MS bg-2019-276, Kunert et al.: Highly active and stable fungal ice nuclei are widespread among Fusarium species

We thank referee #1 for his/her comments, questions, and suggestions, which have been taken into account upon revision of our manuscript. The comments and our answers are listed below (referee's comments marked with blue letters).

1. Title:

Referee comment: The title is misleading: the authors' conclusion is that 16% of the tested strains were ice active above -14 °C. I would argue that this percentage does not equate to "widespread". The authors also did substantial work with physical and chemical processing of their material which is not reflected in the title, but could be. For example, something along the lines of "Ice nucleation ability of 65 different Fusarium species: Effects of storage, size and chemical processing"

Author's response: We changed the title to: "Macromolecular fungal ice nuclei in *Fusarium*: Effects of physical and chemical processing" and modified the corresponding parts in the manuscript accordingly.

2. Abstract:

Referee comment: Why did the authors choose -14 °C as their threshold? This discussion should be added here in the abstract (and in the text as well).

Author's response: We thank the referee for pointing this out. We actually meant -12 °C, and we realized that we had a typing error here, which we corrected now.

As *Fusarium* nucleates in a broad temperature range between -1 and -9 °C (Hasegawa et al., 1994; Humphreys et al., 2001; Pouleur et al., 1992; Richard et al., 1996; Tsumuki et al., 1992; Tsumuki and Konno, 1994), and as the water background of LINDA started to freeze at -14 °C, we set the threshold to -12 °C.

Referee comment: The relevance of Fusarium should be explained in the abstract.

Author's response: We thank the referee for this suggestion and included the following sentences in the abstract: "Ice nucleation activity in fungi was first discovered in the cosmopolitan genus *Fusarium*, which is widespread in soil and plants, has been found in atmospheric aerosol and cloud water samples, and can be regarded as the best studied IN-active fungus."

Moreover, we modified the following sentences: "The frequency and distribution of ice nucleation activity within *Fusarium*, however, remains elusive. Here, we tested more than 100 strains from 65 different *Fusarium* species for ice nucleation activity."

3. Introduction:

Referee comment: Lines 16-17: more recent references should be added, especially because of the mention of macromolecules. Also see review by (Knopf et al., 2018).

Author's response: We thank the referee for this comment and included further references in our manuscript.

Referee comment: Lines 18-20: it would be important to mention nonetheless that recent work has made contributions to our understanding of IN and precipitation references by (Petters and Wright, 2015; Stopelli et al., 2015, 2017).

Author's response: We thank the referee for this remark and added the references to our manuscript.

Referee comment: Lines 21-23: the 3+9 references could be better represented by explaining what each one has observed in one or two sentences each. This added discussion could help set the stage for the relevance of the work under review.

Author's response: The discussion of each reference in one or two sentences each would result in a very long introduction with a review character, especially as referee #2 suggested to add additional references here. Adding a detailed discussion of the different types of biological ice nuclei at this point goes beyond the scope and focus of this manuscript and could lead to confusion of the readers. Instead, some of the references are discussed in more detail in the results and discussion section.

Referee comment: Lines 24-26: same comment as above in addition to this reference (Šantl-Temkiv et al., 2015)

Author's response: We included the suggested reference as well as the reference, which was suggested by referee #2 (Failor et al., 2017), but we prefer to not extend the bacterial IN part of the introduction as the focus of the manuscript should be on fungi, particularly *Fusarium*.

We modified the following sentences: "The best characterized biological IN are common plant-associated bacteria of the genera *Pseudomonas*, *Pantoea*, and *Xanthomonas* (Garnham et al., 2011; Govindarajan and Lindow, 1988; Graether and Jia, 2001; Green and Warren, 1985; Hill et al., 2014; Kim et al., 1987; Ling et al., 2018; Schmid et al., 1997; Wolber et al., 1986), and recently, an ice nucleation-active (IN-active) *Lysinibacillus* was found (Failor et al., 2017). The first identified IN-active fungi were strains of the genus *Fusarium* (Hasegawa et al., 1994, Pouleur et al., 1992, Richard et al., 1996, Tsumuki et al., 1992)."

Referee comment: Lines 28-30: when temperatures are reported, what fraction does it represented? The onset? 1%? Temperature when 50% of the droplets are frozen- T50? See (Vali, 2019)

Author's response: As mentioned in Line 28, the temperatures are reported as initial freezing temperatures, which corresponds to the onset freezing temperature: "To date, a few more fungal genera with varying initial freezing temperatures such as *Isaria farinosa* (~ -4 °C), *Mortierella alpina* (~ -5 °C), *Puccinia species* (-4 °C to -8 °C), and *Sarocladium* (formerly named *Acremonium*) *implicatum* (~ -9 °C) have been identified as IN-active (Fröhlich-Nowoisky et al., 2015; Huffman et al., 2013; Morris et al., 2013; Richard et al., 1996)".

Referee comment: Line 39: define the positive selective pressure for IN activity

Author's response: We thank the referee for pointing out the ambiguity of our statement. For clarification, we modified the sentence: "While the factors for a positive selective pressure for ice nucleation activity in *Fusarium* and other fungi have not been directly identified, an ecological advantage of initiating ice formation is easily conceivable." For example, the bioprecipitation feedback cycle can be such a factor, which is discussed in more detail later (Lines 47-49).

Referee comment: It would be useful for the authors to discuss the mode of freezing investigated and why immersion freezing was used and what is its relevance.

Author's response: The droplet freezing assays, which were used in this study, all measure ice nucleation activity in the immersion freezing mode, where the IN is contained inside a liquid droplet when initiating freezing. Biological IN are often proteins, which are surrounded by a hydration shell, so the immersion freezing mode is suitable for biological IN. Thus, the most common techniques to study biological IN are droplet freezing assays (Després et al., 2012; Hoose and Möhler, 2012).

To avoid misunderstanding, we modified the sentence: "Ice nuclei of selected *Fusarium* species were further analyzed in immersion freezing mode using the high-throughput Twin-plate Ice Nucleation Assay (TINA) (Kunert et al., 2018)."

Referee comment: Good overview of bioprecipitation. Great description of the evolutionary reasons for fungal species to be good ice nuclei.
Author's response: We thank the referee for this comment.

4. Materials and Methods
Referee comment: In general, controls and filter blanks are missing from the data description and analysis and the authors are encouraged to show this data (perhaps in supplementary information) and to discuss this data. For example, what was the IN activity of the water background? What was the activity of the filter background? How did the backgrounds differ from LINDA to TINA?
Author's response: We added the information about the negative controls and included the following sentences in the manuscript:

For the thermal cycler: "Aliquots of uninoculated DPY broth were used as negative controls, which did not freeze in the investigated temperature interval."

For LINDA experiments: "As a negative control, a 0.9 % NaCl solution was added to three uninoculated agar plates, and the freezing started below -14 °C."

For TINA experiments: "Pure water samples (0.1 µm filtered) served as a negative control for each experiment. These did not freeze in the observed temperature interval."

Referee comment: It is clear that the authors used two techniques for their experiments, yet their discussion does not include any comparison plots or discussing the differences in the two instruments. Each figure (and Table S1) should also state which instrument was used to acquire the data.
Author's response: As described in Lines 104-105, the initial screening was performed with two independent droplet freezing assays in two laboratories. Strains of the USDA-ARS/Michigan State University were screened with a thermal cycler as described in Fröhlich-Nowoisky et al. (2015) (Lines 106-108). Strains from the Schmale laboratory at Virginia Tech and strains from the Kansas State University Teaching Collection were screened with LINDA (Lines 111-113). Table S1 provides a summary of all tested strains, the strain collection they originate from, and the results of the screening. Table 1 shows the mean freezing temperatures for the positively tested species. All further analyses were performed with TINA.

Referee comment: Line 115: could the authors show the positive control data?
Author's response: We included the following sentence in section 2.3: "The freezing temperatures ranged from -3.46 °C to -4.58 °C."

Referee comment: Lines 119: clarification: can the authors show their calculations here and are the data presented corrected for the freezing point depression or is the 0.5 C part of the overall uncertainty?
Author's response: We added the calculations to the supplementary information.

The data presented here were not corrected for the freezing point depression as highly concentrated *Fusarium* extracts were used for the initial screening. Thus, we cannot exclude that the high concentration of *Fusarium* IN compensates the effect of NaCl on the freezing

temperature. We added this information in the manuscript: "We cannot exclude, however, that the high concentration of IN compensates the effect of NaCl on the freezing temperature. This is supported by the investigations of Stopelli et al. (2014), who did not find a systematic suppression of freezing at this salt concentration in LINDA experiments."

Referee comment: Additional experiment: dilution series of an active strain to see if the behaviour of the IN active material in solution is linear. I would argue that this experiment would be important to help support the seemingly accurate high freezing temperature data observed for certain strains, for example in Figures 3 and 4 and S1.

Author's response: All samples, which were analyzed with TINA, were measured in a dilution series. We described this in Lines 121-123: "The aqueous extracts were serially diluted 10-fold with pure water by a liquid handling station (epMotion ep5073, Eppendorf, Hamburg, Germany), and 96 droplets (3 µL) were tested per dilution with a continuous cooling rate of 1 °C min^{-1} from 0 °C to -20 °C."

For clarification, we optimized the sentences: "The aqueous extract was serially diluted 10-fold with pure water by a liquid handling station (epMotion ep5073, Eppendorf, Hamburg, Germany) to a dilution where droplets remained liquid in the investigated temperature interval. Of each dilution, 96 droplets (3 µL) were tested with a continuous cooling rate of 1 °C min^{-1} from 0 °C to -20 °C."

5. Results and Discussion

Referee comment: It is necessary for the authors to define their reported freezing temperatures. Are they the onset, the equivalent of one well freezing? If so, how do the authors address the recommendations of not using the onset addressed in (Polen et al., 2018)? Reporting freezing temperatures as T10 and T50 would be additionally helpful.

Author's response: Except for the initial screening, we always report the initial freezing temperatures (T_i) for our measurements, which is equivalent to the onset. We first reported the freezing temperatures for the initial screening as initial freezing temperatures, but we actually meant mean freezing temperatures.

We replaced "initial" by "mean" several times in the text, where we talk about the initial screening.

Referee comment: Lines 141-144: could the authors offer a hypothesis to this lack of verifiability?

Author's response: The fungal culture plates, which were used for the initial screening, could not be used for the measurements with TINA, as different laboratories were involved in this study. Moreover, it is well known that some *Fusarium* species can reduce or lose their IN activity after several subcultures (Pummer et al., 2013; Tsumuki et al., 1995). We discussed this in Lines 156-159 in the manuscript: "It is known that *Fusarium* can regulate the gene expression for IN production depending on environmental conditions such as nutrient availability (Richard et al., 1996), and some *Fusarium* species reduce or lose their IN activity after several subcultures (Pummer et al., 2013; Tsumuki et al., 1995)."

Referee comment: The hypothesis of proteinaceous material acting as IN is valid. What about polysaccharides? (Dreischmeier et al., 2017)

Author's response: We cannot exclude that polysaccharides are involved in the ice nucleation of *Fusarium*. To our knowledge, however, there is no published study showing that polysaccharides are involved in the ice nucleation activity of *Fusarium*.

We discussed a potential role in section 3.3: "The remaining activity after the 98 °C treatment, however, could indicate that post-translational modifications like glycosylation and therefore polysaccharides could play a role in the ice nucleation activity of *Fusarium*. Further systematic studies including chemical analyses are needed for elucidation."

We included the following sentence in the conclusion: "An involvement of polysaccharides, however, cannot be excluded."

Referee comment: Line 166: was there any hypothesis associated with the selection of the strains presented in this section?

Author's response: Not all *Fusarium* strains were available for the experiments with TINA, as the initial screening was performed in different laboratories. But we tried to cover as many different species as possible and selected species, which were long known for ice nucleation activity (*F. acuminatum*, *F. avenaceum*) as well as all the newly identified species.

For clarification, we included this information in section 2.3: "Ice nuclei of selected *Fusarium* species, which were long known for ice nucleation activity (*F. acuminatum*, *F. avenaceum*) as well as all the newly identified species, were further analyzed in immersion freezing mode using the high-throughput Twin-plate Ice Nucleation Assay (TINA) (Kunert et al., 2018)."

Referee comment: Size experiments should be compared to (Irish et al., 2019; Wilson et al., 2015) for example. In addition, the Wilson et al., Nature 2015 paper has a nm parameterization that the authors should include in their discussion of their values.

Author's response: We included the following sentence: "Moreover, biological INMs smaller than 200 nm were also found in various organisms e.g., other fungi (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015), leaves, bark, and pollen from birch trees (*Betula* spp.) (Felgitsch et al.,2018; Pummer et al., 2012), leaf litter (Schnell and Vali, 1973), some microalgae (Tesson and Šantl-Temkiv, 2018), strains of *Lysinibacillus* (Failor et al., 2017), and biological particles in the sea surface microlayer (Irish et al., 2019; Wilson et al., 2015)."

Referee comment: Lines 184-185: I do not understand how the authors arrived at this conclusion. According to figure 2, the majority of the IN activity was lost between 300 and 100 kDa. I would have concluded that the best IN are within that size, not smaller than 100 kDa. I agree with the authors nonetheless that there are still IN active material below 100 kDa, but not the most active.

Author's response: As IN were found in all size fractions, we concluded that *Fusarium* IN are likely single proteins smaller than 100 kDa, which can agglomerate to large protein complexes in solution. We did not claim that the single proteins smaller than 100 kDa are the most active ones. Lines 184-185: "We hypothesize that *Fusarium* IN are single proteins smaller than 100 kDa, which agglomerate to large protein complexes in solution."

As explained in Lines 177-178, filtration through a 300 000 MWCO filter unit decreased the cumulative number of IN per gram of mycelium about 50 % to 75 %. Further filtration through a 100 000 MWCO filter unit reduced the IN number to less than 1 % of the initial concentration (Lines 180-181). So, the majority was lost upon 300 000 MWCO filtration, which were the most efficient IN nucleating at the highest temperatures.

Referee comment: For the discussion to flow, it would be important to explain in line 189 why Erickson came to that conclusion.

Author's response: We changed the sentence to: "Erickson (2009) determined the size of proteins based on theoretical calculations. As the interior of proteins is closely packed with no

substantial holes and almost no water molecules inside, proteins are rigid structures with approximately the same density (~1.37 g cm^{-1}). Assuming the protein as a smooth spherical particle, the minimum diameter of the INM would be smaller than 6.1 nm".

Referee comment: The null effect of chemical processing with O3 and NO2 was somewhat surprising. Based on (Borduas-Dedekind et al., 2019; Gute and Abbatt, 2018; Kunert et al., 2018), I would have expected to see oxidation of the proteinaceous material and thus decrease in IN ability. A discussion involving a hypothesis to the resistance of the strains to oxidation is warranted in light of these studies. Did the authors attempt to extend the exposure to longer times to force a reaction? On a pedantic note, I would argue that ozone exposure of 1 ppm over 4h is not equivalent to 200 ppb over 20h. The experiment was done while bubbling ozone into extracts and there are concentration effects to consider as well as the diffusion of the ozone could affect the chemistry. I would simply omit this sentence and just state the concentration with no mention of equivalence.

Author's response: Based on our results, we cannot exclude that post-translational modifications of the *Fusarium* IN protein occurred during oxidation. These potential modifications do not seem to influence the ice nucleation activity of the protein. For example, they could be in parts of the protein, which are not involved in the nucleation process. We agree with the referee that further investigations are necessary, and we will consider these experiments for future studies.

Moreover, we included the suggested references in the manuscript and extended the following sentence: "This is in contrast to other biological IN e.g., bacterial IN (Snomax®) (Kunert et al., 2018), birch and alder pollen (Gute and Abbatt, 2018), and dissolved organic matter (Borduas-Dedekind et al., 2019), where exposure to oxidizing agents reduced the IN activity."

We deleted the statement and modified the following sentence: "Briefly, a mixture of 1 ppm O_3 and 1 ppm NO_2 was bubbled through 1 mL aliquots of aqueous extract for 4 h, and the IN concentration was determined using TINA."

Referee comment: Null results are difficult to present. To further substantiate the authors' conclusion, I would recommend that the authors show material that indeed reacted under their O3 and NO2 conditions. The authors did do a positive control (Lines 205-206) and showing that data would help further support their claim.

Author's response: As the focus of this study is on fungal IN of *Fusarium*, we did not use Snomax in any of the experiments. As described in the manuscript (Lines 205-206), we found a reduction of IN activity upon exposure to O_3 and NO_2 for Snomax in a previous study (Kunert et al., 2018).

Referee comment: Finally, the storage effects were also null results, but did the authors also do a positive control? In any case, these results are very useful for the community.

Author's response: We could not include a positive control in our storage tests as a suitable control for such experiments was not available. We agree that further IN should be tested for effects of storage.

Referee comment: Figure S1 arguably belongs in the text. The reproducibility between fungal culture plates is remarkably the largest change observed compared to other treatments such as O3 and NO2 exposure. A discussion relating this uncertainty to the other analyses would be important.

Author's response: The data in Figure S1 were obtained from three different fungal culture plates, whereas the exposure experiments were performed with the same aqueous extract of the

particular fungal species. The variability of measurement with individual fungal culture plates is higher than measurements of the same aqueous extract, as the differences did not result from the measurements themselves but rather from the fact that we investigated biological samples.

Referee comment: Report the weights of the mycelium measured gravimetrically (for example in Table S1).

Author's response: Table S1 shows the results of the initial screening, which was performed with two different droplet freezing assays, first a thermal cycler and second the LINDA instrument (section 2.3). For the thermal cycler, mycelium was picked and directly transferred into 96-well PCR plates (Lines 108-110), and for LINDA, 0.9 % NaCl solution was added to the fungal culture plates, which were scraped afterwards to obtain a suspension of mycelium and spores (Lines 80-82). As the initial screening was only a yes or no test, it was not deemed necessary to determine the weight of the mycelium.

Referee comment: Is there value in considering the work in the context of food science and cryogenic food storage? Is it more likely that these strains be found in food or in the atmosphere?

Author's response: *Fusarium* species are frequently associated with plant material (Leslie and Summerell, 2006), including many food types, and some of the strains used in the current study were initially isolated from plants. Thus, IN from such fungi could be important in food response to freezing temperatures, which could be worth future investigation. Considering the work in the context of food science and cryogenic food storage, however, would be outside the scope of this manuscript, in which we focus on atmospheric aspects of ice nucleation activity in *Fusarium*.

Referee comment: Table S1 should present quantitative details. The authors should specify what their criteria is for "IN-active" strains. 1/96 wells? Onset? Temperature range? It would also be useful to add a fourth column with the freezing temperatures (T10 or T50 or T90). Did the authors consider making a parameterization with their data as an upper limit of IN activity of Fusarium species?

Author's response: For the initial screening using the thermal cycler, up to seven droplets were investigated for each sample. If the sample was IN-active, all droplets froze in the investigated temperature interval. We included the following sentence: "Up to seven droplets were measured for each sample, and the mean freezing temperature was calculated."

For the initial screening with LINDA, three droplets were investigated for each sample, which was described in the manuscript in Lines 113-114: "Aliquots of 200 µL of each aqueous extract were transferred to three separate 500 µL tubes and placed on ice for 1 h prior to the LINDA experiments." If the sample was IN-active, all droplets froze in the investigated temperature interval. For clarification, we included the following sentence: "The mean freezing temperature for three droplets was calculated."

The suggested fourth column would correspond to Table 1, which already provides more details about the mean freezing temperatures of the initial screening.

We thank the referee for this suggestion, and we will consider a parameterization in a future study.

6. Conclusion

Referee comment: I would revise the statement on line 226 to say that the most IN-active components were actually between 300-100 kDa, but that IN activity still remained smaller than 100 kDa.
Author's response: As described above, the most IN-active components were larger than 300 kDa, and we hypothesize that these are aggregates consisting of individual proteins smaller than 100 kDa.

Technical comments
Referee comment: The authors use upper case Nm which is arguably inconsistent with the literature using lower case nm. See Wex et al., ACP, 2015 - Line 14: "impact" should be replaced by "implication", since the authors did not quantify the water cycle or the climate in their experiments. - The short summary is very good indeed! (although I would recommend changing the statement to 300 kDa, rather than 100 kDa.)
Author's response: We thank the referee for this comment. For consistency reasons with our former studies, we prefer to keep upper case Nm.

As suggested by the reviewer, we changed "impact" to "implication".

References:

Després, V. R., Huffman, J. A., Burrows, S. M., Hoose, C., Safatov, A. S., Buryak, G., Fröhlich-Nowoisky, J., Elbert, W., Andreae, M. O., Pöschl, U., and Jaenicke, R.: Primary biological aerosol particles in the atmosphere: a review, Tellus B: Chemical and Physical Meteorology, 64, 15 598, 2012.

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Humphreys, T. L., Castrillo, L. a., and Lee, M. R.: Sensitivity of partially purified ice nucleation activity of *Fusarium acuminatum* SRSF616, Current Microbiology, 42, 330–338, 2001.

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Richard, C., Martin, J. G., and Pouleur, S.: Ice nucleation activity identified in some phytopathogenic *Fusarium* species, Phytoprotection, 77,83–92, 1996.

Stopelli, E., Conen, F., Zimmermann, L., Alewell, C., and Morris, C. E.: Freezing nucleation apparatus puts new slant on study of biological ice nucleators in precipitation, Atmospheric Chemistry and Physics, 7, 129–134, 2014.

Tsumuki, H. and Konno, H.: Ice nuclei produced by *Fusarium* sp. isolated from the gut of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), Bioscience, Biotechnology, Biochemistry, 1994.

Tsumuki, H., Konno, H., Maeda, T., and Okamoto, Y.: An ice-nucleating active fungus isolated from the gut of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), Journal of Insect Physiology, 38, 119–125, 1992.

Tsumuki, H., Yanai, H., and Aoki, T.: Identification of ice-nucleating active fungus isolated from the gut of the rice stem borer*, Chilo suppressalis* Walker (Lepidoptera: Pyralidae) and a search for ice-nucleating active *Fusarium* species, Annals of the Phytopathological Society of Japan, 61, 334–339, 1995.

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We thank referee #2 for his/her constructive comments and suggestions, which are highly appreciated and have been taken into account upon revision of our manuscript. The comments and our answers are listed below (referee's comments marked with blue letters).

Specific comments:

Abstract:

Referee comment: Indicate the biological relevance of Fusarium and its ice nucleation activity. This is discussed well in the introduction but will help to bridge the first few sentences of the abstract.
Author's response: We thank the referee for this suggestion and included the following sentences in the abstract: "Ice nucleation activity in fungi was first discovered in the cosmopolitan genus *Fusarium*, which is widespread in soil and plants, has been found in atmospheric aerosol and cloud water samples, and can be regarded as the best studied IN-active fungus."

Moreover, we modified the following sentences: "The frequency and distribution of ice nucleation activity within *Fusarium*, however, remains elusive. Here, we tested more than 100 strains from 65 different *Fusarium* species for ice nucleation activity."

Methods 2.1:

Referee comment: How were the initial samples obtained? Could their original environment (crop vs. airborne, etc.) shed light on IN frequency?
Author's response: Samples from the USDA-ARS/Michigan State University were collected from crop tissue (sugar beet), and samples from the Schmale Laboratory at Virginia Tech were collected with unmanned aircraft systems. There is no detailed information available for the sources of the strains for the Kansas State University Teaching collection. We found IN activity in isolates from crop and air samples. For the air samples we cannot draw any conclusions from their original environment. A controlled comparison of IN frequency from samples collected in the air versus crop plants (and maybe even different types of crop plants) would be important, now that more IN-active species are known.

However, we added the following paragraph to section 2.1: "The strains from the USDA-ARS/Michigan State University were collected from crop tissue (sugar beet). All isolates were from field-grown beets and were obtained by hyphal tip transfer. The strains from the Schmale Laboratory at Virginia Tech were collected with unmanned aircraft systems (UASs or drones) equipped with remotely-operated sampling devices containing a *Fusarium* selective medium (e.g., Lin et al., 2013, 2014). All of the Schmale Laboratory strains were collected 100 m above ground level at the Kentland Farm in Blacksburg, Virginia, USA. Detailed information is not available for the sources of the strains for the Kansas State University Teaching collection. However, some of these strains are holotype strains referenced in Leslie and Summerell (2006)."

We extended Table S1 and provided additional information about sampling location and date.

Referee comment: Line 21: Additional, more recent, studies have contributed to this understanding of IN as well. (Failor et. al. 2017, Hanlon et al. 2017, Stopelli et al. 2017, 2015, Joly et al. 2014).

Author's response: We thank the referee for this remark and added the references to our manuscript.

Referee comment: Line 24-6: Failor et al. (2017) further expanded on known gammaproteobacteria IN.

Author's response: We changed the sentences as follows: "The best characterized biological IN are common plant-associated bacteria of the genera *Pseudomonas*, *Pantoea*, and *Xanthomonas* (Garnham et al., 2011; Govindarajan and Lindow, 1988; Graether and Jia, 2001; Green and Warren, 1985; Hill et al., 2014; Kim et al., 1987; Ling et al., 2018; Schmid et al., 1997; Wolber et al., 1986), and recently, an ice nucleation-active (IN-active) *Lysinibacillus* was found (Failor et al., 2017). The first identified IN-active fungi were strains of the genus *Fusarium* (Hasegawa et al., 1994, Pouleur et al., 1992, Richard et al., 1996, Tsumuki et al., 1992)."

Referee comment: Line 118: Was the range of incubation times necessary to reach a specified optical density? If so, that indication would be useful. If not, elaborate of reasoning for the times.

Author's response: Here, we did not mean that we tested these different incubation times. The sentence was meant to indicate the procedure considering all of the different replications that we used. For clarification, we changed "incubated" to "equilibrated".

Referee comment: Line 119: Be specific for the 0.5°C freezing point depression. Is it 0.5°C or 0.5±x °C.

Author's response: We added the calculations to the supplementary information.

We modified the sentence: "Note, that the aqueous extracts were prepared in 0.9 % NaCl solution, which could reduce the freezing temperatures by 0.5 °C based on theoretical calculations."

Results 3.1:

Referee comment: This would be an interesting point to note the original sampling locations for the various strains and could further demonstrate the cosmopolitan nature of these IN-active species should any tends be identified.

Author's response: We thank the referee for this comment, but as described before, we had only a few different sampling locations for both, the USDA-ARS/Michigan State University and samples from the Schmale Laboratory at Virginia Tech. For samples from the Kansas State University, we cannot specify the original sampling locations further as we obtained these samples from a culture collection.

Referee comment: Lines 154-5: This is a risky assumption to make. Prior to the Failor et al. study, all bacterial IN were thought to be proteinaceous. Exposing a selection of the species to high heat could support this claim.

Author's response: As many earlier studies already performed experiments with heat treatment of *Fusarium* IN, we initially refrained from repeating these experiments. The studies of Hasegawa et al. (1994), Pouleur et al. (1992), and Tsumuki and Konno (1994) only investigated some species of the genus *Fusarium*, and we agree with the referee that it is risky to generalize these findings to the newly found IN-active *Fusarium* species. Based on the suggestion of

referee #2 and #3, we performed additional heat treatment experiments with four different *Fusarium* species: *F. acuminatum*, *F. armeniacum*, *F. avenaceum*, and *F. langsethiae*.

We added a new Figure 4, and renumbered the other figures.

We included the following sentence in the abstract: "Heat treatment at 40 °C to 98 °C, however, strongly reduced the observed IN concentrations, confirming earlier hypotheses that the INM in *Fusarium* largely consists of a proteinaceous compound."

We modified the following sentence in the introduction: "Furthermore, the stability of *Fusarium* IN upon exposure to ozone and nitrogen dioxide, under high and low or quickly changing temperatures, and after short- and long-term storage under various conditions was investigated."

We modified the following sentence in section 2.1: "For quantitative analysis, exposure experiments, heat treatments, freeze-thaw cycles, as well as short- and long-term storage tests a selection of IN-active tested strains was grown on full-strength potato dextrose agar (VWR International GmbH, Darmstadt, Germany) first at room temperature for four to six days and then at 6 °C for about four weeks."

We included the following sentences in section 2.2: "For heat treatment experiments, aliquots of aqueous extracts of *F. acuminatum* 3-68, *F. armeniacum* 20970, *F. avenaceum* 2-106, and *F. langsethiae* 19084 were incubated at 40 °C, 70 °C, and 98 °C, respectively, for one hour. The IN concentration was determined using TINA."

We changed the following sentences in section 3.3: "They can be exposed to chemically modifying agents like ozone and nitrogen dioxide, and physical stressors like high and low or quickly changing temperatures. To investigate the stability of *Fusarium* IN, we performed exposure experiments, heat treatments, freeze-thaw cycles, and long-term storage tests."

We included a new paragraph in section 3.3: "The stability of the INM in *Fusarium* was investigated in heat treatment experiments. The ice nucleation activity was reduced significantly at a 40 °C treatment (Fig. 4). Between 40 % and 90 % of IN were lost at this temperature depending on the species, which supports the hypothesis that the INM in *Fusarium* consists of a proteinaceous compound. A heat treatment at 70 °C reduced the ice nucleation activity to less than 0.01 % compared to the initial level. Moreover, the initial freezing temperature was shifted to lower temperatures indicating a breakdown of the large protein aggregates. After a 98 °C treatment, we still found ice nucleation activity for all investigated species except for *F.avenaceum* 2-106. The results are in agreement with previous studies, which also reported a reduction in ice nucleation activity with increasing temperature in heat treatment experiments (Hasegawa et al., 1994; Pouleur et al., 1992; Tsumuki and Konno, 1994). The remaining activity after the 98 °C treatment, however, could indicate that post-translational modifications like glycosylation and therefore polysaccharides could play a role in the ice nucleation activity of *Fusarium*. Further systematic and chemical analysis studies are needed for elucidation."

We included the following sentences in the conclusion: "A heat treatment of 40 °C reduced the IN concentration significantly, supporting the hypothesis that the INM in *Fusarium* largely consists of a proteinaceous compound. An involvement of polysaccharides, however, cannot be excluded."

Referee comment: Lines 184-6: With the drastic decrease in activity after the 300,000 MWCO filter and then again after 100,000, could the protein not be larger, but when damaged or broken still retains some ice nucleation activity?

Author's response: If the INM in *Fusarium* is a single large protein, which breaks into small parts upon filtration, we would expect based on Govindarajan and Lindow (1988) and Pummer et al. (2015) a much lower initial freezing temperature of the filtrate than the temperature, which we obtained in our experiments. The only small shift in the initial freezing temperature after filtration suggests that small IN reassemble again to larger aggregates with similar activity than before filtration. It is unlikely that a damaged or broken IN protein would show a similar activity even if the broken parts would aggregate.

Referee comment: Lines 195-6: Why would single proteins in the atmosphere be unlikely? Please elaborate on this statement.

Author's response: As hypothesized in Lines 184-185, the proteins tend to agglomerate, which make it unlikely that individual proteins will enter the atmosphere. However; if an individual protein would enter the atmosphere it would be in the nucleation mode size range of ~ 6 nm. These particles tend to grow by condensation of gaseous compounds (e.g., semi volatile organic compounds, sulfates, water) and grow to particles in the Aitken mode size range. In this size range further condensation and coagulation takes place and larger agglomerates are formed.

We included the following sentence to our manuscript: "Individual proteins with a diameter of ~ 6 nm which may enter the atmosphere would be in the nucleation mode size range, where particles tend to uptake gaseous compounds and grow to Aitken mode particles, which themselves tend to coagulate to larger agglomerates (Seinfeld and Pandis, 1998)."

Referee comment: Line 216: Change ". . .and the fungus could safe energy." to ". . .and the fungus could save energy.".

Author's response: Changed as suggested.

Referee comment: Figure 1. Inclusion of the positive control SnoMax curve would be beneficial here. Any incidence of spontaneous freezing of the negative control should also be noted (if any occurred with the methods you used).

Author's response: As the focus of this study is on fungal IN of *Fusarium*, we did not use Snomax in any of the TINA experiments. The *Fusarium* strains themselves served as positive controls based on the results of the initial screening (Table S1). Moreover, the correct functionality of TINA including a Snomax curve is presented in Kunert et al. (2018).

For freezing tests, however, a negative control is essential. We added the information about the negative controls and included the following sentences in the manuscript:

For the thermal cycler: "Aliquots of uninoculated DPY broth were used as negative controls, which did not freeze in the investigated temperature interval."

For LINDA experiments: "As a negative control, a 0.9 % NaCl solution was added to three uninoculated agar plates, and the freezing started below -14 °C."

For TINA experiments: "Pure water samples (0.1 µm filtered) served as a negative control for each experiment. These did not freeze in the observed temperature interval."

Pseudomonas syringae CC94 was used as positive control for the initial screening using LINDA as droplet freezing assay. We included the following sentence in section 2.3: "The freezing temperatures ranged from -3.46 °C to -4.58 °C."

Referee comment: Figure 3. You note in the text that SnoMax has been shown to decrease after exposure. Did you see this same result, or did you not use SnoMax because of this interaction?
Author's response: We showed in a previous study that the IN activity of Snomax decreased after exposure to O_3 and NO_2 (Kunert et al. 2018). As this manuscript is focused on the IN activity of *Fusarium*, we refrained from repeating the experiments.

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Govindarajan, A. G. and Lindow, S. E.: Size of bacterial ice-nucleation sites measured *in situ* by radiation inactivation analysis, Proceedings of the National Academy of Sciences of the United States of America, 85, 1334–1338, 1988.

Hasegawa, Y., Ishihara, Y., and Tokuyama, T.: Characteristics of ice-nucleation activity in *Fusarium avenaceum* IFO 7158, Bioscience, Biotechnology, and Biochemistry, 58, 2273–2274, 1994.

Kunert, A. T., Lamneck, M., Helleis, F., Pöhlker, M. L., Pöschl, U., and Fröhlich-Nowoisky, J.: Twin-plate ice nucleation assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples, Atmospheric Measurement Techniques, 11, 6327–6337, 2018.

Pouleur, S., Richard, C., Martin, J.-G., and Antoun, H.: Ice nucleation activity in *Fusarium acuminatum* and *Fusarium avenaceum*, Applied and Environmental Microbiology, 1992.

Pummer, B. G., Budke, C., Niedermeier, D., Felgitsch, L., Kampf, C. J., Huber, R. G., Liedl, K. R., Loerting, T., Moschen, T., Schauperl, M., Tollinger, M., Morris, C. E., Wex, H., Grothe, H., Pöschl, U., Koop, T., and Fröhlich-Nowoisky, J.: Ice nucleation by water-soluble macromolecules, Atmospheric Chemistry and Physics, 15, 4077–4091, 2015.

Stopelli, E., Conen, F., Zimmermann, L., Alewell, C., and Morris, C. E.: Freezing nucleation apparatus puts new slant on study of biological ice nucleators in precipitation, Atmospheric Chemistry and Physics, 7, 129–134, 2014.

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MS bg-2019-276, Kunert et al.: Highly active and stable fungal ice nuclei are widespread among Fusarium species

We thank referee #3 for the review and positive assessment of our manuscript, and we are grateful for the detailed comments, which are very helpful for improving the manuscript. The comments and our answers are listed below (referee's comments marked with blue letters).

Referee comment: A main finding of this study is that filtration experiments suggest that the single cell-free Fusarium is smaller than 100 kDa. This is indeed very interesting and I wonder that the authors do not use the nomenclature of their own paper (Pummer et al., 2015) i.e. ice nucleating macromolecules (INM).
Author's response: We thank the referee for this remark and changed the nomenclature accordingly.

Referee comment: Indeed, water-soluble INMs have also been observed on many other primary biological aerosol particles (PABP) such as leaves, bark, pollen (Felgitsch et al., 2018), algae (Tesson et al., 2018), and bacteria (Failor et al., 2017). The sizes of these INM should be compared among each other, e.g. in a table.
Author's response: A precise comparison of the IN sizes in a table is rather difficult as most studies performed only a 0.2 µm filtration. A conclusion, which can be drawn upon these findings, is, that the IN are cell-free and stay active in solution.

We included the following sentence: "Moreover, biological INMs smaller than 200 nm were also found in various organisms e.g., other fungi (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015), leaves, bark, and pollen from birch trees (*Betula* spp.) (Felgitsch et al.,2018; Pummer et al., 2012), leaf litter (Schnell and Vali, 1973), some microalgae (Tesson and Šantl-Temkiv, 2018), strains of *Lysinibacillus* (Failor et al., 2017), and biological particles in the sea surface microlayer (Irish et al., 2019; Wilson et al., 2015)."

Referee comment: The same is true for the chemical composition and for the stability against oxidation. Also for other PBAPs, proteins and polysaccharides have been found as main components of INM and their stability is extraordinary as well. I also wonder if the authors have carried out heating experiments in order to destroy the ice nucleation activity of the proteins. Eventually, the heating was not successful due to the stability of INMs which would be important information since many colleagues use heating experiments to prove or unprove the presence of PBAP- INPs.
Author's response: Many earlier studies already performed heat treatment experiments on different IN-active *Fusarium* species and strains, including strains of *F. acuminatum* and *F. avenaceum*, consistently showing a small reduction of ice nucleation activity after heating to 40 °C and a bigger loss after heating to 70 °C (Hasegawa et al. (1994), Pouleur et al. (1992), and Tsumuki and Konno (1994)). Thus, we expected similar results from strains and species of the genus *Fusarium* and we initially refrained from repeating these experiments. Based on the suggestion of referee #2 and #3, we performed additional heat treatment experiments with four different *Fusarium* species: *F. acuminatum*, *F. armeniacum*, *F. avenaceum*, and *F. langsethiae*.

We added a new Figure 4, and renumbered the other figures.

We included the following sentence in the abstract: "Heat treatment at 40 °C to 98 °C, however, strongly reduced the observed IN concentrations, confirming earlier hypotheses that the INM in *Fusarium* largely consists of a proteinaceous compound."

We modified the following sentence in the introduction: "Furthermore, the stability of *Fusarium* IN upon exposure to ozone and nitrogen dioxide, under high and low or quickly changing temperatures, and after short- and long-term storage under various conditions was investigated."

We modified the following sentence in section 2.1: "For quantitative analysis, exposure experiments, heat treatments, freeze-thaw cycles, as well as short- and long-term storage tests a selection of IN-active tested strains was grown on full-strength potato dextrose agar (VWR International GmbH, Darmstadt, Germany) first at room temperature for four to six days and then at 6 °C for about four weeks."

We included the following sentences in section 2.2: "For heat treatment experiments, aliquots of aqueous extracts of *F. acuminatum* 3-68, *F. armeniacum* 20970, *F. avenaceum* 2-106, and *F. langsethiae* 19084 were incubated at 40 °C, 70 °C, and 98 °C, respectively, for one hour. The IN concentration was determined using TINA."

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We included the following sentences in the conclusion: "A heat treatment of 40 °C reduced the IN concentration significantly, supporting the hypothesis that the INM in *Fusarium* largely consists of a proteinaceous compound. An involvement of polysaccharides, however, cannot be excluded."

Comment:

Referee comment: The abbreviation "IN" has been used in a confusing way. In the text it means "ice nuclei" but also means "ice nucleation" and "ice nucleating". I recommend using "INP" for "ice nucleating particles" and write the full words in all other cases.

Author's response: We thank the referee for this comment. We clearly defined ice nuclei as IN and ice nucleation-active as IN-active in the abstract and the introduction. We used the abbreviation IN for ice nuclei in our former studies (Després et al., 2012, Fröhlich-Nowoisky et al., 2015, 2016, Kunert et al., 2018, Pummer et al., 2015), and for consistency reasons we

prefer to keep it this way. To avoid misunderstanding, we changed “IN activity” to “ice nucleation activity”.

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Hasegawa, Y., Ishihara, Y., and Tokuyama, T.: Characteristics of ice-nucleation activity in *Fusarium avenaceum* IFO 7158, Bioscience, Biotechnology, and Biochemistry, 58, 2273–2274, 1994.

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List of changes

All changes have been marked in the revised version of the manuscript using latexdiff. The most relevant changes are listed below.

Author list:
The following author was added to the author list:
Kai Tang

The final author list is now: "Anna T. Kunert, Mira L. Pöhlker, Kai Tang, Carola S. Krevert, Carsten Wieder, Kai R. Speth, Linda E. Hanson, Cindy E. Morris, David G. Schmale III, Ulrich Pöschl, and Janine Fröhlich-Nowoisky"

Additional experiments:
We performed additional heat treatment experiments with four different *Fusarium* species: *F. acuminatum*, *F. armeniacum*, *F. avenaceum*, and *F. langsethiae*.

We added a new Figure 4, and renumbered the other figures.

We included the following sentence in the abstract: "Heat treatment at 40 °C to 98 °C, however, strongly reduced the observed IN concentrations, confirming earlier hypotheses that the INM in *Fusarium* largely consists of a proteinaceous compound."

We modified the following sentence in the introduction: "Furthermore, the stability of *Fusarium* IN upon exposure to ozone and nitrogen dioxide, under high and low or quickly changing temperatures, and after short- and long-term storage under various conditions was investigated."

We modified the following sentence in section 2.1: "For quantitative analysis, exposure experiments, heat treatments, freeze-thaw cycles, as well as short- and long-term storage tests a selection of IN-active tested strains was grown on full-strength potato dextrose agar (VWR International GmbH, Darmstadt, Germany) first at room temperature for four to six days and then at 6 °C for about four weeks."

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We changed the following sentences in section 3.3: "They can be exposed to chemically modifying agents like ozone and nitrogen dioxide, and physical stressors like high and low or quickly changing temperatures. To investigate the stability of *Fusarium* IN, we performed exposure experiments, heat treatments, freeze-thaw cycles, and long-term storage tests."

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activity to less than 0.01 % compared to the initial level. Moreover, the initial freezing temperature was shifted to lower temperatures indicating a breakdown of the large protein aggregates. After a 98 °C treatment, we still found ice nucleation activity for all investigated species except for *F.avenaceum* 2-106. The results are in agreement with previous studies, which also reported a reduction in ice nucleation activity with increasing temperature in heat treatment experiments (Hasegawa et al., 1994; Pouleur et al., 1992; Tsumuki and Konno, 1994). The remaining activity after the 98 °C treatment, however, could indicate that post-translational modifications like glycosylation and therefore polysaccharides could play a role in the ice nucleation activity of *Fusarium*. Further systematic and chemical analysis studies are needed for elucidation."

We included the following sentences in the conclusion: "A heat treatment of 40 °C reduced the IN concentration significantly, supporting the hypothesis that the INM in *Fusarium* largely consists of a proteinaceous compound. An involvement of polysaccharides, however, cannot be excluded."

Figures:
Figure 4 was added to the manuscript, and all subsequent figures have been renamed.

Title:
We changed the title from "Highly active and stable fungal ice nuclei are widespread among *Fusarium* species" to "Macromolecular fungal ice nuclei in *Fusarium*: Effects of physical and chemical processing".

Abstract:
We discussed the biological relevance of *Fusarium*. We included the following sentences: "Ice nucleation activity in fungi was first discovered in the cosmopolitan genus *Fusarium*, which is widespread in soil and plants, has been found in atmospheric aerosol and cloud water samples, and can be regarded as the best studied IN-active fungus."

Moreover, we modified the following sentences: "The frequency and distribution of ice nucleation activity within *Fusarium*, however, remains elusive. Here, we tested more than 100 strains from 65 different *Fusarium* species for ice nucleation activity."

Section 2.1:
We included a paragraph about the original environment of the initial samples and additional sampling information: "The strains from the USDA-ARS/Michigan State University were collected from crop tissue (sugar beet). All isolates were from field-grown beets and were obtained by hyphal tip transfer. The strains from the Schmale Laboratory at Virginia Tech were collected with unmanned aircraft systems (UASs or drones) equipped with remotely-operated sampling devices containing a *Fusarium* selective medium (e.g., Lin et al., 2013, 2014). All of the Schmale Laboratory strains were collected 100 m above ground level at the Kentland Farm in Blacksburg, Virginia, USA. Detailed information is not available for the sources of the strains for the Kansas State University Teaching collection. However, some of these strains are holotype strains referenced in Leslie and Summerell (2006)."

We extended Table S1 and provided additional information about sampling location and date.

Section 2.3:
We added information about negative controls for the different freezing assays:

For the thermal cycler: “Aliquots of uninoculated DPY broth were used as negative controls, which did not freeze in the investigated temperature interval.”

For LINDA experiments: “As a negative control, a 0.9 % NaCl solution was added to three uninoculated agar plates, and the freezing started below -14 °C.”

For TINA experiments: “Pure water samples (0.1 µm filtered) served as a negative control for each experiment. These did not freeze in the observed temperature interval.”

Section 2.3:
We clarified the sentence about the freezing point depression: “Note, that the aqueous extracts were prepared in 0.9 % NaCl solution, which could reduce the freezing temperatures by 0.5 °C based on theoretical calculations.”

Additionally, we included the following sentences: “We cannot exclude, however, that the high concentration of IN compensates the effect of NaCl on the freezing temperature. This is supported by the investigations of Stopelli et al. (2014), who did not find a systematic suppression of freezing at this salt concentration in LINDA experiments.”

We added the calculations to the supplementary information.

Section 3.2:
Size experiments were compared to ice nuclei from other biological aerosol particles. We included the following sentence: “Moreover, biological INMs smaller than 200 nm were also found in various organisms e.g., other fungi (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015), leaves, bark, and pollen from birch trees (*Betula* spp.) (Felgitsch et al.,2018; Pummer et al., 2012), leaf litter (Schnell and Vali, 1973), some microalgae (Tesson and Šantl-Temkiv, 2018), strains of *Lysinibacillus* (Failor et al., 2017), and biological particles in the sea surface microlayer (Irish et al., 2019; Wilson et al., 2015).”

~~Highly active and stable~~ Macromolecular fungal ice nuclei ~~are widespread among~~ in *Fusarium*~~species~~: Effects of physical and chemical processing

Anna T. Kunert [1], Mira L. Pöhlker [1], Kai Tang [1], Carola S. Krevert [1], Carsten Wieder [1], Kai R. Speth [1], Linda E. Hanson [2], Cindy E. Morris [3], David G. Schmale III [4], Ulrich Pöschl [1], and Janine Fröhlich-Nowoisky [1]

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Abstract. Some biological particles and macromolecules are particularly efficient ice nuclei (IN), triggering ice formation at temperatures close to 0 °C. The impact of biological particles on cloud glaciation and the formation of precipitation is still poorly understood and constitutes a large gap in the scientific understanding of the interactions and co-evolution of life and climate. ~~To investigate the~~ Ice nucleation activity in fungi was first discovered in the cosmopolitan genus *Fusarium*, which is widespread in soil and plants, has been found in atmospheric aerosol and cloud water samples, and can be regarded as the best studied IN-active fungus. The frequency and distribution of ~~IN activity within the fungal genus~~ ice nucleation activity within *Fusarium*, however, remains elusive. Here, we tested more than 100 strains from 65 different *Fusarium* species ~~were screened~~for ice nucleation activity. In total, ∼11 % of all tested species included ice nucleation-active (IN-active) strains, and ∼16 % of all tested strains showed ~~IN activity above -14~~ice nucleation activity above -12 °C. Besides *Fusarium* species with known ~~IN~~ ice nucleation activity, *F. armeniacum*, *F. begoniae*, *F. concentricum*, and *F. langsethiae* were newly identified as IN-active. The cumulative number of IN per gram of mycelium for all tested *Fusarium* species was comparable to other biological IN like *Sarocladium implicatum*, *Mortierella alpina*, and Snomax®. Filtration experiments ~~suggest that the single~~ indicate that cell-free ice-nucleating macromolecules (INMs) from *Fusarium* ~~IN is~~ are smaller than 100 kDa, and that molecular aggregates can be formed in solution. Long-term storage and freeze-thaw cycle experiments revealed that the ~~*Fusarium* IN remain active in solution for~~ fungal IN in aqueous solution remain active over several months and ~~after~~ in the course of repeated freezing and thawing. ~~Oxidation and nitration reactions, as occurring during atmospheric aging, did~~ Exposure to ozone and nitrogen dioxide at atmospherically relevant concentration levels did also not affect the ~~activity of the *Fusarium* IN . The high frequency of~~ ice nucleation activity. Heat treatments at 40 °C to 98 °C, however, strongly reduced the observed IN concentrations, confirming earlier hypotheses that the INM in *Fusarium* largely consists of a proteinaceous compound. The frequency and the wide distribution of ~~IN~~ ice nucleation activity within the genus *Fusarium*, combined with the ~~high~~ stability of the IN under atmospherically relevant conditions, suggest a ~~significant impact~~ larger implication of fungal IN on the Earth's water cycle and climate than previously assumed.

1 Introduction

Ice particles in the atmosphere are formed either by homogeneous nucleation at temperatures below -38 °C or by heterogeneous nucleation catalyzed by particles or macromolecules serving as ice nuclei (IN) at warmer temperatures ~~*Pruppacher and Klett*, 1997~~(Pruppacher and Klett, 1997; reviewed in detail in *Fröhlich-Nowoisky et* al. [2016] and *Knopf et* al. [2018]). Biological particles in particular are expected to play an important role as IN in the temperature range from -15 °C to 0 °C, but the impact of biological particles on cloud glaciation and the formation of precipitation is still poorly understood [*Coluzza et al.*, 2017]. Several studies suggest a triggering effect of biological IN for cloud glaciation and formation of precipitation [*Creamean et al.*, 2013; *DeMott and Prenni*, 2010; *Failor et* al., 2017; *Hanlon et* al., 2017; *Joly et* al., 2014; *Petters and Wright*, 2015; *Pratt et al.*, 2009; *Stopelli et al.*, 2015, 2017], and former studies have shown that biological particles are more efficient than mineral IN [*DeMott and Prenni*, 2010; *Després et al.*, 2012; *Hill et al.*, 2014; *Hoose and Möhler*, 2012; *Huffman et al.*, 2013; *Möhler et al.*, 2007; *Morris et al.*, 2014; *Murray et al.*, 2012; *Pratt et al.*, 2009].

The best characterized biological IN are common plant-associated bacteria of the genera *Pseudomonas*, *Pantoea*, and *Xanthomonas* [*Garnham et al.*, 2011; *Govindarajan and Lindow*, 1988; *Graether and Jia*, 2001; *Green and Warren*, 1985; *Hill et al.*, 2014; *Kim et al.*, 1987; *Ling et al.*, 2018; *Šantl-Temkiv et* al., 2015; *Schmid et al.*, 1997; *Wolber et al.*, 1986]~~. The first identified~~ , and recently, an ice nucleation-active (IN-active) *Lysinibacillus* was found [*Failor et* al., 2017]. The first identified IN-active fungi were strains of the genus *Fusarium* [*Hasegawa et al.*, 1994; *Pouleur et al.*, 1992; *Richard et al.*, 1996; *Tsumuki et al.*, 1992]. To date, a few more fungal genera with varying initial freezing temperatures such as *Isaria farinosa* (∼ -4 °C), *Mortierella alpina* (∼ -5 °C), *Puccinia* species (-4 °C to -8 °C), and *Sarocladium* (formerly named *Acremonium*) *implicatum* (∼ -9 °C) have been identified as IN-active [*Fröhlich-Nowoisky et al.*, 2015; *Huffman et al.*, 2013; *Morris et al.*, 2013; *Richard et al.*, 1996].

The genus *Fusarium* is cosmopolitan and includes saprophytes and pathogens of plants and animals [*Leslie and Summerell*, 2006; *Nelson et al.*, 1994]. Although they are considered to be primarily soil-borne fungi, many species of *Fusarium* are airborne [*Prussin et al.*, 2014; *Schmale et al.*, 2012; *Schmale and Ross*, 2015], and they were found in atmospheric and cloud water samples [e.g., *Amato et al.*, 2007; *Fröhlich-Nowoisky et al.*, 2009; *Fulton*, 1966]. Some species can cause wilts, blights, root rots, and cankers in agriculturally important crops worldwide [e.g., *Schmale and Gordon*, 2003; *Wang and Jeffers*, 2000]. Other species can produce secondary metabolites known as mycotoxins that can cause a variety of acute and chronic health effects in humans and animals [e.g., *Bush et al.*, 2004; *Ichinoe et al.*, 1983].

~~Whereas the~~ While the factors for a positive selective pressure for ~~IN~~ ice nucleation activity in *Fusarium* and other fungi ~~has~~ have not been directly identified, an ecological advantage of initiating ice formation is easily conceivable. Indeed, most IN-active bacteria and fungi are isolated from regions with seasonal temperatures below 0 °C [*Diehl et al.*, 2002; *Schnell and Vali*, 1972]. Ice nucleation activity at temperatures close to 0 °C could be beneficial for pathogens or might provide an ecological advantage for saprophytic *Fusarium* species by facilitating in the acquisition of nutrients liberated during cell rupture of the host [*Lindow et al.*, 1982]. Furthermore, IN on the surface of the mycelium could avoid physical damage of the fungus by protective extracellular freezing [*Fröhlich-Nowoisky et al.*, 2015; *Zachariassen and Kristiansen*, 2000] or to bind moisture as

ice in cold and dry seasons [*Pouleur et al.*, 1992]. With increasing temperatures, the retained water can be of advantage in early vegetative periods and for bacterial movement on the mycelial water film known as fungal highway [*Kohlmeier et al.*, 2005; *Warmink et al.*, 2011]. Moreover, ~~IN~~ ice nucleation activity might be beneficial for airborne *Fusarium* and for their return to the Earth's surface under advantageous conditions in a feedback cycle known as bioprecipitation [*Després et al.*, 2012; *Morris et al.*, 2013, 2014; *Sands et al.*, 1982]. In addition, once the IN are released into the environment, they can adsorb to clay and might also be available in the atmosphere associated with soil dust particles [*Conen et al.*, 2011; *Fröhlich-Nowoisky et al.*, 2015, 2016; *Hill et al.*, 2016; *O'Sullivan et al.*, 2014, 2015, 2016; *Sing and Sing*, 2010].

The sources, abundance, and identity of biological IN are not well characterized [*Coluzza et al.*, 2017], and it has been proposed that systematic surveys will likely increase the number of IN-active fungal species discovered [*Fröhlich-Nowoisky et al.*, 2015]. *Fusarium* is the best-known IN-active fungus, but the frequency and distribution of ~~IN~~ ice nucleation activity within *Fusarium* is not well known. In this study, more than 100 strains from 65 different *Fusarium* species were tested for ~~IN~~ ice nucleation activity in three laboratories with different freezing methods. A high-throughput droplet freezing assay was used to quantify the IN of selected *Fusarium* species, and filtration experiments were performed to estimate the size of the *Fusarium* IN. Furthermore, the stability of *Fusarium* IN upon exposure to ozone and nitrogen dioxide, ~~during freeze-thaw cycles~~under high and low or quickly changing temperatures, and after short- and long-term storage under various conditions was investigated.

2 Materials and methods

2.1 Origin and growth conditions of fungal cultures

Thirty *Fusarium* strains from USDA-ARS/Michigan State University (L. Hanson, East Lansing, MI, USA), 13 strains from the Schmale Laboratory at Virginia Tech (D. Schmale, Blacksburg, VA, USA), and 69 strains from the Kansas State University Teaching Collection (J. Leslie, Manhattan, KS, USA) were screened for ~~IN~~ ice nucleation activity (Table S1).

The strains from the USDA-ARS/Michigan State University ~~strains were~~ were collected from crop tissue (sugar beet). All isolates were from field-grown beets and were obtained by hyphal tip transfer. The strains from the Schmale Laboratory at Virginia Tech were collected with unmanned aircraft systems (UASs or drones) equipped with remotely-operated sampling devices containing a *Fusarium* selective medium [e.g., *Lin et al.*, 2013, 2014]. All of the Schmale Laboratory strains were collected 100 m above ground level at the Kentland Farm in Blacksburg, Virginia, USA. Detailed information is not available for the sources of the strains for the Kansas State University Teaching collection. However, some of these strains are holotype strains referenced in *Leslie and Summerell* [2006].

The strains from the USDA-ARS/Michigan State University were cultivated on dextrose peptone yeast extract agar, containing 10 g L^{-1} dextrose (VWR, Radnor, PA, USA), 3 g L^{-1} peptone (Difco Proteose Peptone No. 3, Becton, Dickinson and Company, Franklin Lakes, NY, USA), and 0.3 g L^{-1} yeast extract (Merck, Kenilworth, NJ, USA), filtered through a 0.2 µm pore diameter filter (PES disposable filter units, Life Science Products, Frederick, CO, USA). After filtration, 12 g L^{-1} agarose (Certified Molecular Biology Agarose, Bio-Rad, Hercules, CA, USA) was added, and the medium was sterilized by autoclaving at

121 °C for 20 min. The colonies were grown at 22 °C to 24 °C for 7 to 19 days. The strains from the Schmale Laboratory at Virginia Tech and the Kansas State University Teaching Collection were maintained in cryogenic storage at -80 °C and were grown on quarter-strength potato dextrose agar (Difco Laboratories, Detroit, USA) on 100 mm ~~Petri~~ petri plates at ambient room temperature for four days prior to ice nucleation assays.

For quantitative analysis, exposure experiments, heat treatments, freeze-thaw cycles, as well as short- and long-term storage tests a selection of IN-active tested strains was grown on full-strength potato dextrose agar (VWR International GmbH, Darmstadt, Germany) first at room temperature for four to six days and then at 6 °C for about four weeks. For filtration experiments, the fungal cultures were grown at 6 °C for up to six months.

2.2 Preparation and treatments of aqueous extracts

For LED-based Ice Nucleation Detection Apparatus (LINDA) [*Stopelli et al.*, 2014] experiments (see Sect. 2.3), 4 mL of sterile 0.9 % NaCl was added to each of eight petri plates, and the fungal cultures were scraped with the flat end of a sterile bamboo skewer. The resulting suspension of mycelium and spores was filtered through a 100 µm filter (Corning Life Sciences, Reims, France).

For Twin-plate Ice Nucleation Assay (TINA) [*Kunert et al.*, 2018] experiments (see Sect. 2.3) the fungal mycelium was scraped off the agar plate and transferred into a 15 mL tube (Greiner Bio One, Kremsmünster, Austria). The fresh weight of the mycelium was determined gravimetrically. Pure water was prepared as described in *Kunert et al.* [2018]. Aliquots of 10 mL pure water were added before vortexing three times at 2 700 rpm for 30 s (Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY, USA) and centrifugation at 4 500 g for 10 min (Heraeus Megafuge 40, Thermo Scientific, Braunschweig, Germany). For all experiments the aqueous extract was filtered successively through a 5 µm and a 0.1 µm PES syringe filter (Acrodisc®, Sigma-Aldrich, Taufkirchen, Germany), and the aqueous extract contained IN from spores and mycelial surfaces.

For filtration experiments, the 0.1 µm filtrate was further filtered successively through 300 000 MWCO and 100 000 MWCO PES ultrafiltration units (Vivaspin®, Satorius AG, Göttingen, Germany). After each filtration step, the IN concentration was determined using TINA.

For exposure experiments, aqueous extracts of *F. acuminatum* 3-68 and *F. avenaceum* 2-106 were exposed to high concentrations of O_3 and NO_2 as described in *Liu et al.* [2017]. Briefly, a mixture of 1 ppm O_3 and 1 ppm NO_2 was bubbled through 1 mL aliquots of aqueous extract for 4 h, ~~which represents an exposure to an atmospherically relevant amount of approximately 200 ppb of each gas for about 20 h. Afterwards, the~~ and the IN concentration was determined using TINA.

For heat treatment experiments, aliquots of aqueous extracts of *F. acuminatum* 3-68, *F. armeniacum* 20970, *F. avenaceum* 2-106, and *F. langsethiae* 19084 were incubated at 40 °C, 70 °C, and 98 °C, respectively, for one hour. The IN concentration was determined using TINA.

For freeze-thaw cycles, the ~~IN~~ ice nucleation activity of *F. acuminatum* 3-68 was determined shortly after preparation of the aqueous extract and after storage at 6 °C for 24 h using TINA. Then, the aqueous extract was stored at -20 °C for 24 h and thawed again. The ~~IN~~ ice nucleation activity was tested before storage at -20 °C for an additional 24 h. After thawing, the ~~IN~~ ice nucleation activity was determined again.

For long-term storage experiments, the aqueous extract of various *Fusarium* species was stored at 6 °C for about four months or at -20 °C for about eight months, and the ~~IN~~ ice nucleation activity was determined using TINA.

2.3 Ice nucleation assays

Two independent droplet freezing assays conducted in two laboratories were used to investigate the distribution of ~~IN~~ ice nucleation activity within *Fusarium* in an initial screening.

First, a thermal cycler (PTC200, MJ Research, Hercules, CA, USA) was used as described in *Fröhlich-Nowoisky et al.* [2015] to screen 30 *Fusarium* strains from seven species from USDA-ARS/Michigan State University in the temperature range from -2 °C to -9 °C. Mycelium was picked with sterile pipette tips (Eppendorf, Westbury, NY, USA) into 80 µL aliquots of 0.2 µm pore diameter filtered dextrose peptone yeast ~~extract~~ (DPY) broth in sterile 96-well polypropylene PCR plates (VWR International, LLC, Radnor, PA, USA). Up to seven droplets were measured for each sample, and the mean freezing temperature was calculated. Aliquots of uninoculated DPY broth were used as negative controls, which did not freeze in the investigated temperature interval.

Second, the LED-based Ice Nucleation Detection Apparatus (LINDA) was used as described by *Stopelli et al.* [2014] to screen 13 strains from the Schmale Laboratory at Virginia Tech and 69 strains from the Kansas State University Teaching Collection. Aliquots of 200 µL of each aqueous extract were transferred to three separate 500 µL tubes and placed on ice for 1 h prior to the LINDA experiments. LINDA was run from -1 °C to ~~-14~~ -20 °C, and images of the samples were recorded every six seconds. ~~As~~ The mean freezing temperature for three droplets was calculated. Note, that the aqueous extracts were prepared in 0.9 % NaCl solution, which could reduce the freezing temperatures by 0.5 °C based on theoretical calculations. We cannot exclude, however, that the high concentration of IN compensates the effect of NaCl on the freezing temperature. This is supported by the investigations of *Stopelli et* al. [2014], who did not find a systematic suppression of freezing at this salt concentration in LINDA experiments. As a negative control, a 0.9 % NaCl solution was added to three uninoculated agar plates, and the freezing started below -14 °C. As positive control, aqueous suspensions of *Pseudomonas syringae* CC94 from the collection of INRA (Avignon, France) [*Berge et al.*, 2014] (with a final OD_{580} of 0.5 to 0.7, i.e. $\sim 10^9$ bacteria mL^{-1}) were used for each experiment. The bacteria were grown on King's medium B [*King et al.*, 1954] at 22 °C to 25 °C for 48 h, and aqueous suspensions were ~~incubated~~ equilibrated at 4 °C for 1 h to 4 h before LINDA experiments. The ~~aqueous extract was prepared in 0.9~~freezing temperatures of *Pseudomonas syringae* CC94 ranged from -3.46 ~~% NaCl solution, which reduced the freezing temperatures about 0.5~~°C to -4.58 °C~~based on theoretical calculations~~.

Ice nuclei of selected *Fusarium* species~~were further analyzed~~ , which were long known for ice nucleation activity (*F. acuminatum*, *F. avenaceum*) as well as all the newly identified species, were further analyzed in immersion freezing mode using the high-throughput Twin-plate Ice Nucleation Assay (TINA) [*Kunert et al.*, 2018]. The aqueous ~~extract was~~ extracts were serially diluted 10-fold with pure water by a liquid handling station (epMotion ep5073, Eppendorf, Hamburg, Germany) ~~, and~~ to a dilution where droplets remained liquid in the investigated temperature interval. Of each dilution, 96 droplets (3 µL) were tested ~~per dilution~~ with a continuous cooling rate of 1 °C min^{-1} from 0 °C to -20 °C. Pure water samples (0.1 µm filtrated) served as a negative control for each experiment. These did not freeze in the observed temperature interval. The temperature

was measured with an accuracy of 0.2 K [*Kunert et al.*, 2018]. The obtained fraction of frozen droplets (f_{ice}) and the counting error were used to calculate the cumulative number of IN (N_{m}) with the associated error using the Vali formula and the Gaussian error propagation [*Kunert et al.*, 2018; *Vali*, 1971]. For each experiment, the cumulative number of IN was averaged over all dilutions. If the experiment was repeated, the cumulative number of IN was averaged over all experiments, and the standard error was calculated. Three independent experiments with aqueous extract from three individual fungal culture plates of the same strain showed similar results with only slight variation. An example of results is presented for *F. armeniacum* 20970 (Fig. S1).

3 Results and discussion

3.1 IN-active *Fusarium* species

Although several IN-active *Fusarium* species are known, the frequency and distribution of ~~IN~~ ice nucleation activity within the fungal genus *Fusarium* is still not well studied [*Hasegawa et al.*, 1994; *Humphreys et al.*, 2001; *Pouleur et al.*, 1992; *Richard et al.*, 1996; *Tsumuki and Konno*, 1994]. Two initial screenings in the temperature range from -1 °C to ~~-14~~ -20 °C were performed to better evaluate the frequency of ~~IN~~ ice nucleation activity within *Fusarium*. A strain was defined as IN-active, when it initiated ice formation above - 9 °C (thermal cylcer) and -12 °C (LINDA), respectively.

In total, ∼ 16 % (18/112) of the tested strains showed ~~IN activity with initial~~ ice nucleation activity with mean freezing temperatures of -3.5 °C to -11.2 °C (Table 1) in the typical range known for *Fusarium* (-1 °C and -9 °C) (Hasegawa et al., 1994; Humphreys et al., 2001; Pouleur et al., 1992; Richard et al., 1996; Tsumuki et al., 1992; Tsumuki and Konno, 1994). Most formerly reported initial freezing temperatures were obtained with different *Fusarium* strains, growth conditions, and freezing assays, which might explain differences compared to our results. The high proportion of IN-active strains within *F. acuminatum* is consistent with previous reports (Pouleur et al., 1992; Tsumuki et al., 1995). Overall, ∼ 11 % (7/65) of the tested species included IN-active strains. In addition to strains from *Fusarium* species with known ~~IN~~ ice nucleation activity, four *Fusarium* species were newly identified as IN-active: *F. armeniacum*, *F. begoniae*, *F. concentricum*, and *F. langsethiae*. In further experiments, the ~~IN~~ ice nucleation activity of *F. begoniae* and *F. concentricum* could not be verified.

The newly identified IN-active species are cosmopolitan. *Fusarium armeniacum* is a toxigenic saprophyte [*Burgess et al.*, 1993] causing seed and root rot on soybeans [*Ellis et al.*, 2012]. The geographical distribution has been reported as tropical and subtropical [*Leslie and Summerell*, 2006], but it was also found in Minnesota, USA [*Kommedahl et al.*, 1979] and Australia [*Burgess et al.*, 1993]. *Fusarium begoniae* is a plant pathogen of Begonia found in Germany with a potential wider distribution [*Nirenberg and O'Donnell*, 1998]. *Fusarium concentricum* is a plant pathogen, frequently found in Central America and isolated from bananas [*Aoki et al.*, 2001; *Leslie and Summerell*, 2006], and *F. langsethiae* is a broadly distributed cereal pathogen [*Torp and Nirenberg*, 2004]. Some strains of these newly identified IN-active species are known to produce mycotoxins, which can threaten the health of humans and animals [*Fotso et al.*, 2012; *Kokkonen et al.*, 2012; *Wing et al.*, 1993a, b].

The results suggest that the ~~IN~~ ice nucleation activity within *Fusarium* is more widespread than previously known. Not all *Fusarium* species include IN-active strains and not all strains within one species show ~~IN activity.~~ ice nucleation activity. Earlier

studies including experiments suggested that *Fusarium* IN are ~~thought to be~~ proteins or at least ~~to~~ contain a proteinaceous compound [*Hasegawa et al.*, 1994; *Pouleur et al.*, 1992; *Tsumuki and Konno*, 1994]. Their production requires energy, and we might assume that this trait would not be expressed or maintained unless there was an ecological advantage. It is known that *Fusarium* can regulate the gene expression for IN production depending on environmental conditions such as nutrient availability [*Richard et al.*, 1996], and some *Fusarium* species reduce or lose their ~~IN~~ ice nucleation activity after several subcultures [*Pummer et al.*, 2013; *Tsumuki et al.*, 1995]. Thus, we cannot exclude that all *Fusarium* strains have the ability to produce IN. From the phylogenetic distribution of ~~IN~~ ice nucleation activity across the genus *Fusarium*, we can speculate that ~~IN~~ ice nucleation activity is a very old trait, but either the gene expression requires a trigger, which is not yet identified, or the trait might be in the process of being lost. It is unlikely, however, that the age of the genetic determinants of fungal ~~IN~~ ice nucleation activity is older than that in bacteria, since fungi diverged well after the age that has been attributed to the bacterial IN gene [*Morris et al.*, 2014], and the genetic determinants are not the same as those in bacteria.

3.2 Quantification and size determination of IN from selected *Fusarium* species

A selection of IN-active *Fusarium* species was further investigated by extensive droplet freezing assay analysis using TINA. All tested *Fusarium* strains initiated ice nucleation between -3 °C and -4 °C (Fig. 1). Differences in the ~~initial freezing temperature~~ freezing temperatures between the initial screening and the quantitative analysis can be due to different growth conditions and freezing assays. The cumulative number of IN (N_m) per gram of mycelium was in the range between 10^8 g^{-1} and 10^{13} g^{-1}. *Fusarium acuminatum* 3-68 showed the highest ~~IN~~ ice nucleation activity and *F. langsethiae* the lowest per gram of mycelium. The results are comparable to other IN-active microorganisms like *Sarocladium implicatum* (10^8 g^{-1}, Pummer et al., 2015, *Mortierella alpina* (10^9 g^{-1}, Fröhlich-Nowoisky et al., 2015; 10^{10} g^{-1}, Kunert et al., 2018), and the bacterial IN-active substance Snomax® containing *Pseudomonas syringae* (10^{12} g^{-1}, Budke and Koop, 2015; Kunert et al., 2018).

The size of the *Fusarium* IN was investigated by filtration experiments. Filtration through a 5 µm and a 0.1 µm filter did not affect the ~~IN~~ ice nucleation activity (Fig. 2), revealing that *Fusarium* IN are smaller than 100 nm, cell-free, easily removed from the fungus, and stay active in solution. This is ~~consistent with previous~~ in agreement with other *Fusarium* studies [*O'Sullivan et al.*, 2015; *Pouleur et al.*, 1992; *Tsumuki and Konno*, 1994]. Moreover, ~~the IN are smaller than 100~~biological INMs smaller than 200 nm ~~for all tested~~ were also found in various organisms e.g., other fungi [*Fröhlich-Nowoisky et al.*, 2015; *Pummer et al.*, 2015], leaves, bark, and pollen from birch trees (~~*Fusariumstrains*~~ *Betula* spp.) [*Felgitsch et al.*, 2018; *Pummer et al.*, 2012], leaf litter [*Schnell and Vali*, 1973], some microalgae [*Tesson and Šantl-Temkiv*, 2018], strains of *Lysinibacillus* [*Failor et al.*, 2017], and biological particles in the sea surface microlayer [*Irish et al.*, 2019; *Wilson et al.*, 2015]. Filtration through a 300 000 MWCO filter unit decreased the cumulative number of IN per gram of mycelium about 50 % to 75 % depending on the *Fusarium* species, but a tremendous number of IN (10^{10} - 10^{13} g^{-1}) still passed through the filter. The initial freezing temperature was slightly shifted towards lower temperatures. Further filtration through a 100 000 MWCO filter unit reduced the IN number to 10^8 - 10^{10} g^{-1}, which is less than 1 % of the initial IN concentration. Additionally, the initial freezing temperature was shifted about one degree towards lower temperatures.

As ~~IN~~ ice nucleation activity was found in all filtrates, the aqueous extract of *Fusarium* consists of a mixture of IN-active proteins with different sizes. We hypothesize that *Fusarium* IN are ~~single proteins~~ macromolecules (INMs) smaller than 100 kDa, which agglomerate to large protein complexes in solution. Some of these complexes fall apart upon filtration, so that the ~~single IN proteins~~ INMs can pass through the filter. The small shift in the initial freezing temperature suggests that these ~~proteins~~ INMs reassemble again to aggregates after filtration, as larger IN nucleate at warmer temperatures [*Govindarajan and Lindow*, 1988; *Pummer et al.*, 2015]. *Erickson* [2009] determined the size of proteins based on theoretical calculations. As the interior of proteins is closely packed with no substantial holes and almost no water molecules inside, proteins are rigid structures with approximately the same density ($\sim 1.37\,\mathrm{g\,cm^{-1}}$). Assuming the protein as a smooth spherical particle, the minimum diameter of the ~~single IN protein~~ INM would be smaller than 6.1 nm~~according to *Erickson* [2009]~~. Our results are in accordance with *Lagzian et al.* [2014], who cloned and expressed a 49 kDa IN-active protein from *F. acuminatum*.

As *Fusarium* IN are cell-free and can easily be washed off the fungal surface, they can be released in high numbers into the environment. If they are not degraded by microorganisms before, the IN can adsorb to soil dust and be aerosolized attached to these particles [*Conen et al.*, 2011; *Fröhlich-Nowoisky et al.*, 2015; *Hill et al.*, 2016; *O'Sullivan et al.*, 2014, 2015, 2016; *Sing and Sing*, 2010]. This is in good agreement with *Pruppacher and Klett* [1997], who found a positive correlation between IN number concentration and particles in the coarse mode. Other releasing processes cannot be excluded, however, it is unlikely that the ~~single proteins~~ INMs are present in the atmosphere as individual aerosol particles. Individual proteins with a diameter of ~ 6 nm, which may enter the atmosphere, would be in the nucleation mode size range, where particles tend to uptake gaseous compounds and grow to Aitken mode particles, which themselves tend to coagulate to larger agglomerates [*Seinfeld and Pandis*, 1998].

3.3 Stability of *Fusarium* IN

In the atmosphere, IN can interact with other aerosol particles or gases. They can be exposed to chemically modifying agents like ozone and nitrogen dioxide, and physical stressors like ~~low temperatures and~~ high and low or quickly changing temperatures. To investigate the stability of *Fusarium* IN, we performed exposure experiments, heat treatments, freeze-thaw cycles, and long-term storage tests.

The influence of chemical processing on the *Fusarium* IN, in particular oxidation and nitration reactions as occurring during atmospheric aging, was investigated by exposing aqueous extracts from *F. acuminatum* 3-68 and *F. avenaceum* 2-106 to high concentrations of ozone and nitrogen dioxide in liquid phase. Figure 3 shows that for both species neither the initial freezing temperature nor the cumulative number of IN per gram of mycelium was affected by exposure. These results demonstrate a high stability of *Fusarium* IN under oxidizing and nitrating conditions. This is in contrast to other biological IN e.g., bacterial IN (Snomax®) ~~, which were reduced upon exposure~~ [*Kunert et al.*, 2018], birch and alder pollen [*Gute and Abbatt*, 2018], and dissolved organic matter [*Borduas-Dedekind et* al., 2019], where exposure to oxidizing agents reduced the IN activity.

The stability of the INM in *Fusarium* was investigated in heat treatment experiments. The ice nucleation activity was reduced significantly at a 40 °C treatment (Fig. 4). Between 40 % and 90 % of IN were lost at this temperature depending on the species, which supports the hypothesis that the INM in *Fusarium* consists of a proteinaceous compound. A heat treatment

at 70 °C reduced the ice nucleation activity to less than 0.01 % compared to the initial level. Moreover, the initial freezing temperature was shifted to lower temperatures indicating a breakdown of the large protein aggregates. After a 98 °C treatment, we still found ice nucleation activity for all investigated species except for *F. avenaceum* 2-106. The results are in agreement with previous studies, which also reported a reduction in ice nucleation activity with increasing temperature in heat treatment experiments [*Hasegawa et* al., 1994; *Pouleur et* al., 1992; *Tsumuki and Konno*, 1994]. The remaining activity after the 98 °C treatment, however, could indicate that post-translational modifications like glycosylation and therefore polysaccharides could play a role in the ice nucleation activity of *Fusarium*. Further systematic studies including chemical analyses are needed for elucidation.

To study the effects of short-term storage and freeze-thaw cycles on the ~~IN~~ ice nucleation activity of *F. acuminatum* 3-68, IN measurements of the same aqueous extract were performed at different time points (Fig. ~~4~~5). The results of freshly prepared aqueous extract revealed that the highest activity of fungal IN was already developed during preparation of the filtrate and no time for equilibration was required. Storage of aqueous extract at 6 °C for 24 h did not affect the ~~IN~~ ice nucleation activity. Also, further storage at -20 °C for another 24 h, and repeated freeze-thaw cycles had no impact on the ~~IN~~ ice nucleation activity. This means, that, once in the atmosphere, the IN can undergo several freeze-thaw cycles without losing their activity and are still able to influence cloud glaciation and the formation of precipitation. This could be an explanation why not all fungi are always IN-active as their IN are highly stable and quasi recyclable. Ice nuclei might influence the availability of moisture over long times periods, and if enough moisture is available in the environment, the necessity of IN production would be omitted and the fungus could ~~safe~~ save energy.

In addition, the stability of the INM in *Fusarium* ~~IN~~ was studied in long-term storage tests, where aqueous ~~extract~~ extracts of various *Fusarium* species ~~was~~ were stored at different temperatures for a long period of time. Figure ~~5~~ 6 shows that storage at 6 °C for four months and -20 °C for eight months, respectively, did not influence the ~~IN~~ ice nucleation activity of *F. armeniacum* 20970, *F. acuminatum* 1-4, *F. avenaceum* 2-106, and *F. acuminatum* 2-38. The results demonstrate the high stability of the INMs in *Fusarium* ~~IN~~ in liquid and frozen solutions over long time periods, which makes *Fusarium* well applicable for laboratory IN studies. Moreover, the high stability is likely an advantage for these fungi to be linked to atmospheric processes.

4 Conclusions

The frequency and distribution of ~~IN~~ ice nucleation activity within the fungal genus *Fusarium* was investigated in a screening of more than 100 strains from 65 different *Fusarium* species. In total, ∼ 11 % (7/65) of all tested species included IN-active strains, and ∼ 16 % (18/112) of all tested strains showed ~~IN~~ ice nucleation activity, demonstrating the wide distribution of ~~IN~~ ice nucleation activity within *Fusarium*. Filtration experiments suggest that *Fusarium* IN form aggregates consisting of ~~single IN~~ INMs smaller than 100 kDa (∼ 6 nm). Exposure experiments, freeze-thaw cycles, and long-term storage tests revealed a high stability of the INMs in *Fusarium*~~IN~~, demonstrating the suitability of *Fusarium* in laboratory IN studies. Heat treatments at 40 °C to 98 °C reduced the IN concentration significantly, supporting the hypothesis that the INM in *Fusarium* largely consists of a proteinaceous compound. An involvement of polysaccharides, however, cannot be excluded. The wide distribution

of ~~IN~~ ice nucleation activity within the genus *Fusarium* together with the ~~high~~ stability of the INM in *Fusarium* ~~IN~~ under atmospherically relevant conditions, suggest that the ~~impact~~ implication of these IN on the Earth's water cycle and climate might be more significant than previously assumed. Additional research is necessary to characterize the INMs in *Fusarium* ~~IN~~ and processes, which can result in their agglomeration to larger protein complexes. To evaluate the ~~impact~~ implication of these IN on the Earth's climate, additional work is required to study the abundance of *Fusarium* IN in environmental samples on a global scale.

Data availability. All data are available from the corresponding authors upon request.

Author contributions. C.E.M., J.F.-N., U.P. designed the experiments. D.G.S. III and L.E.H. provided fungal cultures. C.E.M., D.G.S. III, and J.F.-N. performed the initial screenings. A.T.K., K.T., C.S.K., C.W., and K.R.S. performed the experiments. A.T.K., J.F.-N., M.L.P., and U.P. discussed the results. A.T.K. and J.F.-N. wrote the manuscript with contributions of all co-authors.

Competing interests. The authors declare that they have no conflict of interest.

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Table 1. Ice nucleation-active *Fusarium* strains with corresponding ~~initial~~ mean freezing temperatures of the initial screening. The newly identified IN-active *Fusarium* species are marked with an asterisk (*).

Species	Strain	$T_{\text{~~i~~}}$ (°C)
F. acuminatum	1-3	-5.6
F. acuminatum	1-4	-5.0
F. acuminatum	1-5	-5.6
F. acuminatum	1-24	-3.5
F. acuminatum	2-38	-5.0
F. acuminatum	2-48	-5.6
F. acuminatum	2-109	-5.6
F. acuminatum	3-48	~~-3.7~~ -5.0
F. acuminatum	3-68	-3.5
F. acuminatum	20964	-6.2
*F. armeniacum**	20970	-5.3
F. avenaceum	2-106	-5.0
F. avenaceum	11440	-7.6
*F. begoniae**	10767	-11.2
*F. concentricum**	10765	-4.6
*F. langsethiae**	19084	-9.4
F. tricinictum	20990	-7.3

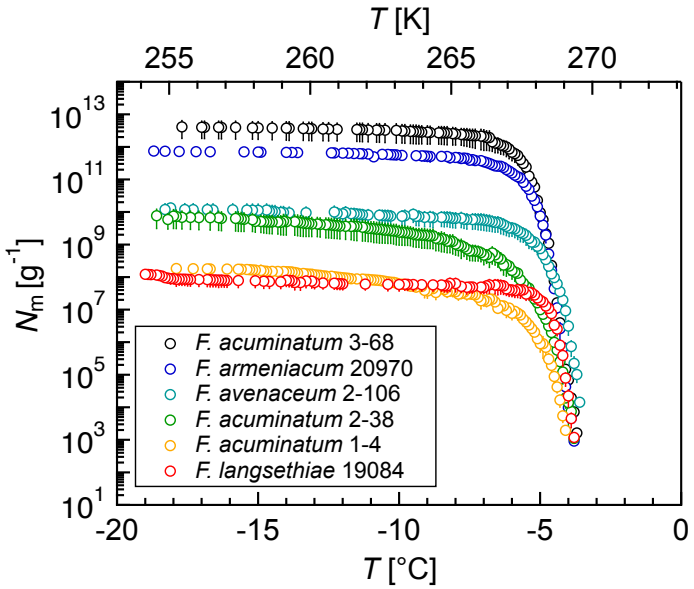

Figure 1. Overview of ~~IN~~ ice nucleation activity for selected *Fusarium* species and strains: cumulative number of IN (N_m) per gram of mycelium plotted against the temperature (T); arithmetic mean values and standard error of three independent experiments with aqueous extracts from different fungal culture plates.

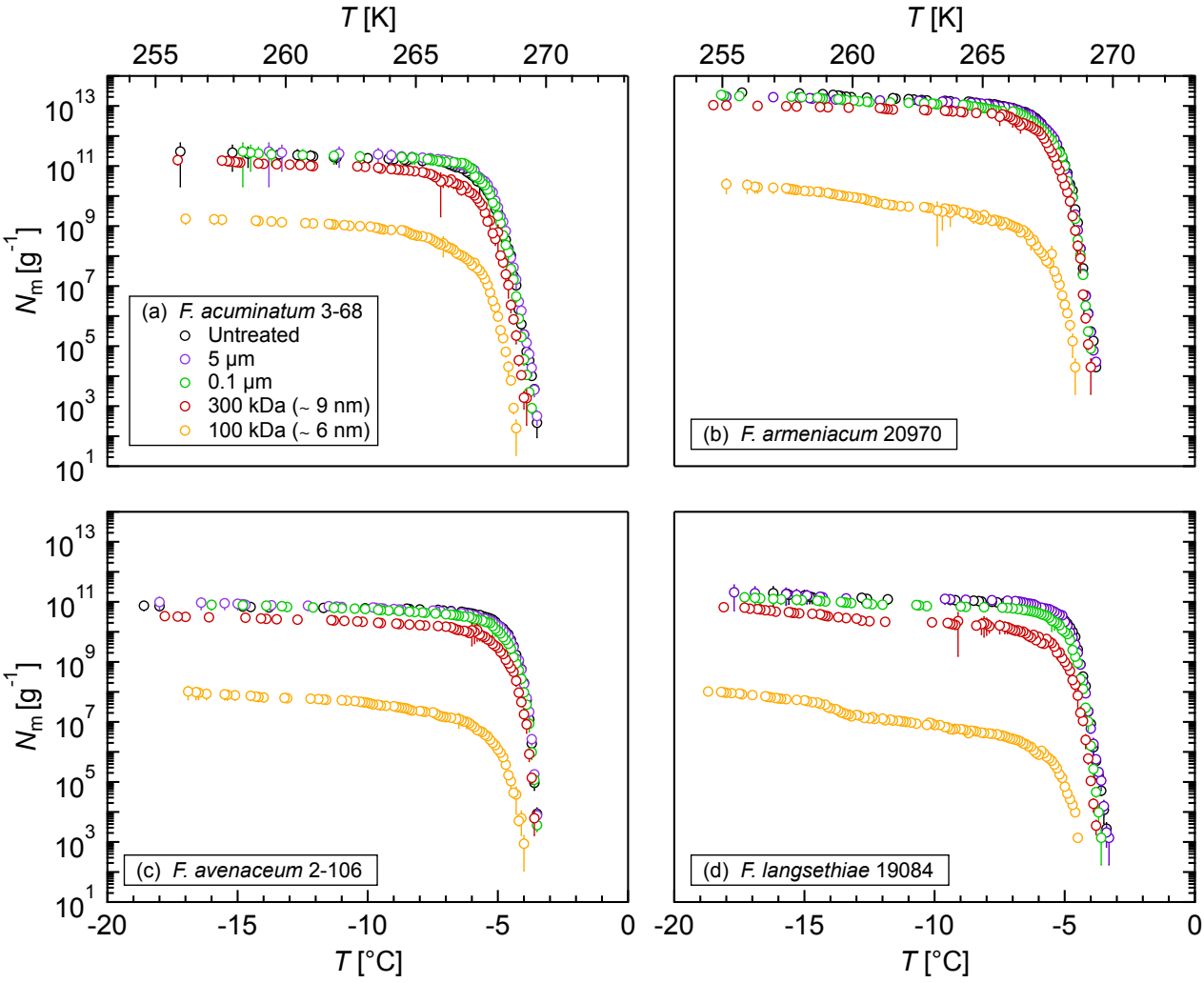

Figure 2. Size determination of the *Fusarium* IN upon filtration: cumulative number of IN (N_m) per gram of mycelium plotted against the temperature (T) for (a) *F. acuminatum* 3-68, (b) *F. armeniacum* 20970, (c) *F. avenaceum* 2-106, and (d) *F. langsethiae* 19084. The error bars were calculated using the counting error and the Gaussian error propagation.

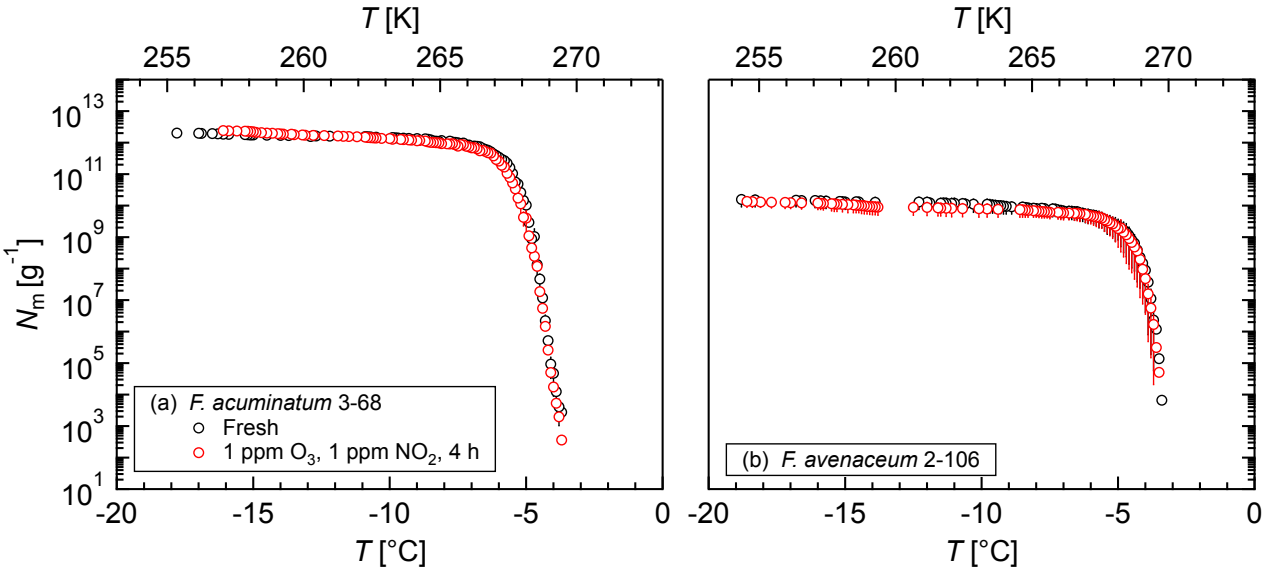

Figure 3. Exposure of aqueous extract from *Fusarium* to ozone and nitrogen dioxide: cumulative number of IN (N_m) per mass of mycelium plotted against the temperature (T) for (a) *F. acuminatum* 3-68 and (b) *F. avenaceum* 2-106; arithmetic mean values and standard error of two independent experiments with aqueous extracts from different fungal culture plates.

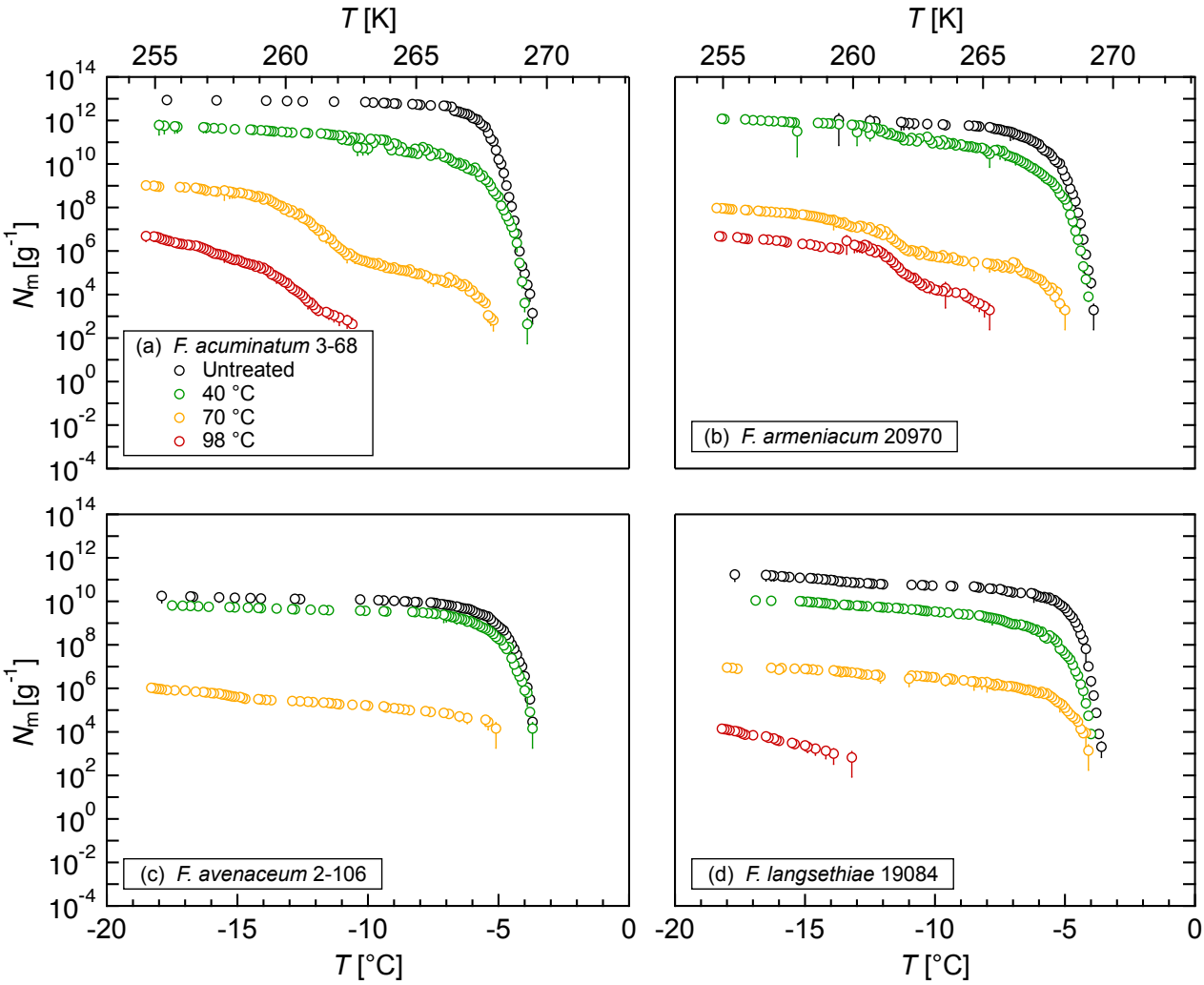

Figure 4. Effects of high temperatures on the ice nucleation activity of *Fusarium*: cumulative number of IN (N_m) per gram of mycelium plotted against the temperature (T) for (a) *F. acuminatum* 3-68, (b) *F. armeniacum* 20970, (c) *F. avenaceum* 2-106, and (d) *F. langsethiae* 19084. The error bars were calculated using the counting error and the Gaussian error propagation.

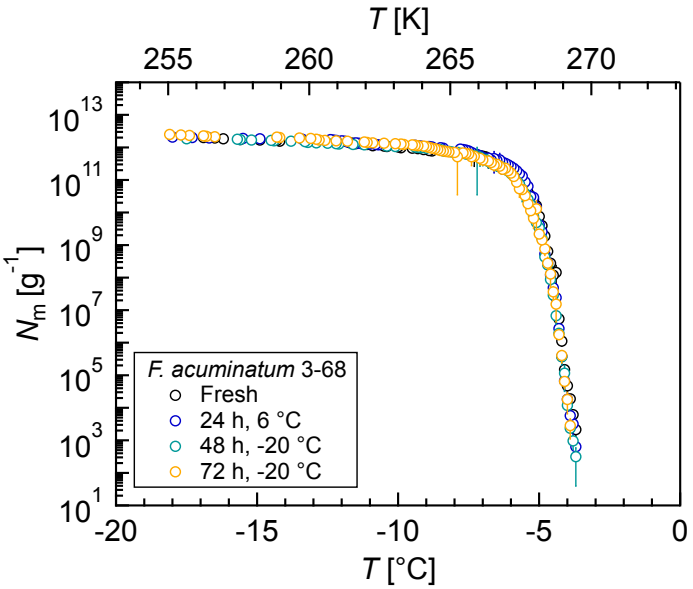

Figure 5. Effects of short-term storage and freeze-thaw cycles on the ~~IN~~ ice nucleation activity of *Fusarium acuminatum* 3-68: cumulative number of IN (N_m) per gram of mycelium plotted against the temperature (T). The same aqueous extract was measured immediately after preparation (black), after storage at 6 °C for 24 h (blue), after another 24 h stored at -20 °C (total 48 h; green), and after another 24 h stored at -20 °C (total 72 h; yellow). The error bars were calculated using the counting error and the Gaussian error propagation.

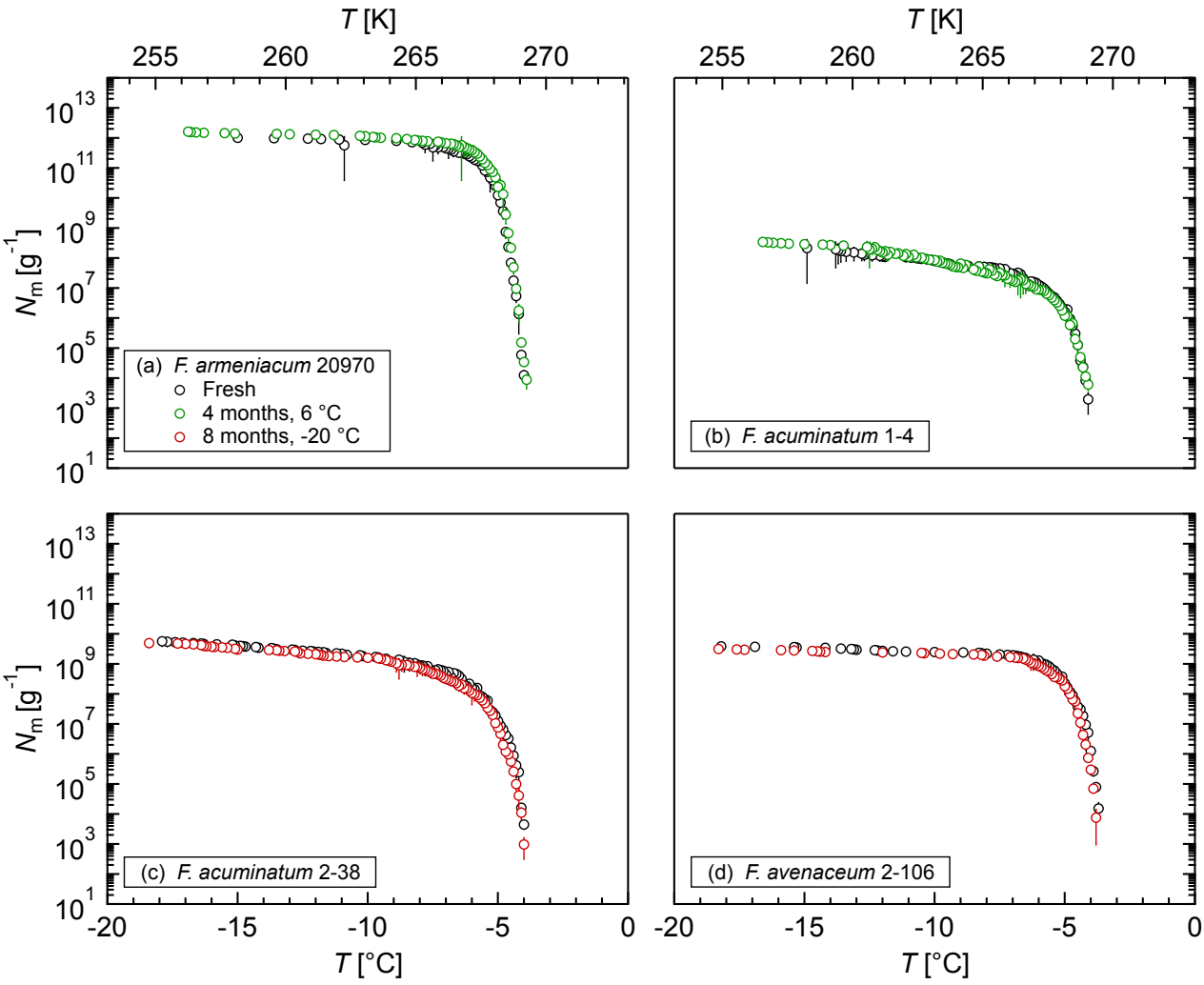

Figure 6. Effect of long-term storage on the ~~IN~~ ice nucleation activity of (a) *F. armeniacum* 20970, (b) *F. acuminatum* 1-4, (c) *F. acuminatum* 2-38, and (d) *F. avenaceum* 2-106: cumulative number of IN (N_m) per gram of mycelium against the temperature (T). The error bars were calculated using the counting error and the Gaussian error propagation.