Science of the Total Environment 800 (2021) 149442

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Seasonal ice nucleation activity of water samples from alpine rivers and lakes in Obergurgl, Austria

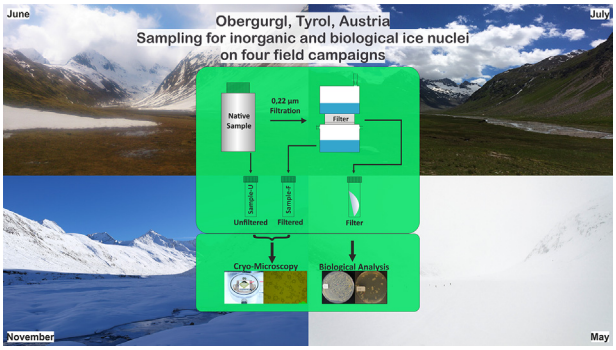
Philipp Baloh [a], Regina Hanlon [b], Christopher Anderson [b], Eoin Dolan [a], Gernot Pacholik [a], David Stinglmayr [a], Julia Burkart [a,c], Laura Felgitsch [a], David G. Schmale III [b], Hinrich Grothe [a,*]

[a] Institute of Materials Chemistry, TU Wien, Vienna, Austria
[b] School of Plant and Environmental Sciences, Blacksburg, VA, USA
[c] Faculty of Physics, University of Vienna, Vienna, Austria

HIGHLIGHTS

- Submicron ice nucleating particles were found to be present in all sampled water systems.
- Ice nucleating particles from the sampled water sources show a significant seasonal trend.
- In winter months ice nucleation active bacteria make up the dominant share of cultured bacteria in the utilized growth medium.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 11 April 2021
Received in revised form 29 June 2021
Accepted 30 July 2021
Available online 5 August 2021

Editor: Christian Herrera

Keywords:
Ice nucleation
Alpine habitats
Freshwater
Water systems
Climate
Ice nucleating particles
Seasonal
Artificial snow production

ABSTRACT

Heterogeneous ice nucleation plays an important role in many environmental processes such as ice cloud formation, freezing of water bodies or biological freeze protection in the cryosphere. New information is needed about the seasonal availability, nature, and activity of ice nucleating particles (INPs) in alpine environments. These INPs trigger the phase transition from liquid water to solid ice at elevated subzero temperatures. We collected water samples from a series of alpine rivers and lakes (two valleys and their rivers, an artificial pond, and a natural lake system) in Obergurgl, Austria in June 2016, July 2016, November 2016, and May 2017. Each alpine river and lake was sampled multiple times across different seasons, depending on site access during different times of the year. Water samples were filtered through a 0.22 µm membrane filter to separate microbial INPs from the water, and both fractions were analyzed for ice nucleation activity (INA) by an emulsion freezing method. Microorganisms were cultured from the filters, and the cultures then analyzed for INA. Portions of the filtered samples were concentrated by lyophilization to observe potential enhancement of INA. Two sediment samples were taken as reference points for inorganic INPs. Sub-micron INPs were observed in all of the alpine water sources studied, and a seasonal shift to a higher fraction of microbial ice nucleators cultured on selective media was observed during the winter collections. Particles larger than 0.22 µm showed INA, and microbes were cultured from this fraction. Results from 60 samples gave evidence of a seasonal change in INA, presence of submicrometer INPs, and show the abundance of culturable microorganisms, with late spring and early summer showing the most active biological INPs. With additional future research on this topic ski resorts could make use of such knowledge of geographical and seasonal trends of microbial INPs in freshwater habitats in order to improve the production of artificial snow.

* Corresponding author.
E-mail address: hinrich.grothe@tuwien.ac.at (H. Grothe).

https://doi.org/10.1016/j.scitotenv.2021.149442

1. Introduction

Freshwater habitats are known as possible sources of ice nucleating particles (INPs) for the atmosphere. Wave action in lakes was shown to produce a considerable amount of aerosol (Slade et al., 2010) with inorganic (Axson et al., 2016; May et al., 2016), and biological (May et al., 2018) particles alike. Broader rivers and streams have wave action and often other aerosol forming mechanisms come into play for them as well. Weirs have been reported to produce aerosol from rivers (Larsen et al., 2017; Knackstedt et al., 2018), and fast flow over natural steps and rocks likely produces similar results. Benson et al. (2019) developed a drone water sampling system to study INPs in remote freshwater environments, and Bieber et al. (2020) designed a drone air sampling system to collect airborne INPs. Systems such as these could help probe INPs in remote alpine environments in the future.

When INPs become aerosolized they can be transported over long distances, and seed ice clouds, a mechanism of particular importance for rain formation over land (Creamean et al., 2013; Mülmenstädt et al., 2015). Another technological process where ice nucleation (IN) is involved is the forced aerosolization of water for artificial snow production. INPs have an impact on this process and commercial additives for snowmaking, like Snomax® make use of this effect. Freshwater systems are a source of such airborne INPs, yet little is known about their diversity, composition, and seasonal availability.

The phase transition from water to ice is a thermodynamic process, that in general, does not need any other factors than decreasing temperature below 0 °C at atmospheric pressure. The freezing process however can behave very differently depending on water purity, volume, and presence or absence of ice nucleation active materials (Langham and Mason, 1958; Koop and Zobrist, 2009; Kanji et al., 2017). For pure water, so-called homogeneous freezing takes place, which is a stochastic process, where the energy activation barrier has to be overcome by the fluctuation of intermolecular bonds between water molecules to form a stable cluster that can be thought of as the first nanoscopic ice crystal that acts as the nucleus to trigger further ice formation (Pruppacher and Klett, 1997). The fact that the water just needs to be cooled below 0 °C for this to happen is thermodynamically true, yet small water droplets have been observed to persist in the liquid phase down to temperatures as low as −37 °C (DeMott et al., 1998). A kinetic inhibition takes place, that leads to supercooled water in the liquid phase. This can be observed especially well in isolated droplets, as one frozen droplet does not propagate ice formation in other supercooled droplets due to their physical separation (Murray, 2008a, 2008b). Depending on the size of the droplets, pure water can be supercooled down to much lower temperatures than the melting point of water at 0 °C. This is an important factor in natural droplet aggregations such as clouds, but also for artificial droplets as produced by snow makers for snow production.

When water is not free of particles, another mechanism is dominant that is called heterogeneous ice nucleation where INPs come into play. Heterogeneous nucleation is a non-stochastic process that is determined by the activity of the INPs. Certain INPs, if present, cause a droplet to freeze at a defined temperature - characteristic for the INP - independent of the droplets volume and the time it already persisted in a supercooled state.

Many different materials can act as INPs. The most abundant INPs in the atmosphere are of inorganic nature in the form of mineral dust (Creamean et al., 2013; Cziczo et al., 2013; DeMott et al., 2003). Carbonaceous materials can act as INPs as well, as does e.g. cellulose (Hiranuma et al., 2015, 2019) which is ubiquitous in the form of decayed plant matter, or soot (DeMott, 1990; Gorbunov et al., 2001; Häusler et al., 2018) that can be found in many remote places due to anthropogenic pollution. Intact biological matter such as some pollen (Diehl et al., 2001, 2002; Pummer et al., 2012), fungi (Duman and Olsen, 1993; Kondo et al., 2012), and bacteria (Maki et al., 1974; Vali et al., 1976), have very strong ice nucleation activity and recent field measurements showed biological material to be present in cloud droplets (Pratt et al., 2009; Creamean et al., 2013). Biological materials are the most active known INPs (Kanji et al., 2017; Murray et al., 2012); they allow water to freeze at the highest temperatures. The bacterium *Pseudomonas syringae* is known to increase the nucleation temperature to around −2 °C (Maki et al., 1974). Some strains of the bacterium are pathogens of plants. Many strains of *P. syringae* are ubiquitous in aquatic environments (Morris et al., 2013), and recent works evaluated the potential for this bacterium to become aerosolized from freshwater (Pietsch et al., 2015) and showed a feedback mechanism with rainwater (Morris et al., 2017).

P. syringae is used as a commercial INP in artificial snow production, available under the trade name Snomax®. The use of this product has generated considerable debate regarding its potential impact on natural ecosystems (Cochet and Widehem, 2000; Lagriffoul et al., 2010; Ward and DeMott, 1989). In several countries including Austria, the addition of any foreign substances to the water used for snow production is not allowed (Das Tiroler Naturschutzgesetz, 2005 - *TNSchG 2005, LGBl. Nr. 26/2005 idF | 293/20*, n.d.). Even without the addition of any commercial INPs, natural water usually contains a certain amount of INPs of differing activity (Benson et al., 2019). Inorganic INPs are certainly present in most terrestrial waters yet their ice nucleation activity (INA) is dependent on their composition, which depends largely on local geology. Assessment of river sediments' INA can give an insight on their usability and availability. Furthermore *Pseudomonas* ssp. are known to be ubiquitous in aquatic environments (Morris et al., 2008, 2010), along with other microorganisms that show INA. Besides this technologically inspired topic, the composition of INPs in alpine water systems is of fundamental interest for investigating possible feedback mechanisms by precipitation (Joly et al., 2014; Morris et al., 2017), and therefore for ice cloud formation in alpine areas.

We hypothesized that the nature, activity, and availability of INPs in alpine environments varies with season. To test this hypothesis, we collected and analyzed water samples from a series of alpine rivers and lakes (two valleys and their rivers, an artificial pond, and a natural lake system) in Obergurgl, Austria in June 2016, July 2016, November 2016, and May 2017. Each alpine river and lake was sampled multiple times across different seasons, depending on site access during different times of the year. Water samples were filtered through a 0.22 μm membrane filter to separate microbial INPs from the water, and both fractions were analyzed for INA by an emulsion freezing method. The specific objectives of our work were to: (1) determine the types and concentrations of INPs present in freshwater alpine environments in Obergurgl, Austria, (2) examine potential spatial effects of varying water sources on INPs, (3) study potential seasonal effects of INPs across the different freshwater alpine environments, and (4) consider how new knowledge from this study could be used to enhance artificial snow production at ski resorts by incorporating native INPs. For this work, we focused on the inorganic and the biological INPs. Ski resorts can make use of new knowledge of geographical and seasonal trends of microbial INPs in freshwater habitats in order to improve the production of artificial snow.

2. Materials and methods

2.1. Sampling sites

Four major sites were studied in Obergurgl, Tyrol, Austria. Not all spots could be visited continuously over the year due to accessibility and safety issues in the winter months and only seasonal activity of smaller streams. The two sampled rivers were in the Gaißbergtal (GAI, ~2280 m above sea level (a.s.l.)) and in the Rotmoostal (ROT, ~2275 m a.s.l.), two valleys that are next to each other as seen in Fig. 1. The valleys have different geomorphologies as GAI is a steeper valley with one single main river whereas ROT is a much broader valley with a river that meanders over a larger area often parting and reuniting over the length

of the valley. The artificial pond (A-Pond, ~2270 m a.s.l.), that was sampled regularly lies at the base of the valley ROT flows through and is fed by its water at certain times of year but remains stagnant when full and not in use for artificial snow production. The natural pond system (N-Pond, ~2670 m a.s.l) is on the opposite mountain range of the valleys and is fed by meltwater, rain, and springs. Different pools have formed there on a pan like area that contain more water in spring/early summer and are more shallow and stagnant in summer. It is not a fully lentic system as there is a significant flow in and out of the ponds especially when sampling took place in July 2016, however since the pools are wide there is far less flow and disturbance in their water than in the rivers. Fig. 1 shows an overview map of the sampled water system and their locations. Pictures of the four areas are shown in Fig. 2.

2.2. Collection of water samples

Water samples were collected in Obergurgl, Austria during four field campaigns (FCs) (FC1 (1-2 June 2016), FC2 (6-7 July 2016), FC3 (29-30 November 2016), and FC4 (3-4 May 2017)). Samples from streams and rivers were collected with sterile 1 L wide-mouth bottles (Thermofisher cat # 2105-0032). Samples from ponds and lakes were collected with a LaMotte water sampler (Number 1077, Model JT-1) about 1 m from the shoreline, and 1 m below the surface of the water. GPS waypoints were recorded on a smartphone on Google Maps for each sample location. A sampling table with time and GPS data, and a figure showing the locations of all the collected samples are found in Supplement Table S1, and Supplement Fig. S2.

2.3. Collection of river sand

Sand samples were taken on Field Campaign 2 in July 2016 from the sediment of the two rivers. The samples were collected in 50 mL centrifuge tubes (Brand cat # 114823) GPS waypoints were recorded for these samples as well. The samples were kept cool until the end of the sampling day and then stored in the freezer at −20 °C until further processing.

2.4. Processing of water samples

Following collection, samples were placed in a cooler bag with frozen gel packs for transport to the laboratory for processing. Water samples of 250 mL were filtered through a 0.22 μm filter (Millipore cat #9004-70-0) on the same day of collection using a water jet pump (VWR cat # 159600) and a filter receiver unit (Thomas cat #300-4100). Negative controls were done with sterilized ultrapure water for tests of filters and filtrate. Filters (containing particles >0.22 μm) were placed into 15 mL conical tubes and shipped in a cooler with frozen gel packs to the Schmale Laboratory, Virginia Tech, Blacksburg, VA, USA for culturing of microbes and ice nucleation activity assays (INA-assays) (e.g., (Hanlon et al., 2017; Jimenez-Sanchez et al., 2018; Benson et al., 2019; Garcia et al., 2019). Approximately 40 mL from every unfiltered sample and its filtrate were transferred into 50 mL sterile centrifuge tubes (Brand cat # 114823) and kept frozen at −20 °C until analysis by cryo-microscopy.

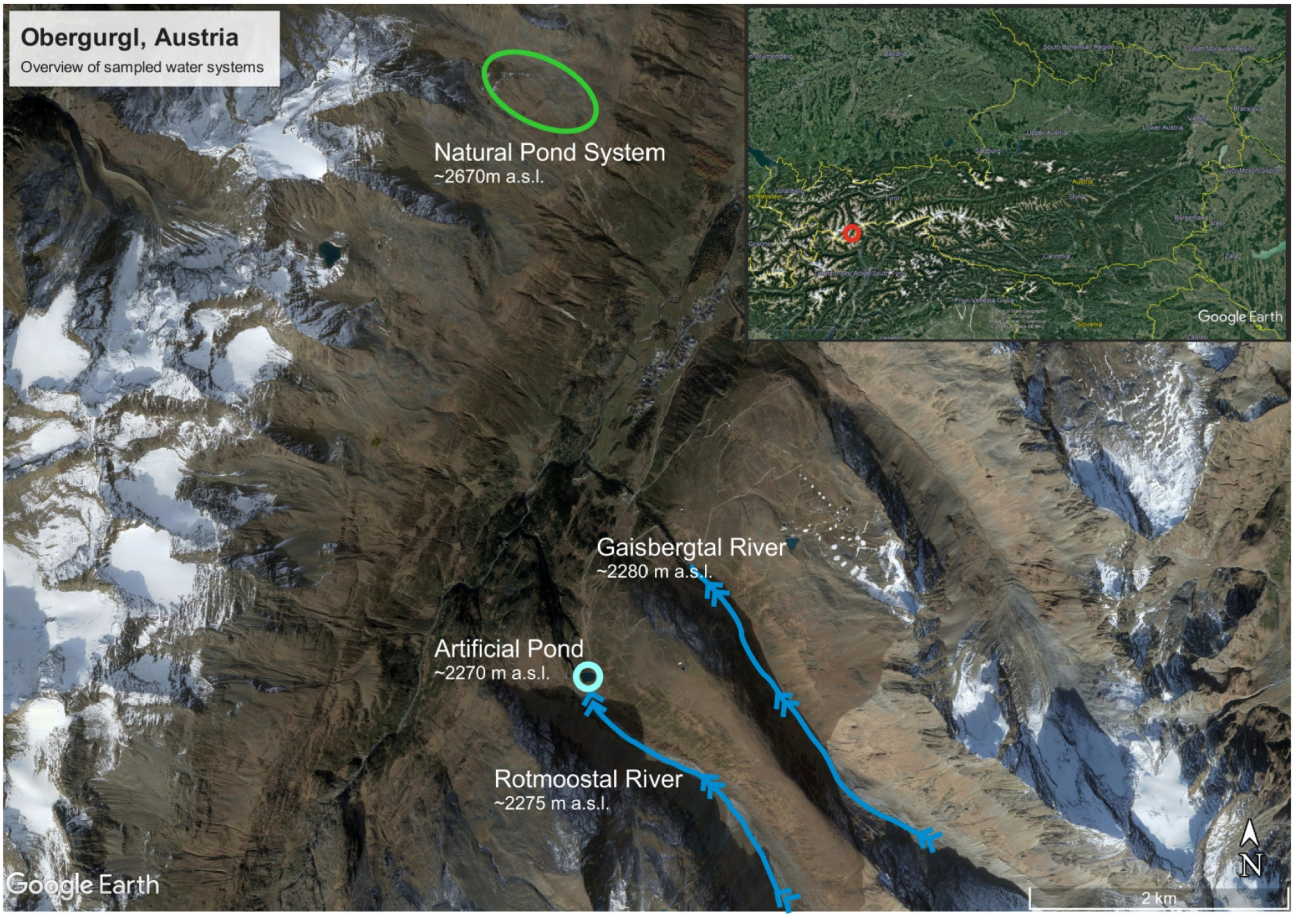


Fig. 1. Overview of the sampled water systems in Obergurgl, Austria. A map of Austria is shown in the upper right corner, and the red dot indicates the study site. The green circle shows the area where the natural ponds are located (N-Pond, ~2670 m a.s.l.), the light blue circle is where the artificial pond/water reservoir is located (A-Pond, elevation 2270 m a.s.l.), and the blue lines show the location and flow of the two sampled rivers (GAI, 2280 m a.s.l. and ROT ~2275 m a.s.l.). Adapted from image sources: "Austria", 47.594° N and 14.125° E, Google Earth pro. 2019, Accessed January 22, 2020; "Obergurgl" 46.847° N, 11.031° E, Google Earth pro. October 17, 2017. Accessed January 22, 2020. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

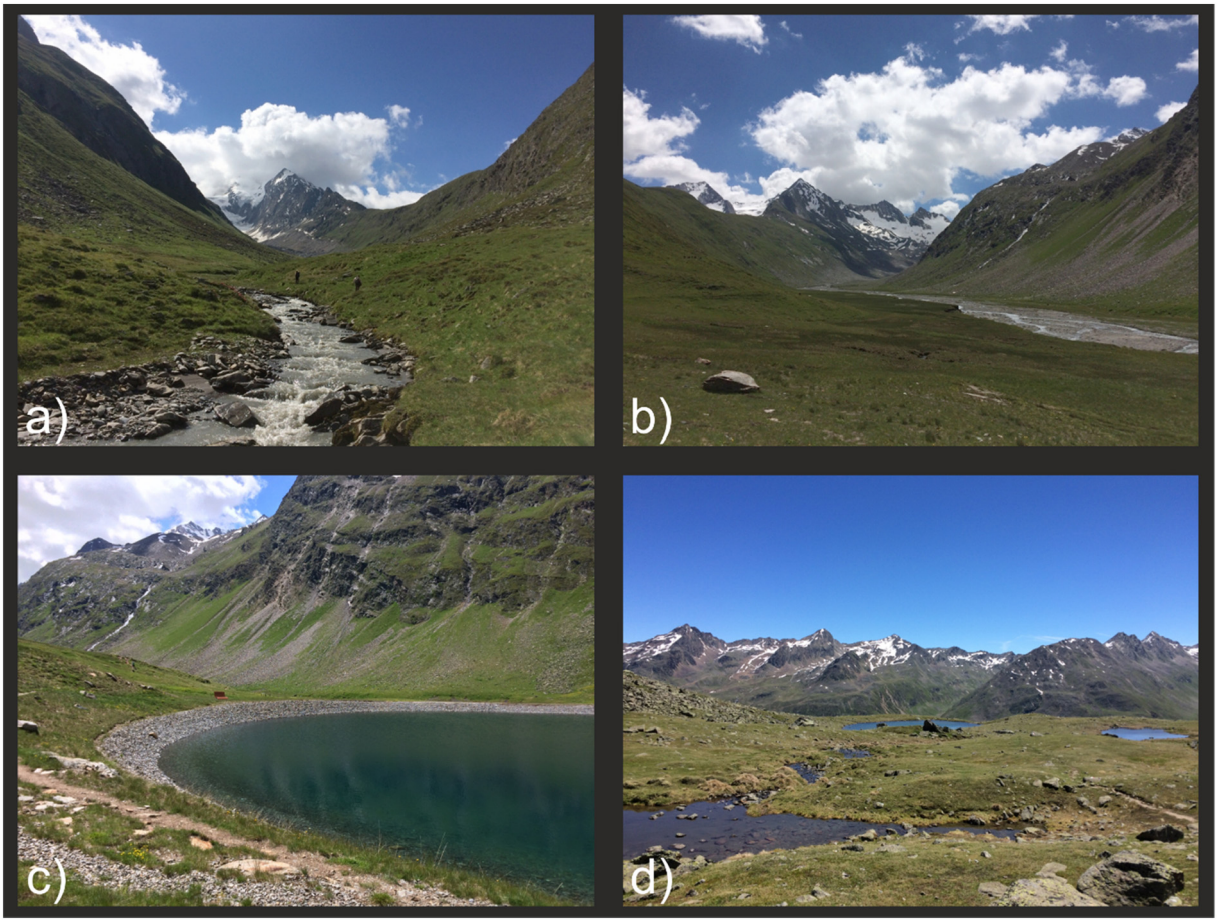


Fig. 2. Images of field sites in Obergurgl, Tyrol, Austria: a) Gaißbergtal river (GAI), b) Rotmoostal river (ROT), c) artificial pond (A-Pond), d) natural pond system (N-Pond).

2.5. Ice nucleation activity by cryo-microscopy for unfiltered and filtered water samples

After the sampling and processing, the unfiltered and the filtered water samples were kept frozen at −20 °C. For analysis, the samples were thawed in a cold-water bath, shaken to counter sedimentation and 0.5 mL pipetted into sterile Eppendorf tubes for immediate use. Ice nucleation activity of these samples was determined microscopically with the Vienna optical droplet crystallization analyzer (VODCA), an emulsion freezing method previously described in other publications (Felgitsch et al., 2018; Häusler et al., 2018; Pummer et al., 2012). The setup consists of an optical microscope (Olympus BX51M) equipped with a cryo-cell for low temperature measurements. The cryo cell consists of a custom made, gas tight Teflon® cell equipped with a Peltier element (Quick-cool QC-31-1.4-3.7M) for cooling purposes, and a glass window in the cover to enable microscopy. The Peltier element is mounted on a copper cooling block which is cooled by circulating cold water thus enabling the Peltier element to cool the cold side down to −50 °C. Temperature in the cell is recorded with a thermocouple type-K situated next to the sample area on the ceramic surface. The emulsion samples are prepared by creating a water in oil emulsion where the watery phase is the sample material and the oil phase is made of 85 wt% paraffin oil and 15 wt% Lanolin as an emulsifier. Similar mixtures were used and proven useful for droplet freezing (Hauptmann et al., 2016; Murray, 2008b). The emulsion is created by pipetting a small excess of oil phase next to a drop of sample on a cleaned glass lid and then mixing it by hand with the disposable micropipette tip. This method, yet very simple produces good and reproducible results and was favored over mixing with a Vortex laboratory mixer or an ultrasonic bath. The emulsified sample was transferred into the cryo-cell, which was then closed and flushed with nitrogen gas for one minute to remove excess humidity. The temperature program was set to a cooling rate of 0.6 K/s until 253 K and 0.1 K/s until 233 K. Every sample was measured at least three times resulting in approximately 75-140 counted droplets per sample. The freezing droplets are counted manually and only the droplets in the size range of 25 ± 10 μm are registered. This results in the droplets volume between 1.8 and 22.4 pL with the mean droplet size of 25 μm having a volume of 8.2 pL. For all samples, the median freezing temperature (T_{50}) and the initial freezing temperature/onset temperature (T_O) were determined. The median freezing temperature T_{50} is the temperature where 50% of the recorded droplets are frozen. T_O is the temperature where the first droplet of the sample freezes. T_O is generally less reliable as it is a single value and therefore prone to outliers. However, in order to screen a large set of samples with presumably low concentration of INPs, for the most active ones, it is a useful value. T_{50} mean values and their Standard Deviation (SD) were determined by a minimum of three runs and for T_O values the highest onset temperature of the several runs was determined. For the T_O values we calculated a SD from previous refreezing experiments of reference material. The cumulative nucleus concentrations K(T) for T_{50} and T_O values were calculated according to Vali (2019, 1971). With a mean droplet size of 25 μm, $K(T_{50})$ can be calculated as 8.45×10^7 mL^{-1} for the resembling T_{50} values.

2.6. Freeze drying of water samples

Water samples that showed a high T_{50} and/or T_O were picked for freeze drying along with several inactive ones for reference. The freeze

drying was done to concentrate the INPs without destroying any active composites. For this process 12 mL of a sample were freeze dried (Christ Alpha 1-4 LDplus; 24 h at 1 mbar and −55 °C and 4 h at −55 °C and 10^{-2} mbar) and the residues were dispersed in 200 μL of ultrapure water (produced with Millipore® SAS SIMSV0001). The samples were then analyzed for ice nucleation activity with the procedure described in Section 2.5.

2.7. Treatment of sand samples

Sand samples were thawed and filtered through a standard cellulose filter (VWR cat # 516-0802) to remove excess water. After this step, the sand was transferred into glass Petri dishes and heat treated in a laboratory oven at 300 °C for 24 h to remove water and destroy possible organic/biological material. At this temperature most biological INPs will be denatured (Pouleur et al., 1992) and organic material combusts or evaporates (Kristensen, 1990). Once cooled, the samples were milled in a ball mill (Retsch MM400) with a tungsten carbide ball for 90 s at 30 Hz to produce a homogeneous mineral dust for further analysis. The dust was kept in sterile 50 mL centrifuge tubes (Brand cat # 114823) until measurement by cryo-microscopy.

2.8. Ice nucleation activity by cryo-microscopy of river sand

Cryo-microscopy of the sand samples was carried out the same way as for the water samples. For the sample preparation 5 mg of the minerals dust were suspended in 1 mL of ultrapure water with the aid of a laboratory vortex mixer. The samples were kept in an ultrasonic bath to counter sedimentation and briefly mixed with the vortex mixer before measurement.

2.9. Culturing of microorganisms from filters

Culturing of microbes from filters was conducted following similar protocols to Benson et al. (2019). Briefly, filters were resuspended in 5 or 10 mL of sterile water in 100 mL sterile glass bottles. A single sterile magnetic stir bar was added to each bottle, and a Cimarec stir plate (cat #SP131635) was used to resuspend material on the filters following a gentle spin (setting #4) for 10 min. Following resuspension, 0.2 mL of this concentrated sample was plated in triplicate on KBC selection plates (Kings B medium made as 15 g/L proteose peptone, 1.5 g/L anhydrous K_2HPO_4, 10 mL/L 100% glycerol, 6 mM $MgSO_4$) with 24 mM H_3BO_3, cephalexin (10 mg/L), and cycloheximide (50 mg/L)) (Mohan and Schaad, 1987). Subsequent dilutions of the resuspension were plated in triplicate on KBC and TSA + cycloheximide 50 mg/L (10% TSA, 1.8 g/L tryptone soya broth). The abundance of bacterial growth, or number of colony forming units (CFU), for each plate was recorded. To normalize the concentration of bacteria across the data set, the concentration or dilution used for plating was converted to mL and CFU/ mL was determined for each sample. The abundance of CFU counts on individual plates was categorized as low (1-19), moderate (20-399), high (400-900), and tmtc (too many to count) (999). Plates with moderate growth were used to record CFU values when possible.

2.10. Ice nucleation assays for microorganisms cultured from water samples

Colonies were picked from KBC plates and tested for INA using a droplet freezing method (Hanlon et al., 2017). Briefly, sterile toothpicks were used to transfer single colonies to 140 μL of sterile DI water in 96-well plates. Plates were covered in pierceable foil, vortexed for 30 s, and stored at 4 °C for 1 h. Following this incubation step, 12 μL droplets were loaded in duplicate onto a PARAFILM® M (Sigma P6543, 20 in. × 50 ft) float assembly placed in a cooling bath held at −6 °C. The cooling bath consisted of ethylene glycol coolant fluid (Air gas RAD64000246) in a Lauda Alpha RA 12 (LCKD 4908) cooling unit (LAUDA-Brinkmann, LP, Delran, NJ, 08075). Temperature was decreased twice, in 1-min intervals to −8 °C, and then held at −8 °C for 10 min. Pictures of each cryo-float assembly were recorded after the 10-min incubation.

2.11. Statistical analyses

To test for significant differences of T_{50}, T_O, CFU/mL, and INA fraction, Analysis of Variance (ANOVA) was applied on these sample sets. One-way ANOVA was applied for each pair of campaign, characteristic, filtered/unfiltered as factors and T_{50}, T_O, CFU/mL, and INA fraction, as data with a significance level of 0.05. Tukey's Honest Significant Difference (HSD) was used to test for means differences with a significance level of 0.05. The program *Origin Pro 2019b* was used for this.

3. Results and discussion

3.1. INA of river and pond samples

The results of all samples are summarized in Supplement Tables S2 and S3 with sections for cryo-microscopy and biological analyses. A total of 60 water and 2 sand samples were collected. For the cryo-microscopy, 60 each of unfiltered/filtered water samples were analyzed, 0.22 μm filters and two pretreated and re-dispersed sand samples. On the overall view most samples are very close to the homogeneous nucleation temperature of the cryo-microscopy setup which is at 237 K. T_O often differs by several degrees from T_{50}, evidence for the strong dilution of INPs in the samples and therefore in the water sources. The lowest value for T_{50} was at 233.8 K (OBA48) and the highest at 243.3 K (OBA9). For T_O the lowest value was at 236.3 K (OBA48) and the highest at 264.5 K (OBA9). The highest and lowest freezing temperatures were observed within the river sample set. The cumulative nucleus concentration $K(T_{50})$ is 8.45×10^7 mL^{-1} for all resembling T_{50} values. $K(T_O)$ values are summarized in Supplement Table S3.

TSA, a general growth medium was used to confirm culturable microbe counts for bacteria at all locations. All locations had growth (ranging from low to tmtc) on TSA plates (data not shown). KBC is a semi-selective growth medium used to select for *Pseudomonas* and highly related genera. Bacterial colonies from KBC plates were used for the INA screen to determine the fraction of INA positive bacteria for each sample location. For FC1, FC2, FC3, and FC4, the mean concentration of culturable microbes on KBC was 73, 1102, 0.563, and 1.98 CFU/mL, respectively. Concentration of growth on TSA plates for the four campaigns followed the same trend with mean concentrations of 3968, 26852, 38, and 144, respectively.

The fraction of bacteria with the ice+, or INA phenotype was determined by number of colonies that froze in the INA-assay. Droplets that froze on the cryo-bath float during the 10-min incubation at −8 °C (265 K) were considered INA positive. The mean % of positive INA for FC1, FC2, FC3, and FC4 was 27%, 5.6%, 86% and 23%, respectively. INA fraction was the lowest for samples taken during FC2 (July2016), and the highest for samples collected during FC3 (November2016). For several samples in FC3, 100% INA was observed (OBA37, 41, 43, 44, 46). One sample in FC4 (OBA58) had 100% INA. These six samples all had low CFU/mL values (mean of 0.178 KBC CFU/ mL) and were all in the low (1-19) abundance of colony growth.

Data for both methods are shown in Figs. 3, 4, and 5, sorted by sampling area (rivers, artificial pond, natural ponds). Fig. 3 shows similar trends between the two rivers GAI and ROT. For FC2, however, no INA positive colonies were cultured from river GAI. In the INA-assays three INA samples were observed (3 of 6 total) from river ROT (Fig. 3b FC2), and one from the N-Pond samples (Fig. 5b OBA30). For FC3, the CFU/mL was low, however, the percentage of bacteria that froze in the INA assay was the highest of the four field campaigns with a mean of 86% INA. For FC3, the number of colonies screened ranged from 1 to 33 and the number that froze ranged from 1 to 22. When comparing the results of the cryo-microscopy with the biological activity in Figs. 3, 4, and

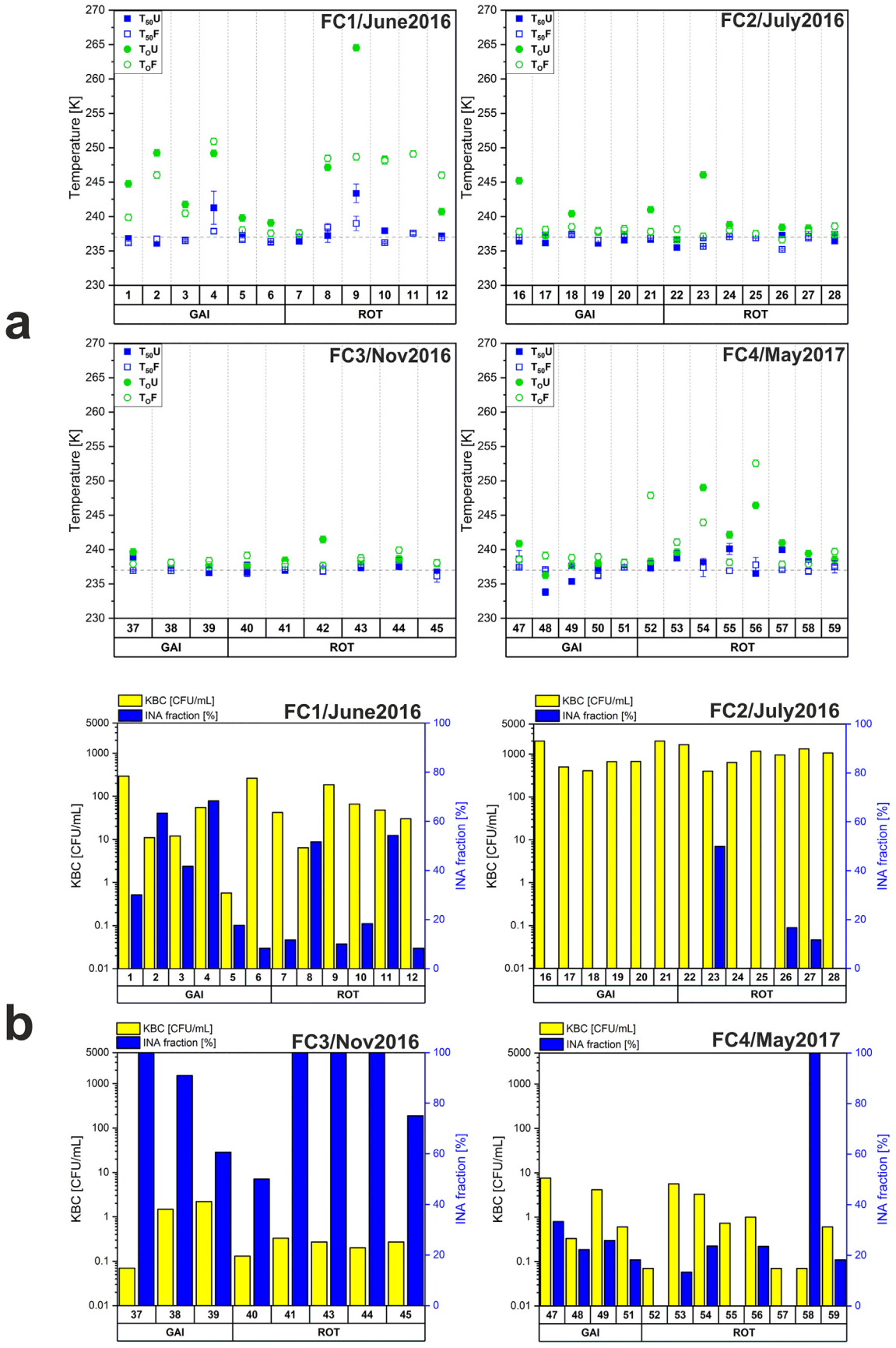


Fig. 3. Results for river water samples from the four field campaigns FC1-FC4. Sample numbers are shown on x-axis without the prefix "OBA" along with the river (GAI/ROT) they were sampled from a.) Shows cryo-microscopy results with T_{50} and T_O temperatures of unfiltered and filtered samples on the y-axis in Kelvin. A reference line is plotted at 237 K which is a threshold for homogeneous nucleation with the used system. b.) Shows results for culturable microorganisms, with CFU/mL grown from KBC media on y-axis 1, and the INA fraction (% INA) on y-axis 2. The CFU/mL on x-axis 1 have different scales according to the results from the four sampling campaigns.

5, some samples bear a similarity between IN active colonies and higher T_O and T_{50} values. Yet others show INA in the INA-assay and no outstanding T_O and T_{50} values and vice versa. A direct link can therefore not be made. A reason for this might stem from the fact that few numbers of bio INPs might go undetected with the cryo-microscopy setup yet can grow manifold in cultivation. The highest average concentration of colonies (KBC) was observed for FC1 and FC2 with a mean of 73 CFU/mL and 1102 CFU/mL, respectively.

The data for the artificial pond are plotted in Fig. 4. Similar to the river samples, the T_{50} values are close to the homogeneous nucleation temperature indicated by the reference line. T_O is often much higher than T_{50}, a sign for low concentration of the INPs. The biological analysis shows CFU/mL to be 1.2 to 3.38 CFU/mL for KBC and 4995 CFU/mL for TSA (FC1), 1998 for KBC and 5000 CFU/mL for TSA (FC2), yet no IN active colonies. FC3 had low growth (0.13 CFU/mL KBC and 6 CFU/mL TSA, however 100% of colonies (2/2) were INA positive, a trend that was observed for the river samples as well. FC4 shows slightly more growth of overall colonies (1.67 CFU/mL KBC and 310 CFU/mL TSA) again with a fraction being INA positive.

Natural pond data are shown in Fig. 5. T_{50} and T_O values of the natural ponds are both very close to the homogeneous nucleation temperature as seen in Fig. 5. The same is observed for the other samples from FC2 (Figs. 3 and 4). The samples OBA32 and OBA35 show an outlying behavior in that the filtered samples have a higher T_{50} and T_O than the unfiltered, which was also observed in some river samples. OBA32 was sampled at the outlet of a semi-stagnant natural pond with lots of visible red algae growth, OBA35 was sampled further down of the same system. The samples OBA33 and OBA34 were sampled in between in the same stream yet did not show this behavior. The biological analysis shows low CFU/mL for most samples with only one sample having high CFU/mL and INA positive species (OBA30). Out of this sample set, OBA30 was collected at the highest elevation of the natural pond samples yet with only one sample showing this effect no conclusion can be drawn from this observation. Future research might look closer into a possible correlation between elevation and INA. Other small scale local differences might play a role as well. Such differences do not seem to extend over the rest of the water system, as all other collected samples from this set lie downstream of the OBA30 sampling spot.

3.2. Concentration of INPs by freeze drying

Unfiltered samples that showed good INA were selected for further concentration by freeze drying along with some lesser active samples for comparison. The results (T_{50},T_O and according K(T)) are shown in Supplement Tables S4 and S5. All samples except four (OBA30, 35, 57, 60) showed an increase in T_{50} temperatures through the concentration step. Freeze-drying did significantly enhance the ice nucleation activity of most samples. Changes of T_{50} of up to 12.3 K (OBA8) were found. T_{50} and T_O for most samples lie closer together, a sign of steeper freezing curves for the concentrated samples. This shows the overall strong dilution of INPs in the native/unconcentrated samples from these alpine water sources as they show much stronger differences between T_{50} and T_O. Four samples have a lower T_{50} with one sample being OBA35 which showed better freezing activity after filtration which could be a sign for the concentration of any compound interfering with ice nucleation. The other three samples with a lower activity after freeze drying did not show any outlying behavior in their native form and OBA33 which also showed better ice nucleation activity after filtration had increased activity after freeze drying. The data is therefore too slim to draw any conclusions. The most important result of the freeze-dried samples is that the highly active samples from FC1 increased their ice nucleation activity to temperatures above the concentrated sediment samples (Sand-ROT, Sand-GAI) showing that inorganic ice nuclei are not the cause of highly active INPs in these samples.

3.3. Seasonal and spatial trends of INPs

The cryo-microscopy results of the unfiltered samples from two rivers, the artificial pond, and the natural ponds were compared to examine potential seasonal and spatial (geographic) trends. With regard to the spatial trends, there were no significant differences found for T_{50} ($p = 0.36$), and T_O ($p = 0.54$) values when comparing characteristics of the samples. For the culturable microbes, site characteristics also showed no significant differences for the number of CFU/mL from KBC media ($p = 0.55$) and no significant differences for the INA positive fraction ($p = 0.21$). Overall, the INA of the samples determined by two different methods showed no significant differences when comparing locations of the two rivers, the artificial pond, and the natural ponds. This leads to the conclusion that INA in this relatively small alpine area does not differ strongly, depending on the characteristic of the water body. For future research a combination of cultivation and subsequent cryo-microscopy might reveal fine differences in spatial bacterial INA yet this cannot be observed in this study. A similar observation was made by Pietsch et al. (2017) which found no significant differences of ice nucleation active strains between different spots and depth of a lake. However, there are outliers among the samples where ice nucleation activity is much higher

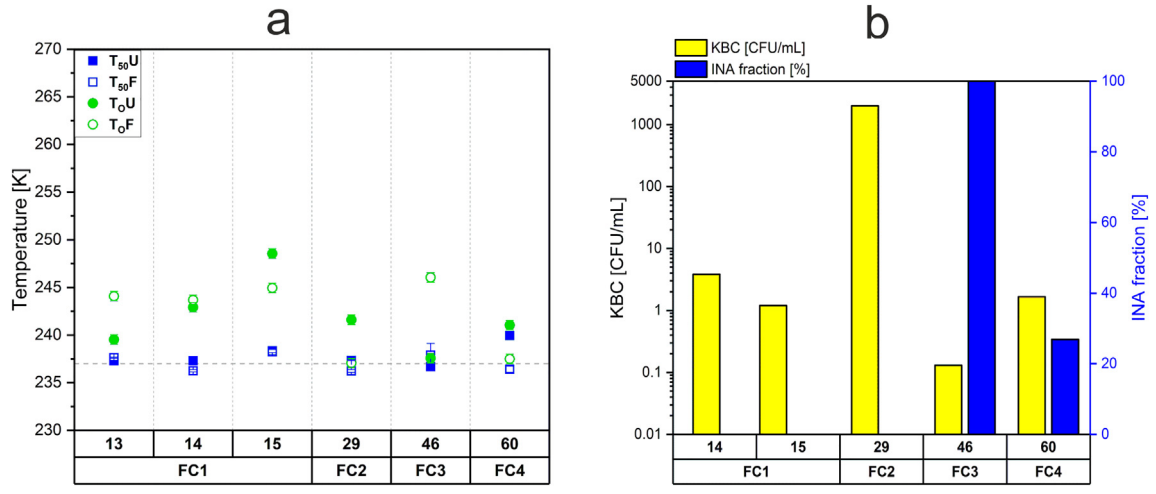


Fig. 4. Artificial pond water samples from the four field campaigns FC1-FC4. Sample numbers are shown on the x-axis without the prefix "OBA" along with the field campaign number they were sampled on. a) Shows cryo-microscopy results with T_{50} and T_O temperatures of unfiltered and filtered samples on the y-axis in Kelvin. A reference line is plotted at 237 K which is a threshold for homogeneous nucleation with the used system. b) Shows results for culturable microorganisms, with CFU/mL grown from KBC media on y-axis 1, and the INA fraction (% INA) on y-axis 2.

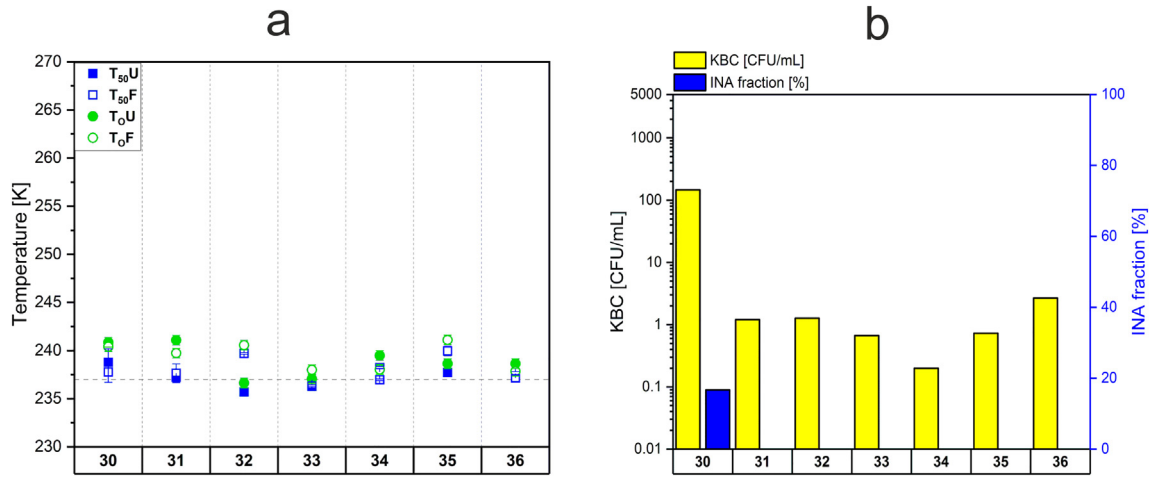


Fig. 5. Natural pond results from field campaign 2 (July 2016). Sample numbers are presented on the x-axis without the prefix "OBA" a) Shows cryo-microscopy results with T_{50} and T_O temperatures of filtered and unfiltered samples on the y-axis. A reference line is plotted at 237 K which is a threshold for homogeneous nucleation with the used system. b) Shows results for culturable microorganisms, with CFU/mL grown from KBC media on y-axis 1, and the INA fraction (% INA) on y-axis 2.

than the mean of a certain body of water. Locations with much higher INA could be caused by small scale differences as bacterial colonies/films on rocks (Morris et al., 2007), small feeder streams with higher concentrations of incorporated soil and decayed plant matter (Schnell and Vali, 1976) or other highly local factors that lead to these results. With larger scale sampling as it was done for this study, single locations with strong ice nucleation activity do not contribute enough to impact the mean of one water mass much stronger than the other to show a significant difference. These small-scale factors change over the course of a year as some of the spots with very good IN activity were revisited at exact coordinates and did not show exceptional IN activity on other sampling campaigns.

With regard to the seasonal trends, the T_{50} values are not significantly different ($p = 0.22$) partly due to the broad spread of the values. T_O values showed a significant difference ($p = 5 \times 10^{-4}$) for the different sampling campaigns with a trend that is also observable for the T_{50} values (Supplement Fig. S3). A plot for the mean T_O values is shown in Fig. 6a. The graph shows a trend with the highest T_O values for FC1 (June) lowering for FC2 (July) and FC3 (November), and an increase again for FC4 (May). For the culturable microbes, the campaigns revealed a significant difference for the CFU/mL from KBC media ($p = 3 \times 10^{-12}$) and significant differences for their INA fraction ($p = 4 \times 10^{-7}$). The plotted mean values of the four sampling campaigns are shown in Fig. 6b and c. Fig. 6b shows that there is a medium to low amount of CFU/mL present for FC1 with some variability in concentration. For samples from FC2 (July) there is a strong increase in CFU/mL albeit with quite some variety within the sample set. Samples from FC3 (November) and FC4 (May) then show a stark decrease in CFU/mL with a much smaller variety. In Fig. 6c, the mean INA fraction from the grown colonies is plotted. FC1 samples do show some INA with around 30% of INA colonies. FC2 samples behave inversely to the CFU/mL with the lowest observed INA of around 6% for the sample set. FC3 also shows such inverse behavior as the CFU/mL were observed as the lowest of all field campaigns yet INA fraction is highest with around 75% being INA. FC4 samples are very similar to FC1 with a slightly lower mean INA fraction yet not really distinguishable.

This trend could be explained in part by different transport mechanisms of materials in glacier fed streams (as GAI and ROT are) over the course of a year, which changes the physical-chemical composition of the water and even more important, the amount of particles transported in the stream (Füreder et al., 2001). This could increase the number of potential ice nuclei, especially inorganic ones, transported in the water. The cryo-microscopy results of the concentrated sediments from the two streams show the ice nucleation potential of the inorganic particles can account for freezing events up to a T_{50} of 247.2 K and T_O of 250.4 K (see Supplement Table S2). Highly active samples show even higher activity, some in native form and some after freeze drying. Freezing events higher than the T_{50} and T_O of the sediment samples are likely not caused by inorganic but by biological INPs. Due to snow melt, many feeder streams are present in late spring and early summer (represented by samples from FC1 and FC2 in this study) with water flowing over the meadows with a possible uptake of microorganisms (Morris et al., 2007), soil (O'Sullivan et al., 2014; Zolles et al., 2015), and plant material (Pummer et al., 2015; Schnell and Vali, 1972). This has an impact on the general number of particles and microbes transported in these waters which shows in the T_{50} and T_O values and CFU/mL concentrations of the water samples. Precipitation might also re-/introduce bacterial INPs into the water systems as it was observed for *Pseudomonas* ssp. in rain (Morris et al., 2014, 2017) and cloud water (Joly et al., 2013). For snow only negligible biological INP concentrations were observed for a snowfall event in Obergurgl, 2018 (Baloh et al., 2019). We assume that the largest share of ice nucleation active microbes in our water samples is of terrestrial origin similar to other recent observations (Larsen et al., 2017; Knackstedt et al., 2018). The samples from FC1 show high CFU/mL with a mean IN activity of around one third of these cultivated colonies. This is consistent with the high mean T_O results for FC1. In general, those samples with a high ice nucleation activity in cryo-microscopy often show good growth of colonies and activity in the microbial INA-assay as well. There are however some samples where this does not line up as they show INA with the cryo-microscopy yet none in the INA-assay. As it is often the case with natural samples, probably more than one factor contributes to our results and here *Pseudomonas* and related genera seem to contribute to the INA of many but not all samples tested. It is hard to determine which other INPs are present may it be material from plants, fungi, or even yet unculturable bacteria but they seem to follow a seasonal trend.

We speculate that at warmer temperatures (FC1/June 2016 and FC2/July 2016), overall abundance of microbes is naturally larger with some share of those INA bacteria which grew on the KBC media in late spring (FC1). In the summer (FC2), the - from the TSA media observed - generally large microbial abundance seems to outcompete those IN active species with only little IN active colonies observed in the sample set. When temperatures grow cold in November/December (FC3), observed microbial abundance, is much lower, yet with a striking amount of IN active species. In the winter months the colder temperatures and lower amount of nutrients in the water (Füreder et al., 2001) lead to a competitive advantage for those IN active bacteria. When temperatures are slowly getting warmer in May (FC4), overall observed abundance begins to increase from an average of 0.533 CFU/mL to 1.98 CFU/mL (KBC) and 37.7 CFU/mL to 143.6 CFU/mL (TSA). However, the INA

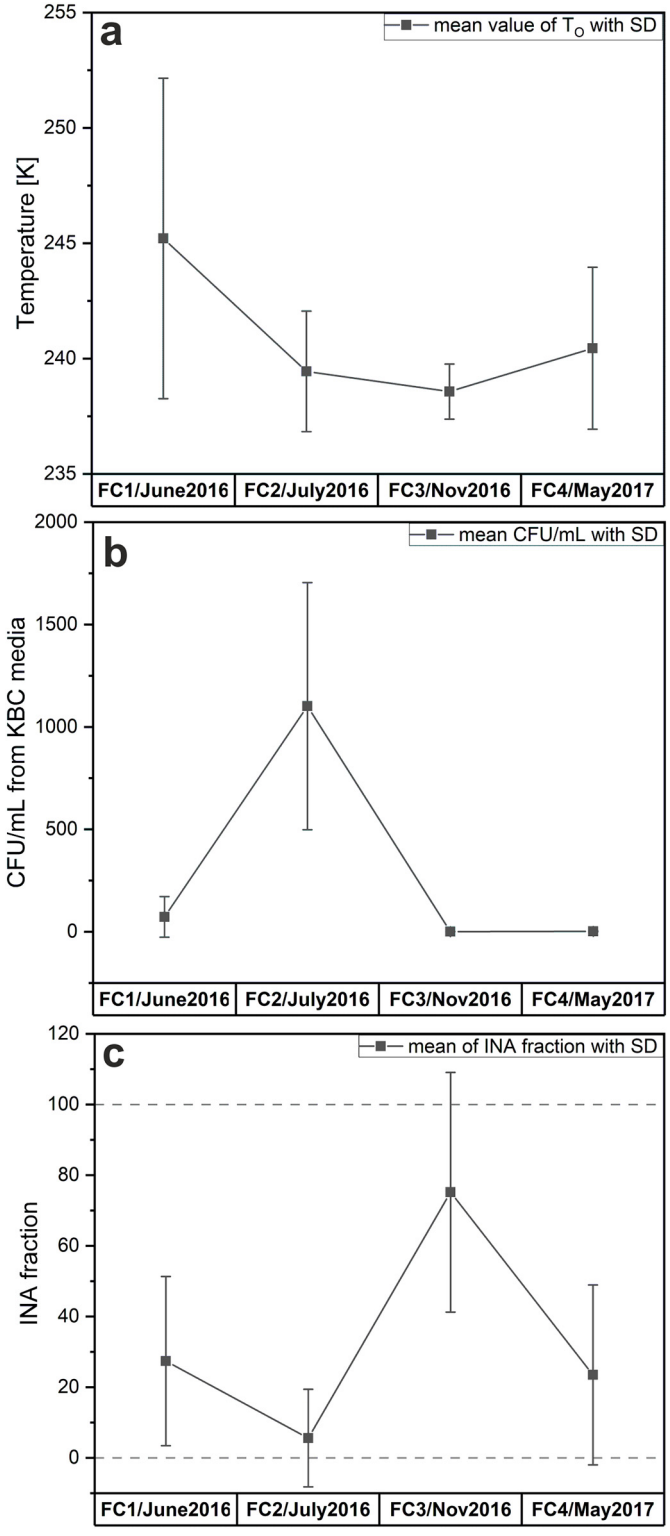


Fig. 6. Mean values of the campaigns with the campaign number and month on the x-axes. a) Shows mean T_O temperatures of a campaign with standard deviation (SD) on the y-axis, b) Shows the mean number of CFU/mL grown on KBC media from a campaign with standard deviation (SD) on the y-axis, c) Shows the mean INA fraction (percentage of INA) of colonies from different campaigns with standard deviation (SD) on the y-axis with a reference line for the maximum of 100 and the minimum of 0.

fraction of bacteria from KBC media dramatically shifts from 75.2% down to 23.5%. We speculate that the best conditions for those IN active species which can grow on KBC media are probably sometime in spring/early summer, likely due to their selective survival over winter they then benefit from the warmer temperatures and better nutrient availability before they are outcompeted by other, less IN active species in the summer. The INA phenotype could provide a potential survival advantage similar to typical psychrophilic bacteria that have been shown to produce ice binding proteins (Bar Dolev et al., 2016; Raymond et al., 2008) which include antifreeze- as well as ice nucleating proteins (Dhaulaniya et al., 2019) for their survival. If bacteria that produce ice nucleating proteins, but are not typically psychrophilic, as the *P. syringae* species, have better survival rates at subzero temperatures, is an intriguing question that to our knowledge has not been investigated yet. Overall, the activity of INPs (inorganic and biotic) was observed to change significantly with the time of year. The number of inorganic particles transported in the river changes over the year, especially at snowmelt. Yet besides the particle load, the composition of the transported sediments is unlikely to change fundamentally over the year and our results show INPs exceeding the activity of the sediment samples. One explanation is the presence of certain INA bacteria as we could show with our results. But as mentioned above this does not seem to be the whole story and for some samples other INA factors seem to contribute, which we cannot determine from our experiments. Candidates are plenty, from plants to fungi to yet uncultured microbes and the metabolites and secondary products of all of these. More specific research on such single materials will hopefully shed light on other possible INPs contributing to INA in natural water sources.

3.4. Effect of 0.22 μm filtration on INA

ANOVA of the 0.22 μm filter step showed no significant differences for T_{50} ($p = 0.44$), and T_O ($p = 0.52$) values between unfiltered and filtered samples. In fact, when plotted, the differences look very similar to a normal distribution as seen in Supplement Fig. S2. This is evidence for submicron particles altering INA after the filtration step. Not all samples of our sample set showed the same behavior. With a closer look the samples can be grouped into three categories. 1.) Samples with an already very low T_{50} that does not change significantly after the filtration step. Those samples have a very low concentration of particles that alter ice nucleation and consequently ice nucleation activity does not change much after filtering these samples. 2.) Samples with a considerable ice nucleation activity that have a significantly lower T_{50} and T_O after filtration. Those samples have microbial and probably inorganic INPs that are effectively held back by the filter and thus their influence cannot be seen in the filtrate which then has a lower T_{50} and T_O. Often, however, ice nucleation is still higher than the homogeneous nucleation temperature after filtration which can be attributed to sub-micron scale particles 3.) Samples with a near homogeneous or even lower T_{50} and T_O that change to higher values after the filtration. Category 3 seems counterintuitive as it is generally considered that a water volume with more particles nucleates faster than with less particles, as the probability for good ice nuclei is higher. Particles above the 0.22 μm size threshold are only present in the unfiltered sample which according to classic theories about INPs (Pruppacher and Klett, 1997) should also lead to much better ice nucleation. This however does not apply to some of the collected samples. Two of the natural pond samples (OBA32, OBA35) and three of the river samples (OBA48, OBA49, OBA56) stand out the most. These samples show a T_{50} of the unfiltered samples that is lower than the reference line for homogeneous nucleation of ultrapure water on our setup and have a higher T_{50} after the filtration. These five samples showed microbial growth in line with the other samples from the respective sampling campaigns. A reasonable explanation is that some material from these samples counter the ice nucleation and the filtration removes them. Antifreeze proteins (AFPs) can cause such an altered freezing behavior and they are known to be produced by various organisms such as fish (Fletcher et al., 2001), insects (Kong et al., 2017), plants (Atici and Nalbantoglu, 2003), and also bacteria (Duman and Olsen, 1993). AFPs have a smaller size between 7.4 and 14 KDa (Davies, 2014) than ice nucleating proteins which vary between 100 up to around 1000 kDa, many of

which are approximately much smaller than the 0.22 μm filter step. AFPs - which would pass the 0.22 μm filter – have recently been found to induce ice nucleation at higher than homogeneous freezing temperatures in lab experiments (Eickhoff et al., 2019). This along with other nanoscale INPs could explain a higher than homogeneous freezing temperature, yet still the question remains what compound is filtered out of the samples that suppresses ice nucleation. Soluble organic and inorganic compounds are known to absorb on solid particles and alter their ice nucleation in both directions (Boose et al., 2019; Chernoff and Bertram, 2010; Möhler et al., 2008; Whale et al., 2018). Since the samples of this study were continuously in aqueous solution the filter step should not interfere with any soluble compounds except if any strong adsorption bonding takes place. Additional investigations of possible microorganisms or other factors contributing to this observation are warranted.

Many samples still show ice nucleation activity after the filter step, a sign for nano-scale particles being active as ice nuclei in these samples. This is consistent with recent findings about INPs from biotic origins that show soluble ice nucleating macromolecules are present in plants (Pummer et al., 2012; Dreischmeier et al., 2017; Felgitsch et al., 2018), fungi (Fröhlich-Nowoisky et al., 2015), and bacteria (Vasebi et al., 2019). Sub-micron sized INPs were also found to be present in natural water samples (Knackstedt et al., 2018; Larsen et al., 2017) consistent with our results. To filter samples with a 0.22 μm filter is a standard method to remove microbial activity from samples (Eykamp, 1997). Along with microbial particles many other carbonaceous and inorganic particles larger than 0.22 μm are held back by this step as well. It would be intuitive to guess that a natural water sample has significant lower INA after filtering it through a 0.22 μm filter, since any INPs larger than that, are held back by this separation step. For the samples analyzed in this study some differ from this assumption and so when evaluating the results for the filter step it further underscores the presence of sub-micron INPs in natural water systems. The topic of sub-micron INPs is of growing interest and more research (lab and field work) will be needed to assess their impact on terrestrial and atmospheric water systems.

3.5. Implications for snowmaking

One part of the motivation for this study came from the idea to screen natural water systems, that are used as sources for artificial snow production, for endemic INPs. This has, to our knowledge, not been done before and we wanted to take a first look onto this subject to see if any patterns exist for the presence of INPs in natural waters. As it is a first look, our findings are far from any immediate application, but it might give some ideas to future applied research projects that look further into this subject. Knowledge about source or time when endemic INPs are present could benefit snow production without the need for additives to the water which is not always desired. We can for sure say that INPs are present in all water sources with some of them also showing relevant INA. We can also say that the choice of source from the sampled systems does not seem make a significant difference. As mentioned above the time of year seems to make a difference, yet since snow productions is mainly done in late fall and winter the flexibility here is limited. The interesting part of our results might be the growing seasonal share of certain INA bacteria grown on KBC media after winter. It would have to be determined what leads to this effect if it may be temperature, nutrient availability, or other factors. This could be a lead as to how, certain already present INPs, could be maximized. For certain, more research in the form of controlled lab and field studies will be needed to elucidate the best ways to use endemic INPs as ice nucleating agents in artificial snow production.

4. Conclusion

We found INPs with a broad range of activity in the investigated water sources ranging from a T_{50} of 233.8 K which is below the homogeneous nucleation temperature of our system up to a T_{50} of 250.8 K and T_O of 264.5 K. The highest T_{50} values of water samples exceed the T_{50} values of suspensions from river sediment underlining the impact of bio INPs to high INA in water.

A 0.22 μm filtration step used on all samples revealed no significant differences for T_{50} values proving the presence of submicron INPs in the investigated water sources.

Single samples showed higher T_{50}/T_O values after 0.22 μm filtration. From these few samples we could not draw any conclusions for the cause, yet it is an intriguing question for future research to determine which particles or adsorbents can cause this effect.

Significant spatial differences of INA between the four sampled water sources were not found. Some single samples differed far from the mean T_{50}/T_O of a source to higher IN temperatures. This points to small scale local factors that must play a defining role for this higher than usual INA.

Comparison of the four sampling campaigns revealed significant differences in INA between the campaigns with FC1 (Spring/Early Summer) having the highest overall INA in T_{50} and T_O and FC3 (Winter) having the highest fractions of IN active species grown in KBC media, yet overall low numbers of CFU/mL. We draw the conclusion that these culturable IN active species show better survival rates during the colder months compared to non-IN active species from KBC media.

Funding sources

Hinrich Grothe and Philipp Baloh gratefully acknowledge support by the FFG (Austrian Research Promotion Agency) for funding under project no. 850689 (EarlySnow). This research was supported in part by grants to D. Schmale from the National Science Foundation (NSF) under Grant Number DEB-1241068 (Dimensions: Collaborative Research: Research on Airborne Ice-Nucleating Species, RAINS) and the Institute for Critical Technology and Applied Science (ICTAS) at Virginia Tech. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NSF or ICTAS.

CRediT authorship contribution statement

PB, DGS, and HG designed the study. PB, DGS, LF, JB collected samples. PB, ED, DS, GP conducted cryo-microscopy experiments. RH, CA conducted biological ice nucleation experiments, PB, RH analyzed results. PB led the writing of the manuscript and did statistical analyses. RH, DGS, HG contributed to, and edited the manuscript. DGS and HG provided funding for the project.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the Alpine Research Centre Obergurgl of the University of Innsbruck for lab access and accommodation during the sampling campaigns. PB and HG would like to thank Prof. Thomas Konegger from TU Wien for access to his labs' freeze drier. We would also like to thank the Obergurgl lift company, especially Dr. Werner Hanselitsch and Alex Schmid for access to the artificial pond and logistical help on sampling campaigns. The authors acknowledge TU Wien Bibliothek for financial support through its Open Access Funding Programme.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149442.

References

Atici, Ö., Nalbantoglu, B., 2003. Antifreeze proteins in higher plants. Phytochemistry 64, 1187–1196. https://doi.org/10.1016/S0031-9422(03)00420-5.

Axson, J.L., May, N.W., Colón-Bernal, I.D., Pratt, K.A., Ault, A.P., 2016. Lake spray aerosol: a chemical signature from individual ambient particles. Environ. Sci. Technol. 50, 9835–9845. https://doi.org/10.1021/acs.est.6b01661.

Baloh, P., Els, N., David, R.O., Larose, C., Whitmore, K., Sattler, B., Grothe, H., 2019. Assessment of artificial and natural transport mechanisms of ice nucleating particles in an Alpine Ski Resort in Obergurgl, Austria. Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.02278.

Bar Dolev, M., Bernheim, R., Guo, S., Davies, P.L., Braslavsky, I., 2016. Putting life on ice: bacteria that bind to frozen water. J. R. Soc. Interface 13, 20160210. https://doi.org/10.1098/rsif.2016.0210.

Benson, J., Hanlon, R., Seifried, T.M., Baloh, P., Powers, C.W., Grothe, H., Schmale, D.G., 2019. Microorganisms collected from the surface of Freshwater Lakes using a drone water sampling system (DOWSE). Water 11, 157. https://doi.org/10.3390/w11010157.

Bieber, P., Seifried, T.M., Burkart, J., Gratzl, J., Kasper-Giebl, A., Schmale, D.G., Grothe, H., 2020. A drone-based bioaerosol sampling system to monitor ice nucleation particles in the lower atmosphere. Remote Sens. 12, 552. https://doi.org/10.3390/rs12030552.

Boose, Y., Baloh, P., Plötze, M., Ofner, J., Grothe, H., Sierau, B., Lohmann, U., Kanji, Z.A., 2019. Heterogeneous ice nucleation on dust particles sourced from nine deserts worldwide – part 2: deposition nucleation and condensation freezing. Atmos. Chem. Phys. 19, 1059–1076. https://doi.org/10.5194/acp-19-1059-2019.

Chernoff, D.I., Bertram, A.K., 2010. Effects of sulfate coatings on the ice nucleation properties of a biological ice nucleus and several types of minerals. J. Geophys. Res. Atmos. 115, D20205. https://doi.org/10.1029/2010JD014254.

Cochet, N., Widehem, P., 2000. Ice crystallization by pseudomonas syringae. Appl. Microbiol. Biotechnol. 54, 153–161.

Creamean, J.M., Suski, K.J., Rosenfeld, D., Cazorla, A., DeMott, P.J., Sullivan, R.C., White, A.B., Ralph, F.M., Minnis, P., Comstock, J.M., Tomlinson, J.M., Prather, K.A., 2013. Dust and biological aerosols from the Sahara and Asia influence precipitation in the Western U.S. Science 339, 1572–1578. https://doi.org/10.1126/science.1227279.

Cziczo, D.J., Froyd, K.D., Hoose, C., Jensen, E.J., Diao, M., Zondlo, M.A., Smith, J.B., Twohy, C.H., Murphy, D.M., 2013. Clarifying the dominant sources and mechanisms of cirrus cloud formation. Science 340, 1320–1324. https://doi.org/10.1126/science.1234145.

Das Tiroler Naturschutzgesetz, 2005. TNSchG 2005, LGBl. Nr. 26/2005 idF | 293/20. Austrian Legislature. https://www.ris.bka.gv.at/eli/lgbl/TI/2020/80/20200720.

Davies, P.L., 2014. Ice-binding proteins: a remarkable diversity of structures for stopping and starting ice growth. Trends Biochem. Sci. 39, 548–555. https://doi.org/10.1016/j.tibs.2014.09.005.

DeMott, P.J., 1990. An exploratory study of ice nucleation by soot aerosols. J. Appl. Meteorol. 29, 1072–1079. https://doi.org/10.1175/1520-0450(1990)029<1072:aesoin>2.0.co;2.

DeMott, P.J., Rogers, D.C., Kreidenweis, S.M., Chen, Y., Twohy, C.H., Baumgardner, D., Heymsfield, A.J., Chan, K.R., 1998. The role of heterogeneous freezing nucleation in upper tropospheric clouds: inferences from SUCCESS. Geophys. Res. Lett. 25, 1387–1390. https://doi.org/10.1029/97GL03779.

DeMott, P.J., Sassen, K., Poellot, M.R., Baumgardner, D., Rogers, D.C., Brooks, S.D., Prenni, A.J., Kreidenweis, S.M., 2003. African dust aerosols as atmospheric ice nuclei. Geophys. Res. Lett. 30.

Dhaulaniya, A.S., Balan, B., Kumar, M., Agrawal, P.K., Singh, D.K., 2019. Cold survival strategies for bacteria, recent advancement and potential industrial applications. Arch. Microbiol. 201, 1–16. https://doi.org/10.1007/s00203-018-1602-3.

Diehl, K., Quick, C., Matthias-Maser, S., Mitra, S.K., Jaenicke, R., 2001. The ice nucleating ability of pollen: part I: laboratory studies in deposition and condensation freezing modes. Atmos. Res. 58, 75–87. https://doi.org/10.1016/S0169-8095(01)00091-6.

Diehl, K., Matthias-Maser, S., Jaenicke, R., Mitra, S.K., 2002. The ice nucleating ability of pollen: part II. Laboratory studies in immersion and contact freezing modes. Atmos. Res. 61, 125–133. https://doi.org/10.1016/S0169-8095(01)00132-6.

Dreischmeier, K., Budke, C., Wiehemeier, L., Kottke, T., Koop, T., 2017. Boreal pollen contain ice-nucleating as well as ice-binding 'antifreeze' polysaccharides. Sci. Rep. 7, 41890. https://doi.org/10.1038/srep41890.

Duman, J.G., Olsen, T.M., 1993. Thermal hysteresis protein activity in bacteria, fungi, and phylogenetically diverse plants. Cryobiology 30, 322–328. https://doi.org/10.1006/cryo.1993.1031.

Eickhoff, L., Dreischmeier, K., Zipori, A., Sirotinskaya, V., Adar, C., Reicher, N., Braslavsky, I., Rudich, Y., Koop, T., 2019. Contrasting behavior of antifreeze proteins: ice growth inhibitors and ice nucleation promoters. J. Phys. Chem. Lett. 10, 966–972. https://doi.org/10.1021/acs.jpclett.8b03719.

Eykamp, W., 1997. Membrane separations in downstream processing. In: Goldberg, E. (Ed.), Handbook of Downstream Processing. Springer Netherlands, Dordrecht, pp. 90–139 https://doi.org/10.1007/978-94-009-1563-3_5.

Felgitsch, L., Baloh, P., Burkart, J., Mayr, M., Momken, M.E., Seifried, T.M., Winkler, P., Schmale III, D.G., Grothe, H., 2018. Birch leaves and branches as a source of ice-nucleating macromolecules. Atmos. Chem. Phys. 18, 16063–16079. https://doi.org/10.5194/acp-18-16063-2018.

Fletcher, G.L., Hew, C.L., Davies, P.L., 2001. Antifreeze proteins of teleost fishes. Annu. Rev. Physiol. 63, 359–390. https://doi.org/10.1146/annurev.physiol.63.1.359.

Fröhlich-Nowoisky, J., Hill, T.C., Pummer, B.G., Yordanova, P., Franc, G.D., Pöschl, U., 2015. Ice nucleation activity in the widespread soil fungus Mortierella alpina. Biogeosciences 12, 1057.

Füreder, L., Schütz, C., Wallinger, M., Burger, R., 2001. Physico-chemistry and aquatic insects of a glacier-fed and a spring-fed alpine stream. Freshw. Biol. 46, 1673–1690. https://doi.org/10.1046/j.1365-2427.2001.00862.x.

Garcia, E.B., Hanlon, R., Makris, M.R., Powers, C.W., Jimenez-Sanchez, C., Karatum, O., Marr, L.C., Sands, D.C., Schmale, D.G., 2019. Microbial diversity of individual raindrops collected from simulated and natural precipitation events. Atmos. Environ. 209.

Gorbunov, B., Baklanov, A., Kakutkina, N., Windsor, H.L., Toumi, R., 2001. Ice nucleation on soot particles. J. Aerosol Sci. 32, 199–215. https://doi.org/10.1016/S0021-8502(00)00077-X.

Hanlon, R., Powers, C., Failor, K., Monteil, C.L., Vinatzer, B.A., Schmale, D.G., 2017. Microbial ice nucleators scavenged from the atmosphere during simulated rain events. Atmos. Environ. 163, 182–189. https://doi.org/10.1016/j.atmosenv.2017.05.030.

Hauptmann, A., Handle, K.F., Baloh, P., Grothe, H., Loerting, T., 2016. Does the emulsification procedure influence freezing and thawing of aqueous droplets? J. Chem. Phys. 145, 211923.

Häusler, T., Gebhardt, P., Iglesias, D., Rameshan, C., Marchesan, S., Eder, D., Grothe, H., 2018. Ice nucleation activity of graphene and graphene oxides. J. Phys. Chem. C 122, 8182–8190. https://doi.org/10.1021/acs.jpcc.7b10675.

Hiranuma, N., Möhler, O., Yamashita, K., Tajiri, T., Saito, A., Kiselev, A., Hoffmann, N., Hoose, C., Jantsch, E., Koop, T., Murakami, M., 2015. Ice nucleation by cellulose and its potential contribution to ice formation in clouds. Nat. Geosci. 8, 273–277. https://doi.org/10.1038/ngeo2374.

Hiranuma, N., Adachi, K., Bell, D.M., Belosi, F., Beydoun, H., Bhaduri, B., Bingemer, H., Budke, C., Clemen, H.-C., Conen, F., Cory, K.M., Curtius, J., DeMott, P.J., Eppers, O., Grawe, S., Hartmann, S., Hoffmann, N., Höhler, K., Jantsch, E., Kiselev, A., Koop, T., Kulkarni, G., Mayer, A., Murakami, M., Murray, B.J., Nicosia, A., Petters, M.D., Piazza, M., Polen, M., Reicher, N., Rudich, Y., Saito, A., Santachiara, G., Schiebel, T., Schill, G.P., Schneider, J., Segev, L., Stopelli, E., Sullivan, R.C., Suski, K., Szakáll, M., Tajiri, T., Taylor, H., Tobo, Y., Ullrich, R., Weber, D., Wex, H., Whale, T.F., Whiteside, C.L., Yamashita, K., Zelenyuk, A., Möhler, O., 2019. A comprehensive characterization of ice nucleation by three different types of cellulose particles immersed in water. Atmos. Chem. Phys. 19, 4823–4849. https://doi.org/10.5194/acp-19-4823-2019.

Joly, M., Attard, E., Sancelme, M., Deguillaume, L., Guilbaud, C., Morris, C.E., Amato, P., Delort, A.-M., 2013. Ice nucleation activity of bacteria isolated from cloud water. Atmos. Environ. 70, 392–400. https://doi.org/10.1016/j.atmosenv.2013.01.027.

Jimenez-Sanchez, C., Hanlon, R., Aho, K.A., Powers, C., Morris, C.E., Schmale, D.G., 2018. Diversity and Ice Nucleation Activity of Microorganisms Collected With a Small Unmanned Aircraft System (sUAS) in France and the United States. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.01667.

Joly, M., Amato, P., Deguillaume, L., Monier, M., Hoose, C., Delort, A.-M., 2014. Quantification of ice nuclei active at near 0 °C temperatures in low-altitude clouds at the puy de Dôme atmospheric station. Atmos. Chem. Phys. 14, 8185–8195. https://doi.org/10.5194/acp-14-8185-2014.

Kanji, Z.A., Ladino, L.A., Wex, H., Boose, Y., Burkert-Kohn, M., Cziczo, D.J., Krämer, M., 2017. Overview of ice nucleating particles. Meteorol. Monogr. 58, 1. https://doi.org/10.1175/amsmonographs-d-16-0006.1.

Knackstedt, K.A., Moffett, B.F., Hartmann, S., Wex, H., Hill, T.C.J., Glasgo, E.D., Reitz, L.A., Augustin-Bauditz, S., Beall, B.F.N., Bullerjahn, G.S., Fröhlich-Nowoisky, J., Grawe, S., Lubitz, J., Stratmann, F., McKay, R.M.L., 2018. Terrestrial origin for abundant riverine nanoscale ice-nucleating particles. Environ. Sci. Technol. 52, 12358–12367. https://doi.org/10.1021/acs.est.8b03881.

Kondo, H., Hanada, Y., Sugimoto, H., Hoshino, T., Garnham, C.P., Davies, P.L., Tsuda, S., 2012. Ice-binding site of snow mold fungus antifreeze protein deviates from structural regularity and high conservation. PNAS 109, 9360–9365. https://doi.org/10.1073/pnas.1121607109.

Kong, C.H.Z., Leung, I.K.H., Sarojini, V., 2017. Synthetic insect antifreeze peptides modify ice crystal growth habit. CrystEngComm 19, 2163–2167. https://doi.org/10.1039/C7CE00232G.

Koop, T., Zobrist, B., 2009. Parameterizations for ice nucleation in biological and atmospheric systems. Phys. Chem. Chem. Phys. 11, 10839–10850. https://doi.org/10.1039/B914289D.

Kristensen, E., 1990. Characterization of biogenic organic matter by stepwise thermogravimetry (STG). Biogeochemistry 9, 135–159.

Lagriffoul, A., Boudenne, J.L., Absi, R., Ballet, J.J., Berjeaud, J.M., Chevalier, S., Creppy, E.E., Gilli, E., Gadonna, J.P., Gadonna-Widehem, P., Morris, C.E., Zini, S., 2010. Bacterial-based additives for the production of artificial snow: what are the risks to human health? Sci. Total Environ. 408, 1659–1666. https://doi.org/10.1016/j.scitotenv.2010.01.009.

Langham, E.J., Mason, B.J., 1958. The heterogeneous and homogeneous nucleation of supercooled water. Proc. R. Soc. A 247, 493–504. https://doi.org/10.1098/rspa.1958.0207.

Larsen, J.A., Conen, F., Alewell, C., 2017. Export of ice nucleating particles from a watershed. R. Soc. Open Sci. 4, 170213. https://doi.org/10.1098/rsos.170213.

Maki, L.R., Galyan, E.L., Chang-Chien, M.-M., Caldwell, D.R., 1974. Ice nucleation induced by pseudomonas syringae. Appl. Microbiol. 28, 456–459.

May, N.W., Axson, J.L., Watson, A., Pratt, K.A., Ault, A.P., 2016. Lake spray aerosol generation: a method for producing representative particles from freshwater wave breaking. Atmos. Meas. Tech. 9, 4311–4325. https://doi.org/10.5194/amt-9-4311-2016.

May, N.W., Olson, N.E., Panas, M., Axson, J.L., Tirella, P.S., Kirpes, R.M., Craig, R.L., Gunsch, M.J., China, S., Laskin, A., Ault, A.P., Pratt, K.A., 2018. Aerosol emissions from Great Lakes harmful algal blooms. Environ. Sci. Technol. 52, 397–405. https://doi.org/10.1021/acs.est.7b03609.

Mohan, S.K., Schaad, N.W., 1987. An improved agar plating assay for detecting pseudomonas syringae pv. syringae and P. s. pv. phaseolicola in contaminated bean seed. Phytopathology 77, 1390. https://doi.org/10.1094/Phyto-77-1390.

Möhler, O., Benz, S., Saathoff, H., Schnaiter, M., Wagner, R., Schneider, J., Walter, S., Ebert, V., Wagner, S., 2008. The effect of organic coating on the heterogeneous ice nucleation efficiency of mineral dust aerosols. Environ. Res. Lett. 3, 025007. https://doi.org/10.1088/1748-9326/3/2/025007 (8pp).

Morris, C.E., Kinkel, L.L., Xiao, K., Prior, P., Sands, D.C., 2007. Surprising niche for the plant pathogen pseudomonas syringae. Infect. Genet. Evol. 7, 84–92. https://doi.org/10.1016/j.meegid.2006.05.002.
Morris, C.E., Sands, D.C., Vinatzer, B.A., Glaux, C., Guilbaud, C., Buffière, A., Yan, S., Dominguez, H., Thompson, B.M., 2008. The life history of the plant pathogen pseudomonas syringae is linked to the water cycle. ISME J. 2, 321–334. https://doi.org/10.1038/ismej.2007.113.
Morris, C.E., Sands, D.C., Vanneste, J.L., Montarry, J., Oakley, B., Guilbaud, C., Glaux, C., 2010. Inferring the evolutionary history of the plant pathogen pseudomonas syringae from its biogeography in headwaters of rivers in North America, Europe, and New Zealand. MBio 1, e00107-10. https://doi.org/10.1128/mBio.00107-10.
Morris, C.E., Monteil, C.L., Berge, O., 2013. The life history of pseudomonas syringae: linking agriculture to earth system processes. Annu. Rev. Phytopathol. 51, 85–104. https://doi.org/10.1146/annurev-phyto-082712-102402.
Morris, C.E., Conen, F., Huffman, J.A., Phillips, V., Pöschl, U., Sands, D.C., 2014. Bioprecipitation: a feedback cycle linking earth history, ecosystem dynamics and land use through biological ice nucleators in the atmosphere. Glob. Chang. Biol. 20, 341–351. https://doi.org/10.1111/gcb.12447.
Morris, C.E., Soubeyrand, S., Bigg, E.K., Creamean, J.M., Sands, D.C., 2017. Mapping rainfall feedback to reveal the potential sensitivity of precipitation to biological aerosols. Bull. Am. Meteorol. Soc. 98, 1109–1118. https://doi.org/10.1175/BAMS-D-15-00293.1.
Mülmenstädt, J., Sourdeval, O., Delanoë, J., Quaas, J., 2015. Frequency of occurrence of rain from liquid-, mixed-, and ice-phase clouds derived from A-train satellite retrievals. Geophys. Res. Lett. 42, 6502–6509. https://doi.org/10.1002/2015GL064604.
Murray, Benjamin J., 2008. Enhanced formation of cubic ice in aqueous organic acid droplets. Environ. Res. Lett. 3, 025008. https://doi.org/10.1088/1748-9326/3/2/025008.
Murray, B.J., 2008. Inhibition of ice crystallisation in highly viscous aqueous organic acid droplets. Atmos. Chem. Phys. 8, 5423–5433. https://doi.org/10.5194/acp-8-5423-2008.
Murray, B., O'Sullivan, D., Atkinson, J.D., Webb, M.E., 2012. Ice nucleation by particles immersed in supercooled cloud droplets. Chem. Soc. Rev. 41, 6519–6554. https://doi.org/10.1039/C2CS35200A.
O'Sullivan, D., Murray, B.J., Malkin, T.L., Whale, T.F., Umo, N.S., Atkinson, J.D., Price, H.C., Baustian, K.J., Browse, J., Webb, M.E., 2014. Ice nucleation by fertile soil dusts: relative importance of mineral and biogenic components. Atmos. Chem. Phys. 14, 1853–1867. https://doi.org/10.5194/acp-14-1853-2014.
Pietsch, R.B., III, D.G.S, David, R.F., Marr, L.C., Vinatzer, B., 2015. Aerosolization of two strains (ice and ice−) of pseudomonas syringae in a collison nebulizer at different temperatures. Aerosol Sci. Technol. 49, 159–166. https://doi.org/10.1080/02786826.2015.1010636.
Pietsch, R.B., Vinatzer, B.A., Schmale, D.G.I., 2017. Diversity and abundance of ice nucleating strains of pseudomonas syringae in a freshwater Lake in Virginia, USA. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.00318.
Pouleur, S., Richard, C., Martin, J.-G., Antoun, H., 1992. Ice nucleation activity in fusarium acuminatum and fusarium avenaceum. Appl. Environ. Microbiol. 58, 2960–2964.
Pratt, K.A., DeMott, P.J., French, J.R., Wang, Z., Westphal, D.L., Heymsfield, A.J., Twohy, C.H., Prenni, A.J., Prather, K.A., 2009. In situ detection of biological particles in cloud ice-crystals. Nat. Geosci. 2, 398–401. https://doi.org/10.1038/ngeo521.
Pruppacher, H.R., Klett, J.D., 1997. Microphysics of Clouds and Precipitation. 2nd edition. Kluwer Academic Publishers New York, Boston, Dordrecht, London, Moscow.
Pummer, B.G., Bauer, H., Bernardi, J., Bleicher, S., Grothe, H., 2012. Suspendable macromolecules are responsible for ice nucleation activity of birch and conifer pollen. Atmos. Chem. Phys. 12, 2541–2550. https://doi.org/10.5194/acp-12-2541-2012.
Pummer, B., Budke, C., Augustin-Bauditz, S., Niedermeier, D., Felgitsch, L., Kampf, C., Huber, R., Liedl, K., Loerting, T., Moschen, T., et al., 2015. Ice nucleation by water-soluble macromolecules. Atmos. Chem. Phys. 15, 4077–4091.
Raymond, J.A., Christner, B.C., Schuster, S.C., 2008. A bacterial ice-binding protein from the vostok ice core. Extremophiles 12, 713–717. https://doi.org/10.1007/s00792-008-0178-2.
Schnell, R.C., Vali, G., 1972. Atmospheric ice nuclei from decomposing vegetation. Nature 236, 163. https://doi.org/10.1038/236163a0.
Schnell, R.C., Vali, G., 1976. Biogenic ice nuclei: part I. terrestrial and marine sources. J. Atmos. Sci. 33, 1554–1564. https://doi.org/10.1175/1520-0469(1976)033<1554:BINPIT>2.0.CO;2.
Slade, J.H., VanReken, T.M., Mwaniki, G.R., Bertman, S., Stirm, B., Shepson, P.B., 2010. Aerosol production from the surface of the Great Lakes. Geophys. Res. Lett. 37. https://doi.org/10.1029/2010GL043852.
Vali, G., 1971. Quantitative evaluation of experimental results an the heterogeneous freezing nucleation of supercooled liquids. J. Atmos. Sci. 28, 402–409. https://doi.org/10.1175/1520-0469(1976)033<1565:BINPIB>2.0.CO;2.
Vali, G., 2019. Revisiting the differential freezing nucleus spectra derived from drop-freezing experiments: methods of calculation, applications, and confidence limits. Atmos. Meas. Tech. 12, 1219–1231. https://doi.org/10.5194/amt-12-1219-2019.
Vali, G., Christensen, M., Fresh, R.W., Galyan, E.L., Maki, L.R., Schnell, R.C., 1976. Biogenic ice nuclei. Part II: bacterial sources. J. Atmos. Sci. 33, 1565–1570.
Vasebi, Y., Mechan Llontop, M.E., Hanlon, R., Schmale III, D.G., Schnell, R., Vinatzer, B.A., 2019. Comprehensive characterization of an aspen (Populus tremuloides) leaf litter sample that maintained ice nucleation activity for 48 years. Biogeosciences 16, 1675–1683. https://doi.org/10.5194/bg-16-1675-2019.
Ward, P.J., DeMott, P.J., 1989. Preliminary experimental evaluation of snomax (TM) snow inducer, nucleus pseudomonas syringae, as an artificial ice for weather modification. J. Weather Modif. 21, 9–13.
Whale, T.F., Holden, M.A., Wilson, T.W., O'Sullivan, D., Murray, B.J., 2018. The enhancement and suppression of immersion mode heterogeneous ice-nucleation by solutes. Chem. Sci. 9, 4142–4151. https://doi.org/10.1039/C7SC05421A.
Zolles, T., Burkart, J., Häusler, T., Pummer, B., Hitzenberger, R., Grothe, H., 2015. Identification of ice nucleation active sites on feldspar dust particles. J. Phys. Chem. A 119, 2692–2700.