amounts of pyruvic acid.

2.3 Treatment Experiments

2.3.1 Sonication

Lignin solutions concentrated at 20 mg C L⁻¹and 200 mg C L⁻¹ were sonicated in 10 mL volumetric flasks for up to 60 min in 145 a sonicator (Ultrasonic Cleaner, VWR, USA) at 30° C.

2.3.2 Heating

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Lignin was heated as a powder in 20 mg aliquots for 3 h at temperatures ranging from room temperature (21 °C) to 300 °C. After heating, background water was subsequently added to reach a carbon content of 200 mg C L⁻¹. It was necessary to heat lignin in powder form, as heating in solution would have led to unquantified concentration changes due to the evaporation of water. To test the ice nucleating ability of the insoluble by-products generated during the heating, vacuum filtration (0.22 μ m

MCE Membrane, MF-MilliporeTM) and sterile syringe filtration (0.22 μ m PES Membrane, TPP, Switzerland) were used to separate the soluble from the insoluble heated lignin (see Figure 6).

2.3.3 Hydrogen peroxide reaction

The chemical treatment with hydrogen peroxide (H₂O₂) was adapted from Paramonov et al., 2018. Solid lignin samples reacted with H₂O₂ (35 wt% aqueous solution, Sigma-Aldrich) in ratios varying from 1:5 to 1:750 (g lignin : mL H₂O₂) in a glass vial. Using a carbon content of lignin of 50%, based on the information from the supplier, these ratios approximately equate to 1.2 times to 180 times molar excess of H₂O₂ to carbon. The samples were left to react overnight and then diluted accordingly with background water to reach a concentration of 200 mg C L⁻¹ before determining the IN activity of the solution. To ensure the

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160 reaction with hydrogen peroxide was complete and no additional change to the IN activity was introduced, one set of samples (ratios 1:5, 1:50, 1:500) was left to react for an additional time period of 3 days before dilution to 200 mg C L⁻¹.

2.3.4 Size filtration

To classify the size fraction of the lignin polymer responsible for IN activity, lignin sample solutions were filtered with sterile syringes through 0.02 μ m (inorganic membrane, Whatman, Anotop) and 0.22 μ m (PES Membrane, TPP, Switzerland) filters.

165 The filters were first rinsed with 30 mL milli-Q water and then rinsed with 10 mL background water prior to filtration of 10 mL of sample. Freezing experiments with cellulose acetate filters (0.2 μm and 0.45 μm, VWR, USA) and a PTFE membrane filter (0.2 μm, BGB, USA) showed persistent IN activity similar to lignin's at 20 mg C L⁻¹ in our ice nucleation setup after rinsing (see SI, Fig. S2). Therefore, the use of these filters was discontinued for this study.

2.4 Atmospheric processing experiments

170 2.4.1 Photochemical experiment

For the photochemical experiments, 9 mL of a 20 mg C L⁻¹ lignin solution was pipetted in 10-mL borosilicate test tubes (Pyrex, 15 × 85 mm, disposable) and placed inside a motorized turn-table of a commercial photoreactor (Rayonet, Southern New England Ultraviolet Co). The photoreactor was equipped with 8 UVB light bulbs (UVB – 3000 Å from Southern New England Ultraviolet Co.) and provided an irradiation as a function of wavelength spectrum peaking at 310 nm (Fig. S3). The temperature

175 inside the photoreactor is kept stable with a fan at 30-32 °C for the duration of the experiment (up to 25h). At each time-point, test tubes containing sample solution were replaced with test tubes containing either more sample solution or pure milli-Q water to ensure a constant light path.

2.4.2 Actinometry

- To quantify the light intensity in the photochemical setup, we conducted an actinometry experiment with the chemical actinometer pyridine/*p*-nitroanisole (PNA) following the method in Laszakovits et al. (2016) and Borduas-Dedekind et al. (2019). Briefly, a solution containing 20 μM of recrystallized PNA and 0.25 mM of pyridine in nanopore water was irradiated for 6 h under the same conditions as the lignin solutions. At different timepoints, the PNA was quantified via high-pressure liquid chromatography (UltiMate 3000 HPLC, ThermoFisher Scientific). The system was equipped with a reverse-phase C18 column (Ascentis express, 90 Å C18, 15 cm x 4.6 mm, 5 μm), its guard column, and a UV detector. The analyses were
- 185 performed in isocratic mode using as eluent 50:50 A:B, where A is 100% acetonitrile (ACN) and B is 90% acetate buffer at pH 6 with 10% ACN. The eluent was delivered at a flow rate of 1 mL min⁻¹, while the sample injection volume was $20 \,\mu$ L. In these conditions, PNA eluted at 2.98 min and was detected at 316 nm. A plot of ln(c[PNA]/c[PNA]_0) versus time (SI, Fig. S4) provided the pseudo first-order degradation constant for PNA of $k_{deg,PNA} = (0.568 \pm 0.004) h^{-1}$, where the error is the standard deviation of triplicate experiments. Based on $k_{deg,PNA}$ and the measured spectral irradiance of the used light bulbs, we calculated
- 190 the absolute spectral irradiance I_{λ} of this photochemistry setup as I_{λ} = (109.94 ± 0.85) W m⁻² (further calculation details are described in the SI, Sect. S3). Additionally, we determined a conversion factor of 3.14 from the irradiation time in the photoreactor into the equivalent irradiation time in natural sunlight. With this factor, 25 h irradiation in the photoreactor equates to 6.5 days of sunlight in the environment, consistent with the atmospheric lifetime of organic aerosols.

2.4.3 Ozone oxidation setup

- 195 Ozone (O₃) was generated in situ reaching target concentrations of 100 ppbv and 1 ppmv O₃. The O₃ concentration was monitored with an ozone monitor (932, BMT Berlin Messtechnik GmbH). In detail, compressed ambient air filtered (Druckluft-Filter, Center Diehl) from particles and volatile organic compounds (VOCs) was led with 1 L min⁻¹ into the O_3 generator (A2Z Ozone, Inc. USA) where O₃ was generated in a fan-cooled Corona Discharge tube from atmospheric oxygen. The highly concentrated O₃-containing outflow was diluted accordingly with pure nitrogen and then bubbled for up to 6.5 h at
- 200 a flowrate of 0.1 L min⁻¹ through 20 mL of lignin solutions using a teflon tube. Sample solutions with lignin concentrated at 20 mg C L⁻¹ and background water for controls were prepared in 50 mL round-bottom flasks and stirred with a magnetic stirrer on a stir plate throughout the oxidation experiment. The background controls ensured that no additional IN active contamination was introduced in the oxidation setup (SI, Fig. S14). The mass loss through evaporation of solvent following bubble bursting at the solution surface was monitored, but remained < 0.5 wt%. The effect on the final solution concentration 205 was therefore minor and not further considered.

2.5 Ice nucleation experiments

2.5.1 Ice nucleation setup

We used our home-built Freezing Ice Nuclei Counter (FINC) to quantify the heterogeneous ice nucleation of aqueous lignin through immersion freezing (Miller* et al., 2020). Briefly, FINC works by using an ethanol bath to cool sample solution and

- 210 sample freezing is detected based on a change in light intensity in successive pictures captured by a mounted camera throughout cooling. Each experiment yields 288 freezing temperatures, from 20 µL aliquots of sample solution pipetted into 288 piko PCR tray wells. Sample trays are prepared in a laminar flow hood to minimize sources of contamination. FINC's limit of detection is at - 23.9 ± 0.6 °C, determined based on repeated freezing experiments with background water (calculation details in SI, Sect. S4). For lignin samples concentrated at 20 mg C L⁻¹ in 20 μ L aliquots, FINC's uncertainty in the reproducibility is
- 215 ± 0.2 °C based on one standard deviation of the T₅₀ values of 7 experiments (SI, Fig. S7). Additionally, we report a temperature uncertainty of 0.5 °C in the freezing temperature of each well in FINC resulting from the uncertainty in bath temperature and the spread over the three piko PCR trays. This temperature uncertainty was determined based on thermocouple calibration experiments with ethanol inside the piko PCR tray wells (Miller et al., 2020). See SI, Sect. S4 for more details about FINC including a picture of the instrument, an exemplary recorded tray picture during a sample run and details regarding data 220 processing.

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2.5.2 Normalization to organic carbon content

Freezing temperatures were normalized to total organic carbon (TOC) content following Eq. 1 according to Vali (1971, 2008, 2019). The calculation requires the frozen fraction (FF as value between 0 and 1), the concentration of non-purgeable carbon (TOC in mg C L⁻¹), and the sample aliquot in each well (V_{well} is 20 μ L), and results in the n_m value representing the ice-active mass site density (Figure 3). Without dividing by TOC, Eq. 1 results in the number of IN sites, also plotted in Figure 3, Note

that the TOC content was calculated based on the 50 % carbon content by mass as specified from the vendor's elemental analysis (Sect. 2.1). Uncertainties in the TOC content were quantified based on sample preparation, and included the balance $(\pm 0.01 \text{ mg})$ and the volumetric flask $(\pm 0.06 \text{ mL})$ and illustrated as error bars in Figure 4c. An additional discussion of errors related to n_m can be found in the SI (Sect. S5),

Eq. 1: $n_m = -\frac{\ln (1-FF)}{TOC * V_{well}}$ 230

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2.5.3 Data visualization

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FINC data analysis was conducted in MATLAB®. Additionally to two-dimensional FF curve graphs and n_m , boxplots are used to visualize freezing data. The advantage of boxplots is that one dimension is sufficient per experiment to show temperature dependent freezing events which enables a clear and concise data presentation of experiments side by side (Figure 3) as established in e.g. Brennan et al. (2020).

3. Results and literature comparison

3.1 Lignin as an IN macromolecule

Lignin was active as an IN macromolecule in the temperature range relevant for mixed-phase clouds. This observation led us to conduct the list of experiments visualized in Figure 2, to further characterize lignin's IN ability.

265 3.1.1 Concentration dependence of lignin's IN ability

The 50% frozen fraction (T₅₀) of lignin solution ranged between – 18.8 ± 0.15 °C and – 22.6 ± 0.26 °C for a concentration between 200 mg C L⁻¹ (40 μ mol lignin L⁻¹) and 2 mg C L⁻¹ (0.4 μ mol lignin L⁻¹), respectively (Figure 4). Indeed, this dilution series spans two orders of magnitude where the expected decreasing trend in IN ability was observed (Figure 4a). All T₅₀ freezing temperatures were above the instrument's limit of detection, derived from averaging ten background water

- 270 experiments (black line, Figure 4a). The frozen fraction values were then normalized by organic carbon content, known from the mass of lignin weighed while making the solutions, to obtain n_m values between 7.6 °C to 26.2 °C. The n_m values (Figure 4c) increased exponentially with decreasing freezing temperature and covered more than 5 orders of magnitude between 1 and 10⁵ IN sites per mg C.
- P75 Interestingly, we observe a dilution effect after normalization to organic carbon content throughout the series (Figure 4). In other words, the normalized n_m values of lignin are higher for lower carbon concentrations (Fig. 4c). For example, the n_m values of lignin at 2 mg C L⁻¹ are a factor of 10 higher than the n_m values of lignin at 200 mg C L⁻¹. In fact, the T₅₀ values increased exponentially with an asymptote reaching approximately 19 °C with lignin concentrations higher than 50 mg C L⁻¹ (Fig. 4b). We interpret this result as a change in chemistry of the ice active sites of the macromolecules due to dilution effects and suspect that changes in the supramolecular structure are occurring (Sect. 4.1).

When comparing our obtained n_m values for lignin's dilution series, we note that the data fits very well with the lignin parametrization developed in Miller et al. (2020) based on a 20 mg C L⁻¹ lignin solution. This agreement underscores the reproducibility within lignin's IN activity and FINC at the same concentration. In addition, the n_m values have the same slope

285 as the IN parametrization for biogenic particles in sea-spray aerosols from Wilson et al. (2015) (Figure 4c), but are two orders of magnitude lower than the parametrization. In comparison with n_m values from river and swamp dissolved organic matter (Borduas-Dedekind et al., 2019), lignin's IN ability is also lower. Both comparisons indicate that although lignin may contribute to the IN activity in organic matter, it is not the most active component within organic matter. This finding is also consistent with Steinke et al. (2019), who identified lignin to have lower IN activity than other plant material collected from

290 dry leaf debris from either spruce or maple trees and agricultural dust after rye and wheat harvests.

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300 3.1.2 Size dependence of lignin's IN ability

Filtering aqueous solutions of lignin through sterile syringe filters with pore sizes of 0.22 μ m and 0.02 μ m reduced the IN activity (Figure 5). Specifically, the T₅₀ freezing temperatures of the filtrate of the 200 mg C L⁻¹ lignin solution decreased by 0.6 °C with the 0.22 μ m filter and by 1.9 °C with the 0.02 μ m filter compared to the unfiltered sample. On the other hand, the 20 mg C L⁻¹ solutions showed a decrease of 1.3 °C in the T₅₀ value only after filtration through 0.02 μ m. Notably, these 0.02

305 μm-filtered lignin solutions still showed IN activity higher than the water background by 1.3 °C, indicating that the remaining macromolecules of sizes < 0.02 μm were still active in nucleating ice via immersion freezing. In fact, all solutions after filtering remained IN active above the water background. This result illustrates how the polymers vary in size beyond the limit of the filters used and that every size bin investigated here contributes to lignin's IN activity. Thus, the decrease in IN activity after filtering can be attributed to the loss of mass, i.e. a concentration decrease (Sect. 3.1.1), rather than to the loss of particularly</p>

B10 active size fractions of lignin.

Further, we highlight the contribution of the lignin components $< 0.02 \ \mu m$ in comparison to similar filtering experiments of INP samples collected in the field. In samples collected from the sea-surface microlayer (Irish et al., 2017; Wilson et al., 2015), IN activity was retained after filtering through 0.22 μm filters, but reduced to the background level after filtering through 0.02

315 μ m. The ice-nucleating material was further characterized as likely biogenic. The same reduction to the background level after filtering through 0.02 μ m was also observed Brennan et al. (2020), who investigated the IN ability of alpine snowmelt samples. In these studies, the INP size range was confined to 0.22 – 0.02 μ m, which means lignin was not present in appreciable concentrations due to the lack of activity below 0.02 μ m. However, we could now show that this lower limit of 0.02 μ m cannot be generalized to describe IN macromolecules from complex organic samples universally. If IN activity remains after filtering

through $0.02 \,\mu$ m, this activity could be attributed to fractions of lignin as a particular subcomponent of organic matter.

3.2 Effects of physicochemical treatments on lignin's IN ability

We investigated the effects of physicochemical treatments typically used in atmospheric ice nucleation research to deconvolute the source identities of ambient INP samples, including sonication, heating, and reaction with hydrogen peroxide. In particular, heating and the reaction with hydrogen peroxide have been used frequently to remove organic material including biological material from IN samples (e.g., (Conen et al., 2011; Hill et al., 2016; Paramonov et al., 2018; Perkins et al., 2020; Tobo et al., 2014).

3.2.1 Effect of sonication

Sonication is a common extraction tool to remove particulate matter from filters. As Miljevic et al. (2014) showed, this process
 can produce reactive radicals which may impact the aerosol's chemical composition. We therefore investigated if sonication could affect lignin's IN activity. Aqueous lignin solutions concentrated at 200 mg C L⁻¹ and 20 mg C L⁻¹ were sonicated up to 60 min. After 60 min, this treatment did not introduce a distinct change on lignin's IN activity resulting from reactive radicals produced (Fig. S10). Based on these observations, the effect of pre-treating or extracting organic aerosol samples using sonication is predicted to have no impact on Jignin's JN activity.

335 3.2.2 Effect of heating

Heating procedures are commonly used to remove the contributions of organic matter <u>including biological material</u> to IN activity in complex samples containing mixtures of heat-labile and heat-stable material. We heated dry lignin in an oven at a

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range of temperatures up to 300 °C for 3 h before dissolving the powder in background water. We only observed a decrease in lignin's IN activity after exposure to temperatures above 180 °C (Figure 6). In particular, at 260 °C, the IN activity was reduced to the background water level with a T_{50} value of -23.7 °C. Therefore, to completely remove the contributions from lignin to

IN activity in ambient samples, a temperature above 260 °C is necessary. It is likely that when heat-stable organic fractions have been observed in complex samples after a heat-treatment < 260 °C, lignin fragments were contributing to the remaining IN activity (e.g. in Hill et al., 2016; Suski et al., 2018). Of note, we observed an unexpected increase of the T_{s0} value to -21.8 °C for a heating temperature of 300 °C, challenging the decreasing trend in IN activity. However, the reason for this increase at 300 °C remains unclear.

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The mass of lignin was weighed before and after the heating treatment, and the weighted mass loss of lignin powder below 0.01 wt%. Thus, the observed decrease in IN activity is not due to a decrease in the total lignin concentration (Sect. 3.1.1). With increasing heating temperatures, the lignin powder visually darkened and became insoluble. As a result, the experimental solutions contained visible suspensions of lignin from exposure to 220 °C and higher. Therefore, heating altered the polymer's

- 360 chemical structure which affected the IN activity and the solubility. Indeed, these temperature-dependent structural modifications have been studied in detail previously. For example, Kim et al. (2014b, 2014a) heated milled wood lignin to between 150 °C and 300 °C and analysed modifications in the functional group composition using chromatography methods, nitrobenzene oxidation, and NMR. Briefly, the results from this study showed that from a heating temperature of 150 °C, first ether bonds connecting methoxyl groups to aromatic rings within the polymer are broken and then bonds to increasingly bigger
- 365 side-chains and terminal functional groups are cleaved. Starting at 250 °C, depolymerisation occurred, including breakage of ether bonds releasing monomeric phenols. Simultaneously, condensation reactions took place, which reconnect the lignin fragments to modified polymeric structures.

To further test whether the soluble or insoluble lignin can both act as the ice nucleating macromolecule or particle, respectively,

- 370 we filtered the suspensions with sterile syringe filtration using 0.22 µm PES Membrane, TPP filters and measured the IN ability of the filtrate on FINC (Figure Q). Note that attempted vacuum filtrations with 0.22 µm MCE Membrane, MF-Millipore filters led to elevated background water freezing temperatures, so their use was discontinued. We find consistently that the T₅₀ values of the filtrate were the same as the unfiltered heated lignin suspension (Figure Q). Therefore, the insoluble mass lost through filtering was not responsible for the remaining IN activity further confirming the role of the soluble fraction to the IN
- β75 activity. We can be sure that the lignin concentration was lower in the filtrate solutions, since the filtration step removed mass. Thus, the heated and filtered soluble lignin had a higher IN activity compared to the insoluble lignin, further distinguishing IN macromolecule and IN particles. This IN activity could be due to changes in the chemical structure or due to the dilution that may change lignin's supramolecular structure in solution, as discussed in Sect. 3.1.1 and 4.1.

3.2.3 Effect of reactions with hydrogen peroxide

- 380 We exposed lignin to increasing concentrations of hydrogen peroxide for 24 h, then diluted the solution with background water to match 200 mg C L⁻¹ and subsequently measured the freezing temperatures with FINC. Additionally, we tested prolonged reaction time up to 4 days, which did not affect the freezing temperature results as illustrated in Fig. S12. This control was necessary as there were no reliable indicators to show the completion of the reaction with hydrogen peroxide even after potential bubbling, frothing, or heating of the sample has subsided (Mikutta et al., 2005; Paramonov et al., 2018). In all, lignin's
- 385 IN ability decreased at a ratio of 1 g lignin : 200 mL H₂O₂ and larger (SI, Fig. S10). Thus, lignin is robust under hydrogen peroxide treatment and an exposure of at least 50 times molar excess of hydrogen peroxide to carbon is required to induce oxidative effects that lead to changes in the polymer's IN activity.

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- 400 The necessary hydrogen peroxide concentration to observe change in lignin is not directly transferable to lignin's decomposition in complex IN samples. In complex ambient organic samples, other hydrogen peroxide reaction pathways e.g. involving metal ion catalysts or other reactive intermediates are potentially available to break down the molecule (Mikutta et al., 2005). With insufficient amounts of oxidant, organic residuals may contain lignin which continues to contribute to observed IN activity after a hydrogen peroxide chemical treatment (e.g. in Paramonov et al., 2018; Suski et al., 2018). Specifically,
- 405 Paramonov et al. (2018) treated their ambient soil IN samples in the ratio of 1 g material : 5 mL H_2O_2 (35 wt%). Even though the samples' carbon content was low with a maximum of 2.3%, these conditions only equate to an excess of ~ 27 times molar excess of hydrogen peroxide to carbon. Thus, that amount of oxidant would likely not have been enough to completely oxidize lignin in the samples to remove its contribution to IN activity. We conclude that lignin's recalcitrance should be considered when developing methods to remove all organic carbon from complex IN samples.
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At concentrations of hydrogen peroxide exceeding the ratio 1 g lignin : 200 mL H_2O_2 , an odd freezing point depression from hydrogen peroxide decreased the T_{50} values rapidly down to the T_{50} value of a pure H_2O_2 background solution which was reached at the ratio of 1 g lignin : 750 mL H_2O_2 (SI, Fig. S10). The cause of this effect is not fully understood (SI, Sect. S7) but hinders more detailed interpretations of the freezing temperature results for this chemical treatment. Attempts to further

415 quantify chemical changes to lignin via ¹H and ¹³C NMR were unsuccessful due to lack of mass and thus signal. Further, in UV/Vis-spectrometer measurements, lignin's signal overlapped the absorbance of H₂O₂ in the relevant wavelength range of 400 – 200 nm (Vaghjiani and Ravishankara, 1989; SI, Fig. S11).

3.3 Effects of atmospheric processing on lignin's IN activity and chemical structure

Organic aerosols have an average atmospheric lifetime of days to weeks. During this time, the aerosols will be subject to atmospheric processing, which can include photochemistry resulting from exposure to sunlight or other reactions involving atmospheric oxidants such as ozone. Atmospheric processing causes aging of the aerosols, altering their physical and chemical properties. For example, photochemical processing degrades chromophores. These changes may subsequently have an impact on their IN activity (Attard et al., 2012; Borduas-Dedekind et al., 2019; Gute and Abbatt, 2018; Kunert et al., 2019). To observe possible impacts of atmospheric processing on lignin's IN activity, we conducted photochemical experiments and reactions

425 with ozone. To track possible structural changes, we measured UV/Vis spectra and followed the production of small lowweight organic acids resulting from photochemical decay with IC.

3.3.1 Effect of photochemistry on lignin's IN activity

Lignin samples concentrated at 20 mg C L⁻¹ were subjected to UVB irradiation for up to 25 h in a photoreactor, corresponding to approximately 6.5 days in the atmosphere (SI, Sect. S3). After this photochemical exposure, the change in T_{50} value was less than - 1 °C (SI, Fig. S13). Overall, we calculated a weak decreasing trend in freezing temperatures with increasing

430 less than -1 °C (SI, Fig. S13). Overall, we calculated a weak decreasing trend in freezing temperatures with increasing irradiation with a weak correlation coefficient of -0.65 (with p = 0.023). A repeated 25-h exposure experiment reproduced these results. We conclude that lignin is recalcitrant to photochemical degradation, despite its ability to act as a chromophore.

We emphasize the lack of decrease in lignin's IN activity by photochemistry as recent studies on pollen (Gute and Abbatt, 2018) and dissolved organic matter have shown otherwise (Borduas-Dedekind et al., 2019). Specifically, Gute and Abbatt (2018) investigated the effect of indirect photochemical oxidation with the hydroxyl radical on the IN activity of birch and grey alder sub-pollen particles. This study linked the exposure to OH radicals to a decrease in IN activity of pollen. Similarly, Borduas-Dedekind et al. (2019) subjected naturally occurring dissolved organic matter to photochemical processing and found that its ability to nucleate ice decreased at a loss rate of - 0.04 °C T₅₀ h⁻¹. In contrast, lignin's IN activity was resistant to

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445 atmospherically relevant photochemical processing and is likely to be retained throughout lignin's lifetime in the atmosphere in the absence of other deactivating and degradation processes.

3.3.2 Effect of <u>ozone exposure</u> on lignin's IN activity

To simulate the atmospheric processing of lignin $\frac{by Q_1}{D}$, we exposed bulk solutions concentrated at 20 mg C L⁻¹ to a gas flow containing 100 ppbv and 1 ppmv of O₃ for up to 2 h. This exposure did not affect lignin's IN activity significantly (SI, Fig.

- 450 S14) the T₅₀ values changed by 0.7 °C with 100 ppbv O₃ and by 0.4 °C with 1 ppmv O₃, <u>values approaching to the temperature</u> uncertainty in FINC. Furthermore, we tested longer exposure times, but we observed no further change in T₅₀ during an exposure duration of 6.5 h with an O₃ concentration of 1 ppmv, <u>indicating lignin's stability towards ozone oxidation</u>.
- We argue that our experimental setup with bulk solutions represent an upper limit for the reactivity of lignin towards O₃ in the atmosphere. The bubbling of a flow of O₃ within the solution is not directly comparable to atmospheric gas phase O₁ partitioning. Indeed, bubbling supplies a larger avater-air interface thereby increasing the partitioning of gaseous O₃ into solution, leading to higher dissolved O₃ concentrations in our experimental setup. Nevertheless, the lack of change in freezing temperatures during these experiments further illustrates the recalcitrance of lignin's IN activity. Tropospheric O₃ concentrations average around 35 – 40 ppbv globally and rarely reach 100 ppbv, even in more polluted regions (Tiwari and
- 460 Agrawal, 2018). Thus, atmospheric processing by O₃ likely does not influence lignin's IN activity. This stability in IN activity after <u>oxidation by O₃ up to 1 ppm and 6 h duration</u> was also observed by Kunert et al. (2019) and <u>by Attard et al. (2012)</u> who investigated fungal and bacterial ice nuclei, respectively. Notably, the oxidative gas mixtures in both these studies contained additionally nitrogen dioxide (NO₂) as a second oxidant in the same concentration as O_{3a} and still no significant effect was observed.

465 3.3.3 Chemical changes in lignin through atmospheric processing

Changes in the absorption spectrum of lignin in the UV/Vis range revealed that both the photochemical processing and the reaction with 1 ppmv O₃ affected the polymer's chemical structure (Figure 7). In both treatments, the absorbance decreased throughout the measured range of wavelengths with longer exposure duration, especially at the distinctive absorbance peaks around 205 nm, 230 nm, and 280 nm (SI, Fig. S15). This decrease indicates the decay of chromophores in lignin, which are mostly conjugated aromatic systems present in the monolignols (Huang et al., 2019). Interestingly, the observed decrease in

470 mostly conjugated aromatic systems present in the monolignols (Huang et al., 2019). Interestingly, the observed decrease in absorbance matches closely in both experiments, indicating that the same functional groups were affected by photochemistry.

The preliminary analysis of the TOC content (SI, Sect. S5) showed a potential average decrease of 25% by weight after 25 h of UVB irradiation. However, we know that this decrease is not large enough to substantially influence our interpretation of

- 475 ice nucleating activity after normalization to carbon content (SI, Fig. S8). Furthermore, we tracked the production of photoproducts formed from the chromophoric decay of the polymer, including formic acid, acetic acid, and oxalic acid. Indeed, these oxidation products increased in concentration with exposure duration (SI, Fig. S16). However, neither the decay of chromophores nor the resulting production of these small low-weight organic acids affected IN characteristics (Sect. 3.3.1 and 3.3.2). Thus, the chromophoric substructures of lignin reactive to UVB light or ozone are not responsible for the observed IN
- 480 activity of lignin. Alternatively, the new structures resulting from the atmospheric processing may be equally active in nucleating ice. Both conclusions underscore how lignin acts as an especially robust IN macromolecule that is recalcitrant to the effect of structural changes.

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4 Discussion

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4.1 Lignin's supramolecular structure in solution

The results of the dilution series (Sect 3.1.1) show that the concentration dependence of lignin's IN activity is non-linear. After normalization to carbon concentration, the n_m values increase with decreasing lignin concentration (Figure 4c). We interpret this result as evidence for changes in lignin's supramolecular structure. Indeed, it has already been shown how the

- supramolecular shape of dissolved molecules can influence important aerosol properties including ice nucleation (Cascajo-Castresana et al., 2020; Pfrang et al., 2017; Qiu et al., 2019). For example, light diffusion and viscosity change depending on the supramolecular structure which in turn has implications for reaction rates within the aerosol and cloud nucleation processes (Pfrang et al., 2017). Further, Cascajo-Castresana et al. (2020) showed how pH-dependent protein aggregation influences the
- 535 freezing temperature regimes observed. The supramolecular structure of lignin in solution specifically is dictated by interactions with the solvent and intra- and intermolecular forces within the polymers (Huang et al., 2019; Vainio et al., 2004). Additionally, the source of lignin, the presence of other solutes, the temperature, and the solution pH exert important influences on the supramolecular structure (Huang et al., 2019) and can lead to the presence of clusters and agglomerates (Norgren et al., 2001; Norgren and Edlund, 2001; Vainio et al., 2004). We hypothesize that lignin is indeed aggregating in our aqueous
- 540 solutions. In a cloud droplet, lignin may interact via hydrogen bonding with water helping stabilize the ice embryo (Kanji et al., 2017) and promote ice nucleation. As the concentration of lignin decreases, the probability for intramolecular interactions within the polymer decreases. The biopolymer is less likely to form aggregates and could instead be unfolding to interact with more water molecules. Thus, at lower concentrations, the relative IN activity is increased as the additional interactions promote ice nucleation. The implication of this result is important for the interpretation of dilution series in immersion freezing
- 545 experiments.

4.2 Use of commercial lignin

One of the caveats of our work is the use of commercially available kraft lignin as our source material. Kraft lignin is not naturally occurring lignin, but rather a by-product of the pulp and paper industry. Thus, there are differences in the structure of the lignin we used compared to native lignin in plants (Giummarella et al., 2020). Notably, every isolation method currently

- 550 available introduces changes to the structure of lignin, complicating the observation of the biopolymer in its native state (Chung and Washburn, 2016; Stark et al., 2016). The kraft process in particular fragments the polymer and increases its solubility compared to natural lignin to separate it from the pulp. Additionally, sulphur is used, which introduces thiol groups as a minor impurity into the polymeric structure. Consequently, there could be differences between the behaviour of natural and kraft lignin as IN macromolecules. Further experiments will be conducted to investigate the importance of this issue. Nonetheless,
- 555 the polymer backbone structures of native and kraft lignin remain highly related and we do not expect large differences in the IN activity. We argue that native lignin would also be recalcitrant in the atmosphere and therefore has an extended lifetime within the aerosol while retaining its ice nucleating ability in immersion mode.

4.3 Atmospheric Implications

Lignin is a subcomponent of organic matter in aerosols and soils and is capable of nucleating ice in mixed-phase cloud conditions. Specifically, n_m values for lignin solutions ranged from 1 to 10⁵ IN sites per mg C between – 7.6 °C to – 26.2 °C, representing 1-2 orders of magnitude lower values than dissolved organic matter samples (Borduas-Dedekind et al., 2019; Knackstedt et al., 2018; Moffett et al., 2018), sea surface microlayer samples (Irish et al., 2017; Wilson et al., 2015) and plant extracts (Gute and Abbatt, 2018; Steinke et al., 2019). However, lignin concentrations in the atmosphere have been estimated to be up to 150 ng m⁻³ after biomass burning related events (Myers-Pigg et al., 2016). Lignin is therefore likely more abundant

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in the atmosphere, especially during biomass burning events, than other plant extracts and bioaerosols, despite being less ice active. Another quantitative estimate for lignin's relevance in the atmosphere is based on quantification of atmospheric plant debris. For example, Sánchez-Ochoa et al. (2007) reported annual average concentrations of plant debris between 33.4 and 363 ng m⁻³ depending on the sampling location. Puxbaum and Tenze-Kunit (2003) reported an average of 750 ng m⁻³ of plant debris and of 374 ng m⁻³ of cellulose over a time series of 9 months at an urban sampling location. As lignin and cellulose are related biopolymers, these values may provide an upper limit for a concentration range of atmospheric lignin.

Furthermore, lignin's IN ability shows resistance to physicochemical treatments and atmospheric processing despite structural changes in the chromophoric substructures that were observed by UV/Vis absorbance. Only harsh treatment conditions such as heating above 260 °C substantially reduced lignin's IN ability in immersion mode freezing to FINC's limit of detection. We

575 as heating above 260 °C substantially reduced lignin's IN ability in immersion mode freezing to FINC's limit of detection. We link the source of lignin's stability to the lack of labile functional groups in its structure (Fig. 1). For example, the carbon backbone of the polymer is strong, and the ester and ether groups would require harsh acid hydrolysis to be broken apart. We emphasize that due to this robustness, lignin can likely be part of heat-stable components that are observed after heating treatments of complex organic INP samples. Overall, lignin's observed stability implies that lignin present in aerosols is a

580 particularly long-lived organic component and may contribute to the aerosols' overall IN activity throughout their atmospheric lifetime. Notably, the IN ability of lignin will change during the aerosol's lifetime, as water evaporation or condensation cycles affect the polymer's concentration and consequently its macromolecular structure.

Data availability

585 Data presented in all figures in the main text and in the supplementary information are deposited in the ETH Research Collection data repository at https://doi.org/10.3929/ethz-b-000422111.

Author contributions

SB and NBD designed the experiments, and SB carried them out. SB and NBD prepared the manuscript together.

Competing interests

590 The authors declare no conflict of interest.

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 $805 \quad \mbox{Figure 2: Overview of conducted experiments to characterize lignin as an IN macromolecule (INM).}$

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Figure 3. Exemplary freezing data visualization based on a 100 mg C L^{-1} lignin sample solution. The left blue y-axis shows the FF curve as a stepwise function of each freezing event, 288 data points are included. The green line curve and the right green y-axis show both the INP number concentration and n_m as the INP number concentration normalized to carbon content. Note that the INP conc. and n_m values differ only by the factor of 100, given by the TOC concentration of 100 mg C L^{-1} in this example. The FF curve on top is consolidated into a boxplot at the bottom. On the boxplot, the red dot shows the median (T_{s0}). The box frame is limited to the 25th and 75th percentile and the box whiskers show the 10th and 90th percentile. The remaining percentiles ($1^{s1}-9^{th}, 91^{th}-100^{th}$) are shown as individual data points with filled blue circles.



 $\begin{array}{c|c} 830 & \mbox{Figure 4: (a) Freezing temperatures for aqueous dilution series as FF curves. The FF curves include the water background (bg water black line) and its uncertainty range (shaded grey area) to describe FINC's limit of detection. <u>The temperature uncertainty in each well is constantly \pm 0.5 °C.</u> (b) Median freezing temperature (Ts0) of the aqueous dilution series as a function of lignin concentration. The Ts0 values include the standard deviation from multiple experiments where applicable (n \geq 2) and are fitted with Ts0 = -3.7*exp(-0.046*x) - 19.46 *exp(-0.00014*x), R^2 = 0.99. Lignin's IN activity in this dilution series remains above the water$

- 835 background, but is decreasing exponentially with decreasing concentration as illustrated by the slope of the T₅₀S. (c) ice-active mass site density n_m as a function of temperature of the aqueous dilution series of lignin. At T_{10} , T_{50} , T_{90} of the 200 mg C/L trace, the freezing temperature x-axis uncertainty of 0.5 °C and uncertainty in nm based on variation in carbon content are included. Note that the y-axis uncertainty is smaller than the limits of the box marker. For comparison, a fit for biogenic particles in sea-spray aerosols from Wilson et al. (2015), a lignin parametrization based on 20 mg C L^{-1} solutions from Miller et al. (2020), where 840 n_m=exp(-0.49*T-1.2), and n_m values from dissolved organic matter (Borduas-Dedekind et al., 2019) are included.



Figure 5. Freezing temperature boxplots for the size filtration series. Aqueous lignin solutions of 200 mg C L⁻¹ and 20 mg C L⁻¹ concentration were filtered through 0.22 µm and 0.02 µm sterile syringe filters. The filtration step reduced the T₅₀ values, however 845 the measured IN activity remained above the background for all samples.



Figure 6. Freezing temperature boxplots of the 200 mg C L⁻¹ lignin solutions directly after heating (blue, unfiltered) and after filtering through a sterile syringe filter of 0.22 µm, PES Membrane, TPP (green, filtered). Bg water refers to background water, RT refers to room temperature, i.e. 20 °C. Irrelevant of the filtering, lignin's IN activity is decreasing beginning from a heating temperature of 180 °C.

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Exposure Duration [h] Figure 7. Wavelength-specific absorption values from UV/Vis spectra of 20 mg C L⁻¹ lignin solutions after atmospheric processing through UVB irradiation and exposure to O₃. The insert is a zoom of the plot section from 0 to 2 h exposure duration. With increasing exposure duration, lignin's absorbance is decreasing. Changes after exposure to 100 ppbv O₃ are minimal.

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