We would like to thank the reviewers for their valuable comments. We have revised the manuscript accordingly and think it has strengthened as a result. Please find our responses to reviewer comments and changes to the manuscript below in blue text. A track changes version is also included.

Reviewer 1

General comments:

Creamean at al. studied ice nucleation activity within the domain Archaea and are the first to report ice nucleation activity of this domain. Up to date, Archaea have not been evaluated as INP. In two out of four investigated species, the ability to induce freezing above -18 ◦ C was found. The authors performed additional experiments (heat treatment, peroxide digestions) to further study the composition of INPs from Archaea. They suggest that the IN activity of intact cells were driven by organic and heat-labile materials. This work provides valuable insights into ice nucleation activity within the domain Archaea and contributes to the understanding of biological ice nucleation as a whole. The results are certainly publishable and are aligned with the scope of the journal. Overall, the manuscript is well-written and -structured, and I recommend to publish it in Biogeosciences after the following comments have been addressed.

Specific comments:

Line 111: Where did you obtain the cell cultures from? Please provide more information.

All cultures were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. This information has been included in section 2.1.

Line 117: How did you determine the salinities?

Salinities were confirmed using a handheld refractometer. This information has been added to section 2.1.

Line 122, Table 1, Table 2: Usually ppt is understood as "parts per trillion". In specific disciplines such as oceanography, however, the abbreviation ppt is commonly used for "parts per thousand". I would recommend additional annotations to minimize misunderstandings.

Thank you for bringing this to our attention. We have clarified in the footnotes for both tables that ppt stands for "parts per thousand".

Line 137: What is the temperature uncertainty of the measurement?

The temperature uncertainty of the IS is less than ±0.2 °C, which is a combination of the uncertainty in the thermocouples and the temperature variation across the blocks due to gradients in cooling. We have added this information to section 2.3.

Line 138: If the freezing of wells was recorded in 0.5 ◦ C steps, how did you receive initial freezing temperatures such as -17.6 ◦ C or -19.2 ◦ C (lines 152-153)? Why do you have often more than two data points within one degree in all figures?

Thank you for pointing this out. We ended up recording and plotting all data points (i.e., at approximately 0.33 °C intervals based on the cooling rate). We have clarified this in section 2.3.

Line 140: How many independent experiments have been performed? Have the results been checked for reproducibility? Are the data presented in the figures arithmetic mean values? Otherwise, how did you calculate the standard error?

Independent experiments were not performed in this initial survey. As the reviewer implies, growth at different temperatures and, especially, on/in different media can significantly affect ice nucleation activity of the well-known species of Gram–ve ice nucleation active (INA) bacteria (e.g., Nemecek-Marshall et al. 1993; Ruggles et al. 1993). With the single known species of bacterium from the Gram +ves, a Lysinibacillus sp., growth on two different media shifted the spectrum by 1 – 3 °C (Failor et al. 2017). Our experience with both INA bacteria and fungi has shown us that growth conditions affect activity within a limited temperature range; more important is the intrinsic ability of an organism to fabricate INA molecules, which tend to have limits over which they activate. A constraint with these haloarchaeal species is that we were limited in choices of media due to their specific growth requirements. In this study we did not find evidence that the isolates were capable of exceptional INA ability (i.e., ≥ –10 °C). If they had, we would have conducted further experiments to explore the effect of growth conditions and salinity upon it.

Failor, K.C. et al. 2017. Ice nucleation active bacteria in precipitation are genetically diverse and nucleate ice by employing different mechanisms. The ISME journal, 11, 2740-2753.

Nemecek-Marshall, M. et al. 1993. High level expression of ice nuclei in a Pseudomonas syringae strain is induced by nutrient limitation and low temperature. J. Bacteriol. 175, 4062–4070.

Ruggles, J. A., et al. 1993. Kinetics of appearance and disappearance of classes of bacterial ice nuclei support an aggregation model for ice nucleus assembly. J. Bacteriol. 175, 7216–7221.

In order to be consistent with our previously published IS data, we now use 95% confidence intervals calculated based on the methodology of Agresti and Coull (1998). This is now clarified in the methods and captions.

Line 142: For simplification, I would recommend to use the terms “heat” and “peroxide” (treatment) throughout the whole manuscript as they are also used in the figure legends.

Done.

Line 172: Is there any proof that the intact cells of H. walsbyi and N. pharaonis would show ice nucleation ability, which can be suppressed by lysis? You could additionally test if the ice nucleation ability of H. morrhuae and H. sulfurifontis can be suppressed upon lysis.

Unfortunately, it is not possible to test the ice nucleation activity of H. walsbyi and N. pharaonis intact cells because they are particularly sensitive to lysis under hyposaline conditions (e.g., Boring et al., 1963). They readily lyse in water roughly 5% NaCl and below. The dilutions required to maintain their intact nature would still result in saline conditions that would significantly depress the freezing point of water. H. walsbyi and N. pharaonis require such high salinities in order not to lyse that the depression would be approaching the lower limit of the IS, so we would not obtain much of a spectrum. This is based on control tests using 10% saline solution controls with artificial seawater (Instant Ocean® Sea Salt) and solutions with archaea whereby freezing would not be observed until < –23 °C and then only reach ~ 0.2 fraction frozen at the IS lower limit. So, we would not have much of a spectrum left if using higher salinities to enable these archaea to remain intact.

We cannot test with H. morrhuae because it does not readily lyse in fresh water and is difficult to lyse without significant treatments that would alter its cellular properties (Leuko et al., 2004). A sufficient dilution would result in most H. sulfurifontis cells lysing but to be consistent with our methodologies with the other species would have resulted in a sample with extremely low cell density.

We have added Leuko et al. (2004) to the introduction where we stated that Halococcus do not lyse in fresh water. We added a statement on the particularly sensitive behavior of H. walsbyi and N. pharaonic to saline conditions to the introduction. We also added the rationale above for the chosen dilutions to the methods.

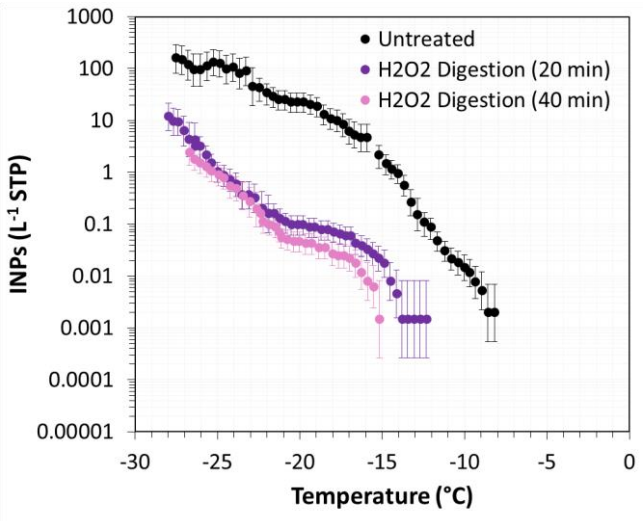
Boring, J., Kushner, D. J., and Gibbons, N. E.: Specificity of the salt requirement of halobacterium cutirubrum, Canadian Journal of Microbiology, 9, 143-154, 10.1139/m63-020, 1963.

Leuko, S., Legat, A., Fendrihan, S., and Stan-Lotter, H.: Evaluation of the LIVE/DEAD BacLight Kit for Detection of Extremophilic Archaea and Visualization of Microorganisms in Environmental Hypersaline Samples, Appl Environ Microbiol, 70, 6884, 10.1128/AEM.70.11.6884-6886.2004, 2004.

Lines 177, 180-182, 187-188: Which temperature do you refer to? Initial freezing temperature? T50?

These refer to the decreases in temperature when comparing the treatments to the standard INP spectra for initial freezing temperatures and when samples reached 100% frozen (i.e., final freezing temperatures). Although, we realize this is confusing. We have changed this so that these decreases (see new values in section 3.2) represent the average drop in temperature from the unamended INPs to the treatments (i.e., by calculating the average freezing temperature over the course of each spectrum and subtracting from the unamended). We have clarified this at the beginning of section 3.2 as well.

Lines 182-186: Have you tried to use an excess of peroxide to remove all organic material, which could serve as INP, so that there are no differences between sample concentrations anymore?



Based on results from many previous studies, we have found that digestion in 10% H_2O_2 provides an excess of oxidant for full decomposition of susceptible organic material. But the reviewer makes a very good point. Very occasionally we see residual activity of warm INPs in atmospheric aerosol samples, which suggests the presence of a population of INPs that are resistant to oxidation (note that we know from the amount of catalase required to neutralize excess peroxide that it had not been used up during the process). With a couple of these samples, we have tried doubling the time of the digestion, as shown at right. We found that this reduced the residual INP population, but did not remove it altogether, suggesting that this residual "hump" was either protected from oxidation or resistant to it (e.g., soot is not oxidized by H_2O_2).

Perhaps the archaea organic INPs are unique and some may be embedded within entities and are somewhat recalcitrant, making it difficult for the peroxide to completely degrade. We hope that any

future investigations of archaeal INPs would include a more rigorous peroxide treatment regimen, akin to that of the recent Bogler et al. (2020) study on lignin biopolymer. We added a statement on our recommendation after this sentence in the revision and included a statement with references on the basis for choosing the amount of peroxide used.

Lines 187-188: Please refer to Figure 3 here.

Done.

Lines 190-191: Please clarify: "Interestingly, H. sulfurifontis INP spectra were more responsive to the heat treatment as opposed to peroxide, . . .". Assuming that the same symbols and gray colors were chosen for figures 2 and 3 (legend is missing), the peroxide treatment shows a stronger reduction of INP spectra than the heat treatment.

We did mix up the symbols and colors and have fixed this. For clarity, we added a legend to Figure 3 as well. This statement is accurate; however, we did revise this paragraph to make this clear. H. morrhuae which was more responsive (i.e., had a larger drop in average freezing temperature) to peroxide than heat while H. sulfurifontis was the opposite in that there was a greater degradation with heat alone than was further enhanced with the addition of peroxide.

Line 197: Can you really negate freezing point depression or is it only a diminution?

Probably cannot completely negate but can reduce to negligible effects. We changed "negate" to "reduce".

Table 1: The word "initial" can be confusing here as it describes the cell concentrations after culturing. Please optimize caption and footnote.

We removed "initial".

Table 2: I would recommend to explain "n/a" in a footnote rather than in the caption.

Done.

Fig. 1: Why do you have sometimes the same frozen fraction for different temperatures (also in the other figures)? I would recommend to show only changes in the frozen fraction. Please explain.

This is because no new drops froze and thus the fraction frozen remained the same between temperatures. However, we understand this may be confusing and have removed redundant data with the same fraction frozen for different temperatures.

Fig. 2: You describe the plain lines in panel (b) as spectra for media controls, which underwent the heat and peroxide treatments. I can only see one plain line in panel (b) additional to the sample spectrum, but two more dashed lines, which have not been described in the caption yet. Please clarify the different lines. I would also recommend to use symbols instead of only colors in the legend. In the legend, you write "peroxide" but in the caption you name it "H2O2". Please be consistent. What kind of error bars did you use here? How do you explain that the heat-treated medium control shows a higher IN activity compared to the unamended control (panel b)?

We meant lines without markers, which we now have updated the caption to reflect this. For clarity, we now provide more comprehensive legends in all figures. We have changed H_2O_2 to "peroxide" and added a statement about the error bars (95% confidence intervals).

Fig. 3: Please add a legend to the figure. Please also consider additional comments of Fig. 2. In this figure, it is the other way around, the peroxide-treated medium control shows a higher IN activity compared to the unamended control. Please explain.

Done.

It would be beneficial for the community to calculate an INP concentration using Vali's equation and compare the results to other biological INP.

We originally considered this; however, this is trickier for haloarchaea versus other microorganisms. Given some samples were subject to lysing (H. walsbyi and N. pharaonic, which would under any hyposaline conditions) and some were not (H. morrhuae and H. sulfurifontis), comparing INPs mL^{-1} of sample, with the inherent assumption that this equate to INPs per cell, for intact versus fragmented cells would be comparing apples to oranges. With the uncertainties introduced by cell lysing, we ultimately kept it as fraction frozen for a more straightforward comparison. Fraction frozen is still shown on occasion (e.g., Adams et al., 2021). We could have calculated the number of active sites like Adams et al. but since we have the issue of intact versus lysed cells in our dilutions, this would still not be realistic when intercomparing the different haloarchaea.

Adams, M. P., Atanasova, N. S., Sofieva, S., Ravantti, J., Heikkinen, A., Brasseur, Z., Duplissy, J., Bamford, D. H., and Murray, B. J.: Ice nucleation by viruses and their potential for cloud glaciation, Biogeosciences Discuss. [preprint], https://doi.org/10.5194/bg-2020-474, in review, 2021.

However, we did like the idea of comparing to other biologically-derivedun INPs, so we have added a figure (Figure 4) that summarizes reported freezing temperature ranges for known biologically-derived INPs and discuss briefly in section 4.

Reviewer 2

There have been no data available on the ice nucleating activities of archaea, and this study examined the capacity of four haloarchaeal species with different cell wall types to serve as INPs. In general, the cells that remained intact after dilution in distilled water incited freezing at the warmest subzero temperatures observed, and additional experiments provided evidence that the activity is mediated by a proteinaceous or organic compound associated with the cells. I suggest a minor revision for the title by replacing "haloarchaea" for archaea since that was the only type of archaeal species they tested and more accurately describes the study. Below are more detailed and specific comments to consider when revising this manuscript. Abstract, Lines 19-22: As written, this sentence implies that thermophiles are prevalent "in other cold niches", which is not where one might expect thermophiles to be prevalent.

Abstract, Lines 22-24: Since the ability of archaea to "become airborne" or "impact cloud formation" was not examined in this study, please consider revising this closing statement in the abstract to more directly reflect the results obtained and their implications.

Done. We changed this sentence to, "Thus, it is important to assess their ability to serve as INPs as it may lead to an improved understanding of biological impacts on clouds."

Line 74: Suggest revising this statement to "up to 40% of the microbial taxa in an ecosystem".

Done.

Lines 118-119; 132-133: That fresh cultures were sent overnight is described, but please indicate how much time passed between receiving the cultures at CSU and performing the ice nucleation assays. This is very important information needed to evaluate the results because it is well established that the phase of growth and culture age affect ice nucleation activity in bacteria (e.g., Nemecek-Marshall et al. 1993, J Bacteriol 175:4062–4070; Fall and Fall 1998, Curr Microbiol 36:370–376; Yankofsky et al. 1983, Current Microbiology 9:263–267).

We ended up removing the information on institutions where the cultures were grown and shipped to as that seemed irrelevant but did change the last sentence of section 2.1 to, "Cultures were measured for their ice nucleation abilities within 1 – 2 days upon achieving log-phase growth."

Lines 122-123: This sentence describes a result and is out of place in the methods.

We think this is important to include here because in general, salinity reductions can cause haloarchaea to lyse. We revised the sentence to make this clear in that it is an effect that is more broadly applicable to haloarchaea: "Reductions in salinity can inherently cause the cells of certain haloarchaeal species to lyse (Boring et al., 1963; Legat et al., 2010; Leuko et al., 2004)."

Lines 124-126; 147-148: These sentences in the methods would be more appropriate for the discussion section.

We moved the sentence from lines 124-126 to the end of section 3.1: "Testing ice nucleation responses from cell lysis is relevant given: (1) cell fragments from fungi and bacteria have been previously observed to serve as INPs (Anderson and Ashworth, 1986; Du et al., 2017; O'Sullivan et al., 2015; Šantl-Temkiv et al., 2015) and (2) a less saline environment is more atmospherically relevant for how archaea might behave once incorporated in a relatively dilute cloud drop prior to immersion freezing. However, the results presented here indicate that of the species studied, lysed haloarchaeal cells (i.e., cell fragments) do not enhance ice nucleation abilities, and possibly even suppress it."

We did not move the sentence on lines 147-148 as that is based on previous work but did change the tense to reflect that and added a couple of references.

Line 128: Please clarify what is meant by "active". Do you mean ice nucleation active? Metabolically active?

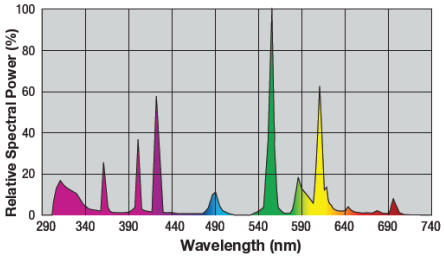
Changed to "metabolically active".

Lines 145-147: Please provide more detail on how the UV exposure was done to irradiate the liquid samples. If this was done by exposing a sample held within a test tube and since UV is opaque to most plastics as well as being attenuated by water and particulates (cells), it is important to explain the composition of materials involved and procedure in more detail (e.g., dose rate of UV source, distance of samples from the source, and if the dense suspension was mixed during exposure). Please also indicate the final concentration of peroxide used in the experiments in v/v. For example, if 0.75 mL of 30% H2O2 + 1.5 mL aliquot of suspension = 2.25 mL, so was it 7.5% H2O2 v/v? Finally, are all the haloarchaea used in this study catalase negative?

Reviewer 2 raises a very valid point. In designing this method, we knew that most suspensions of environmental media already contain species such as manganese dioxide or Fe^{3+}/Fe+ to drive the production of hydroxyl radicals. However, we reasoned that ensuring their production was sensible. We knew that UVC was blocked by glass and plastics, so we chose UVA/UVB-generating bulbs designed to

penetrate terrariums with plastic or glass covers. Efficiency of hydroxyl radical production by UVA/UVB is much lower than with UVC, so we use two 26 W bulbs placed adjacent to the floating samples, as sown. The suspension was not dense, since as noted we used the diluted samples, and were mixed by being agitated by boiling and the large stirrer bar, as shown. We have added the following details and modified the methods to provide more details, thus: "while illuminated with UVA/UVB fluorescent bulbs (Exo Terra Reptile UVB, 2 × 26 W providing ~2,000 µW cm^{-2} UVA and ~300 µW cm^{-2} UVB at the distance used)."

26W	Distance		UVA	UVB	Photo -synth.	UVB Rating	Illumin.
	Inch	cm	µW/cm²	µW/cm²	Vit. D3 IU/Min	See Reptile UVB Chart	LUX
	4	10	1150	195	75	*****	4340
	8	20	360	60	20	***	980
	12	30	150	25	15	***	490
	16	40	60	10	7	**	350
	20	50	40	6	7	**	270
	24	60	30	5	2	*	220
	28	70	20	4	1		200
	32	80	10	3	0		170
	36	90	5	1	0		150
	40	100	2	0	0		150



With reference to the potential production of catalase by the isolates, this is not an issue of concern since immediately after addition of the aliquot of 30% H_2O_2 we immerse the samples in boiling water, which would rapidly denature any catalases, if present.

Line 163: Please clarify what is meant by "markers" and note that polysaccharides are ubiquitous components of archaeal and bacterial cell envelopes.

Meant that they have been used as tracers for INPs as demonstrated in Zeppenfeld et al. (2019). We changed "markers" to "tracers".

Lines 168-169; 171-172: Please consider revising line 168 to state that "lysed cells" of these species do not have ice nucleation activity. The authors should also consider mentioning that it is well known that lysing of ice nucleation active bacterial cells decreases the efficiency at which they are INPs (e.g., Lindow et al. 1989, Mol. PlantMicrobe Interact. 2, 262). Are there any data available from experiments with lysed cells of Halococcus morrhuae and Haloferax sulfurifontis? It would not be surprising if the lysed cells.

Thank you for bringing this to our attention. We changed the first sentence to, "Lysed cells of both H. walsbyi and N. pharaonis did not exhibit ice nucleation activity…". It is true that lysing the well-known INA bacteria greatly lowers their ice nucleation activity. However, this is because their IN proteins are embedded within their outer membrane, where they also agglomerate together, increasing the INA. These archaea are from an entirely different kingdom and do not possess outer membranes. Nor do we know if the INA metabolites are proteins. Hence, we think it would be premature to compare their mechanism of activity with the uniquely-active Gram –ve group, that includes P. syringae.

There are no data of lysed cells of H. morrhuae because these do not lyse in fresh water. A sufficient dilution would result in most H. sulfurifontis cells lysing but to be consistent with our methodologies with the other species would have resulted in a sample with extremely low cell density.

We added the following sentence to the end of section 3.1: "This is analogous to previous work on bacteria, whereby it is well known that lysing of INA bacterial cells decreases the efficiency at which they are INPs (e.g., Lindow et al., 1989)."

Lines 176-179: Figure 2 indicates that INPs active at the warmest temperatures were heat labile, so I'm confused by what is meant by a "more substantial amount" of something else. Since the fraction of samples that froze at each temperature is known, this can be used to calculate the number of INPs at each temperature according to the method of Vali (1971, J Atmos Sci 28:402–409). These data provide context for inferring the fraction of the cell populations that were ice nucleation active at a given temperature/experimental condition.

Even changes in fraction frozen can indicate a more substantial amount (here, for comparing heat-labile versus organic materials), but it is relative since we are talking about fraction frozen. Thus, we changed to, "These results indicate the samples contained some heat-labile, likely proteinaceous, INPs, but contained a relatively larger contribution from other biogenic organic INPs…"

We originally considered calculating INP concentrations using Vali (1971); however, this is trickier for haloarchaea versus other microorganisms. Because we do not have cell concentrations for all dilutions due to lysing, we could not calculate it as something like INP concentration per mL of cell suspension. Given some samples were subject to lysing (H. walsbyi and N. pharaonic, which would under any hyposaline conditions) and some were not (H. morrhuae and H. sulfurifontis), comparing INP concentrations for intact versus fragmented cells would be comparing apples to oranges. This was why we ultimately kept it as fraction frozen: to be able to compare all the species together. Fraction frozen is still shown on occasion (e.g., Adams et al., 2021). We could have calculated the number of active sites like Adams et al. but since we have the issue of intact versus lysed cells in our dilutions, this would still not be realistic when intercomparing the different haloarchaea.

Adams, M. P., Atanasova, N. S., Sofieva, S., Ravantti, J., Heikkinen, A., Brasseur, Z., Duplissy, J., Bamford, D. H., and Murray, B. J.: Ice nucleation by viruses and their potential for cloud glaciation, Biogeosciences Discuss. [preprint], https://doi.org/10.5194/bg-2020-474, in review, 2021.

Lines 182-184: When catalase was added to samples of the less dilute cell suspensions, were oxygen bubbles observed/produced? I follow this argument, but it has me wondering about the "residual organic material" statement. Are the authors suggesting that treatment of the cell suspensions with peroxide oxidizes all macromolecules and organic constituents of the cells completely to CO2?

Yes, they were, liberally. We find that using ≥10% H_2O_2 provides a good excess of oxidant, and that even with "dirty" samples, such as permafrost suspensions, the great bulk of the peroxide remains after the

digestion, requiring the full aliquot (90 µL) of catalase to neutralize it. As mentioned above, in response to Reviewer 1, we do sometimes find aerosol samples that appear to have organic INPs that are resistant to oxidation. Soot and plastics, for example, are not affected by H_2O_2, and organic INPs may be protected by adsorption onto minerals or by being coated by clay particles.

Lines 187-188: I think this section is talking about Figure 3, but on closer inspection, I don't see Figure 3 referred to in the main text.

We have added a reference to Figure 3 in the last paragraph of section 3.2.

Lines 188-190: Please explain how these different behaviors should be interpreted with respect to the properties that can be inferred from the archaeal INPs.

We have added the following: "Collectively, these results indicate that H. morrhuae contained more organic relative to heat-labile INPs, while H. sulfurifontis contained more heat-labile as opposed to organic INPs. These haloarchaea have very different cellular envelop compositions: H. sulfurifontis contains a proteinaceous S-layer while H. morrhuae is devoid of such an S-layer but instead possessed a cell envelope that is composed of highly sulphated heteropolysaccharides. Thus, it would make sense that H. sulfurifontis is more sensitive to heat than peroxide given its proteinaceous cell envelope (assuming those proteins are ice nucleation active) and H. morrhuae is more sensitive to peroxide than heat given its polysaccharide-rich cell envelope."

Thank you very much for leading us to examine this more closely, as the cellular structure of each can help explain their responses to the treatments, which is something we did not realize before!

Lines 198-199: I would not describe the salt concentrations used in these experiments as "low", at least not in comparison to rain, snow, or fresh waters. The average concentration of salt used in the assays was ~1% and is roughly a quarter seawater. Please can the authors describe conditions that would allow cloud droplets to achieve such high ionic strength.

Good point. We have omitted the latter half of this sentence and just specified that we used hyposaline conditions to reduce freezing point depression, which was the primary objective of the dilutions.

Lines 205-206: Please expand on this point as I am not aware of any work that has shown similarities in motifs between gammproteobacterial IN proteins and S-layer proteins.

This point is based on the fact that we found proteins with the domain DUF3494 which has been commonly shown to be involved in ice-binding to be present in at least two haloarchaeal species. This is now explained in section 4.

Ice-binding ability is a characteristic of both INA and antifreeze proteins. Indeed, as Eickhoff et al. (2019) explain, "It has been proposed that both types of proteins interact similarly with ice and that, in principle, they may be able to exhibit both functions. We show that in addition to ice growth inhibition, both can also trigger ice nucleation…, providing unambiguous experimental proof for their contrasting behavior. Our analysis suggests that the predominant difference between AFPs and INPs is their molecular size, which is a very good predictor of their ice nucleation temperature." See also Kobashigawa et al. (2005) and Qiu et al. (2019). We have added the following to the conclusions: "Ice-binding ability is a characteristic of both ice nucleating and antifreeze proteins and is influenced primarily by their size (Eickhoff et al., 2019; Qiu et al., 2019).

Eickhoff, L., Dreischmeier, K., Zipori, A., Sirotinskaya, V., Adar, C., Reicher, N., Braslavsky, I., Rudich, Y. and Koop, T. 2019. Contrasting behavior of antifreeze proteins: Ice growth inhibitors and ice nucleation promoters. The Journal of Physical Chemistry Letters, 10, 966-972.

Kobashigawa, Y., Nishimiya, Y., Miura, K., Ohgiya, S., Miura, A. and Tsuda, S. 2005. A part of ice nucleation protein exhibits the ice-binding ability. FEBS Lett., 579, 1493-1497.

Qiu, Y., Hudait, A. and Molinero, V., 2019. How size and aggregation of ice-binding proteins control their ice nucleation efficiency. Journal of the American Chemical Society, 141, 7439-7452.

Vance, T. D. R., Bayer-Giraldi, M., Davies, P. L., and Mangiagalli, M.: Ice-binding proteins and the 'domain of unknown function' 3494 family, Febs j, 286, 855-873, 10.1111/febs.14764, 2019.

Figure 3: not mentioned in main text.

We now explicitly mention it when we discuss the results from this figure in the last paragraph in section 3.2.

Reviewer 3

Ice nucleation activity of biotic material is an interesting topic and investigation ice nucleating properties pf Archaea is an interesting approach. However, the problem with this study is that I do not feel that there was well thought out experimental design.

1. The authors did not treat all four organisms the same, comparing different dilutions, intact cells from one species vs lysed cells from another.

We actually did treat them all the same. We conducted a 1:15 dilution for all the haloarchaeal species (see Table 2 and Figure 1), the results from which are used when intercomparing all four together. We also subjected all four to the treatments, but as we stated on lines 192-193, H. walsbyi and N. pharaonis showed no response to the treatments because there were little to no INPs in the original dilutions and thus we did not show them in a figure or discuss them further. We added a note of this in the beginning of section 3.2. Given only H. morrhuae and H. sulfurifontis exhibited ice nucleation activity above the media controls, we did show and discuss results from their treatments. Since H. morrhuae was the most active (Figure 1), we further tested it under various dilutions to assess if the activity changes. It is reasonable to conduct different tests on different species given their activities and only intercompare species for the same testing. We did not intercompare species under different testing conditions.

As we indicated in the introduction and, as is generally known, not all microorganisms harbor the same properties. For example, not all bacteria are efficient INPs like P. syringe. These archaea have very different biological makeup, and surface properties and shapes, thus, would behave differently under variable conditions. Naturally, as with other microorganisms like bacteria, certain ones lyse and certain ones do not under hyposaline conditions (e.g., Lindow et al., 1989). The same applies to these haloarchaeal species. However, testing them in their hypersaline suspensions to ensure they remained intact would not make sense as that would significantly decrease the freezing point of water and would not be environmentally representative.

Unfortunately, it is not possible to test the ice nucleation activity of H. walsbyi and N. pharaonis intact cells because they are particularly sensitive to lysis under hyposaline conditions (e.g., Boring et al., 1963). They readily lyse in water roughly 5% NaCl and below. The dilutions required to maintain their intact nature would still result in saline conditions that would significantly depress the freezing point of

water. H. walsbyi and N. pharaonis require such high salinities in order not to lyse that the depression would be approaching the lower limit of the IS, so we would not obtain much of a spectrum. This is based on control tests using 10% saline solution controls with artificial seawater (Instant Ocean® Sea Salt) and solutions with archaea whereby freezing would not be observed until < –23 °C and then only reach ~ 0.2 fraction frozen at the IS lower limit. So, we would not have much of a spectrum left if using higher salinities to enable these archaea to remain intact.

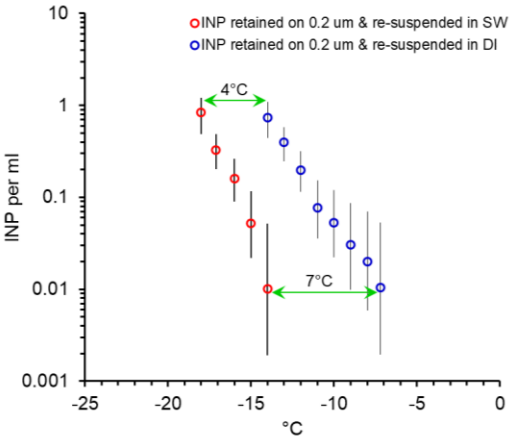
We cannot test with H. morrhuae because it does not readily lyse in fresh water and is difficult to lyse without significant treatments that would alter its cellular properties (Leuko et al., 2004). A sufficient dilution would result in most H. sulfurifontis cells lysing but to be consistent with our methodologies with the other species would have resulted in a sample with extremely low cell density.

We have added Leuko et al. (2004) to the introduction where we stated that Halococcus do not lyse in fresh water. We added a statement on the particularly sensitive behavior of H. walsbyi and N. pharaonic to saline conditions to the introduction. We also added the rationale above for the chosen dilutions to the methods.

Boring, J., Kushner, D. J., and Gibbons, N. E.: Specificity of the salt requirement of halobacterium cutirubrum, Canadian Journal of Microbiology, 9, 143-154, 10.1139/m63-020, 1963.

Leuko, S., Legat, A., Fendrihan, S., and Stan-Lotter, H.: Evaluation of the LIVE/DEAD BacLight Kit for Detection of Extremophilic Archaea and Visualization of Microorganisms in Environmental Hypersaline Samples, Appl Environ Microbiol, 70, 6884, 10.1128/AEM.70.11.6884-6886.2004, 2004.

2. The authors argue correctly that, once airborne, halophiles would be exposed to a dilute environment. However, the salinity of the selected dilution is not representative of cloud droplets. Moreover, why intact cells that did show ice nucleation activity where not further diluted to cause cell lysis, and conversely, cells that were lysed were not tested at lower dilutions to keep cells intact is unclear.



Reviewer 3 is exactly right, that once in a cloud droplet the salinity would be greatly lowered. Also, our experience of marine INPs always shows an increase in INA when diluted in DI water (above the 2 °C due to freezing point depression), as shown at right. The salinity chosen for the diluted culture tests was a reasonable compromise, typically from 0.5 – 1.5% (PBS is around 1% salt, and is not considered a particularly saline solution), to minimize freezing point depression while enabling expression if INA by the cells. As we stated in the introduction, H. morrhuae does not lyse in fresh water. And we did additional dilutions for H. morrhuae (Figure 2). See additional details in response to comment 1. We did omit the statement about how the lowered salinities would be relevant for cloud droplets in sections 2.2, 3.1, and 4.

3. The authors reported that diluting the media reduced survivability of halophiles and, in order to account for that, they determined the number of intact cells by microscopy. Yet, cell numbers reported in Table 1 are simply derived by multiplying cell numbers by the dilution factor (Table 2),

and thus, the study does not account for any losses caused by the dilutions. Undiluted cell suspensions should have been fixed to avoid cell lysis and counted. How can the authors account for a combined effect of intact cells and lysed cell material in these assays?

We reported that diluting the media lysed all H. walsbyi and N. pharaonis. This was confirmed using microscopy. We also reported that diluting did not cause any significant losses to H. morrhuae or H. sulfurifontis. This too was confirmed using microscopy.

4. The purpose of 30% H2O2 treatment is to determine the contribution of abiotic factors. However, the authors question the efficiency of the digestion protocol. If these 'digests' are a mixture of biotic and abiotic compounds, then there is no need for including the data.

We disagree. The fact that not all organic INPs were effectively wiped out for H. morrhuae is interesting as it can indicate that there may be some contribution from inter-cellular materials that the peroxide is not able to reach in the more concentrated dilutions. Note that the only "abiotic compounds" would be from the media, since we know these cultures do not contain mineral dust. We already show the treatments on the media control for 1:15 H. morrhuae, which demonstrated there were no decreases in ice nucleation proficiency from the media (Figure 2b). Thus, it must be something biotic given the cultures only contained the media and the cells.

Based on the comments from Reviewer 1, we modified the end of this paragraph to expand on this point a bit more. We simply used a ratio of peroxide to sample suspension that has been demonstrated to work based on previous IS studies involving dilutions (e.g., Barry et al., 2021; Creamean et al., 2020; Suski et al., 2018). These studies have demonstrated that this ratio of peroxide has been sufficient to essentially knock out all organic INPs. Perhaps the archaea organic INPs are unique and some may be embedded within the cells, making it difficult for the peroxide to reach and thus degrade. We hope that any future investigations of archaeal INPs would include a more rigorous peroxide treatment regimen, akin to that of the recent Bogler et al. (2020) study on lignin biopolymer. We added a statement on our recommendation after this sentence in the revision and included a statement with references on the basis for choosing the amount of peroxide used.

5. There is no statistical evidence presented that any of the organisms/treatments/controls were different albeit 24 replicates.

This is incorrect. A difference in fraction frozen of ≥ 0.25 results in statistically significant differences according to Fisher's Exact test ($p < 0.0479$). That is more than clear for all the spectra in Figure 1. See response below for "Line 174-" for explanation regarding statistical significance of the differences between the treated and unamended spectra.

Again, we did not conduct comparisons between different dilutions or treatments for the four species. It is clear from Figure 1 and the corresponding text, when we do show the species under the same testing conditions, that there is a notable difference in the two intact versus two lysed species, especially when comparing to the DI water and media controls for each.

As intact cells for H. walsbyi and N. pharaonic were not investigated and lysed cells did not show ice nucleation activity, they do not contribute to the study and should be removed.

We disagree. No results are still results and warrant reporting in the literature; removal would simply be cherry picking results. See responses above regarding the inherent effects of dilutions and lysing for these two species.

Methodology: L116: It is confusing and unclear why it is relevant the cells were first grown at the College of Charleston and then shipped to Colorado State University.

We agree this is irrelevant and ended up removing the information on institutions where the cultures were grown and shipped.

L116: Unclear why cells were first grown to mid-log phase but subsequently to somewhere during log-phase.

They were not future grown after reaching mid-log phase. We now clarify that we tested the cultures as soon as possible after they reached this stage at the end of section 2.1: ". All cultures were grown at 37 ˚C and 100 rpm until mid-log phase (i.e., midway through the period of exponential cell growth). The purity and cell density were monitored optically at 1000× magnification throughout. Table 1 provides the cell concentrations and salinities of all four prepared cultures. Cultures were measured for their ice nucleation abilities within 1 – 2 days upon achieving log-phase growth (i.e., the period characterized by cell doubling)."

L117: Please provide more detail no monitoring cell density. What microscope, cell counts, add reference on microscopy-cell abundance procedure. Also, the objective was most likely a 100 x with an extra 10x magnification within the eyepiece or camera. Unclear why cell density would be of relevance prior to shipping?

Cell densities were obtained prior to shipping to ensure that the cultures were in mid-log phase. Also, to confirm that no appreciable growth had occurred between shipping and sample analysis.

The cells were counted using a Leica DM750 microscope (https://us.leica-camera.com/) at 1000x magnification with a 100x oil immersion objective lens. Cells were counted on a Petroff-Hausser 3900 counting chamber (http://hausserscientific.com/). This information is now included in section 2.1.

L117: reference to Table 1: cells were shipped to a different university and additional experiment where performed at this university I assume. Cell numbers always remained constant during shipping, storage, and time passed until experimental setup?

We monitored the cell numbers throughout, including once they arrived in Colorado immediately prior to the ice nucleation measurements, which we now provide more detail on in section 2.1. The numbers reported in Table 1 are from the most recent count.

Table 1: Is salinity presented as gram NaCl? I am asking because e.g. DSMZ media 97 contains 250 g NaCl. Should this be 250 ppt and 25%.

Thank you for bringing this to our attention. This culture of H. morrhuae was grown with only 150 g of NaCl. The alteration to media 97 is now included in the methods.

L127: If the current study truly determined the lysis thresholds, why did the authors not in include dilutions for H. walsbyi and N. pharaonic. Cells were diluted 1:15 one time not serially. Dilution resulted in the lysis of two out of four selected organisms. Reason why not including lower dilutions that left cells intact as well as including dilutions for the other two organisms that would have resulted in cell lysis is unclear; particularly since the authors determined lysis thresholds.

See response to comment 1.

L128: Why were cells grown again if they already reached the desired cell density prior to shipping (L117)?

The cells were not grown again. To prevent confusion, we deleted "active log-phase".

Ll29: What was the reason for selecting two more dilutions for H. morrhuae but not the other three organisms? It is well known that cell density has an effect on ice nucleation.

The goal was to test the most active of the four species, which we now clarify at the beginning of section 3.2: "Dilutions were only applied to H. morrhuae given its relatively high ice nucleating ability compared to the controls and the other haloarchaea tested."

On the contrary, according to our results in Figure 2, dilutions and thus cell density did not influence ice nucleation (i.e., the unamended spectra are roughly the same between the three dilutions.

L130: add detail on microscopy.

Done.

L138: what was the coolant?

Syltherm XLT. Now added to section 2.3.

L142-148: (i) Heat treatment and 30% H2O2 amendments were intended to determine the effect lysed cell material and inorganic molecules on ice nucleation. If the authors think that the digestion was ineffective, they should have altered the protocol rather than hinting at the need for it. (ii) a general problem throughout the experimental design is the comparison of different cell 'material' i.e., intact, lysed, intact/digested, lysed/digested. It appears that the initial intact vs. lysed was an artifact and the authors went with it, but essentially, the study compares apples and oranges.

(i) This is an inaccurate statement. Treatments were conducted on all four species, intact and lysed. We also did not think the digestion was ineffective, rather was only partially effective for only one species (H. morrhuae). See response to comment 4 above. (ii) See response to comment 1 above. The last statement in this comment is a crude speculation.

Results and discussion: As the results and discussion section will largely change after removing a large portion of the dataset here are some general comments. When did controls freeze? H. morrhuae is atypical compared to all Archaea or the ones investigated?

We are not removing a "large portion of the dataset" as contended in our responses above. Also, the controls are already provided in all the figures except for the 1:6 and 1:30 dilutions of the H. morrhuae media control, which we did not dilute further given the 1:15 dilution did not exhibit any decreases in freezing with the treatments. This makes sense given media 97 is 90% salts by weight, which is diluted down in 1000 mL of water and then further diluted to 1:15 here.

We do realize without reading the captions, it may not have been clear the media controls were already included, thus we revised to include more detailed legends in the figures.

L167-172: Irrelevant as lyses cells are compared to intact cells. This is a study on INPs. No sure why it is 'interesting or relevant' to discuss cell lyses. As mentioned before low dilutions should have been used to not lyse cells.

We disagree. See response to comment 1 above.

L176-175: H2O2 treatment should provide information in the abiotic fraction not organic.

The residual remaining is the abiotic fraction, yes, but the INPs removed are the organic INPs. Peroxide treatments (in addition to heat) remove stable organics and afford the contribution from inorganic materials or biological materials resistant to these treatments (Barry et al., 2021; Creamean et al., 2020; Hill et al., 2016; McCluskey et al., 2018; Perkins et al., 2020a; Suski et al., 2018; Tobo et al., 2014).

L174-: Are any of these reported changes in freezing temperatures statistically different from intact cells?

It is not clear what the reviewer is referring to here—H. morrhuae dilutions are all intact cells since it does not lyse in fresh water. For the differences in unamended to heat to peroxide, most of these differences are statistically significant according to Fisher's Exact test except for some of the heat treatment data for the 1:15 dilution (i.e., when approaching fraction frozen of 0 or 1 where there is less of a difference in temperature). A decrease in fraction frozen of ≥ 0.25 results in statistically significant differences ($p < 0.0479$). Certainly, all the peroxide spectra have a much higher decrease from the unamended spectra and thus are statistically significant differences. The statistical significance for each of the treated compared to unamended spectra is not discussed in section 3.2.

L183-186: Discussion on ineffective H2O2 treatment. Effectively separating or removing specific fractions when investigating ice nucleation properties is essential. This section does not strengthen the manuscript.

We disagree. See response to comment 4 above.

L192-193: Why is N. pharaonis now only partially lysed contradicting previous statements?

Thank you for bringing this to our attention, this was an error and in reference to a different dilution threshold. The text has been corrected.

Conclusion: L209-213: seems more suited for an introduction.

We do have this information in the introduction. It is reiterated here for emphasis on possible broader implications.

Figure 1: Please remove the shading. The reader gets the impression that the area under the curve is of importance. Why on a log scale?

Done. We also changed to linear scale.

Figure 2: Why are there no controls for the dilutions 1:6 and 1:30? As shown in Table 2 the salinity ranges from 2.7%-0.5% in these samples.

See response to the first general results and discussion comment above.

Other edits: Check for missing punctuations

Done.

L10: delete 'from microorganisms'

Done.

L15-16: change ‘of a subset of archaeal cells from Haloarchaea’ to ‘selected genera of the class Haloarchaea’

Done.

L16-18. Reason for comparing intact cells two lysed cells from different genera is unclear.

See response to comment 1 above.

L17: without comparing to the freezing temperature of an abiotic control, I would not consider -18C warm. Please rephrase.

As indicated above, the controls were included. However, given –18 ˚C is not warm on a broader INP scale, we changed this to “at temperatures up to –18 ˚C”.

L18: What are warm temperature INPs? Please rephrase.

We changed “warm temperature” to “immersion”.

L23: ‘necessary to improve’. These are extremely strong words. How about ‘intriguing’

We instead rephrased to, “Thus, it is important to assess their ability to serve as INPs as it may lead to an improved understanding of biological impacts on clouds.”

L40: delete ‘approximately’

Done.

L54: replace ‘however’ with ‘further’

Done.

L57-61: please split this running sentence.

Done. Changed to, “Even though many laboratory and field-based investigations have alluded to the importance of biologically-derived INPs, the relatively limited available observational data have caused models to produce equivocal results regarding the global significance of biological ice nucleation in cloud and precipitation formation (Burrows et al., 2013; Hoose et al., 2010b; Hummel et al., 2018; Phillips et al., 2009; Sesartic et al., 2012; Twohy et al., 2016; Vergara-Temprado et al., 2017). This modelling issue is further complicated by a very limited understanding and representation of secondary ice formation processes and their links to biologically-derived INPs in clouds.”

L64: Do all minerals except for feldspars function as INPs? I suggest deleting ‘aside some feldspars’

Good question. We have omitted “aside some feldspars”.

L71: delete ‘relatively’

Done.

L82-83: Are there some bacterial cell that produce no peptidoglycan as the authors say ‘nearly ubiquitous’?

For an example, the mycoplasma and some other pathogens lack a cell wall entirely.

L93: ‘possess’

Fixed.

L96: replace ‘it is’ with ‘its’

Done.

L97: For the other three genera cell characteristics were briefly described as a justification to include them in this study. Why not for Natronomonas?

To our knowledge, there is no information in the literature on the cell wall structure for Natronomonas, which is why the description is limited.

L100: change to ‘they are relatively easy to culture compared to other archaeal lineages’

Done.

L105: What do the authors mean by ‘halophiles and hypersaline’

Typo. We removed “and hypersaline”.

L106-109: I recommend removing this paragraph. This paragraph is trying to oversell the importance of this research.

This is the opinion of one reviewer, which we disagree with. Demonstrating broader significance is an important part of any manuscript.

L116: delete ‘(i.e., midway through the period of exponential cell growth)’

Done.

L118: delete ‘(i.e., the period characterized by cell doubling)’

Done.

L141: delete ‘for all species during each experiment.’

We deleted “during each experiment” since we did run controls for all aside from the 1:6 and 1:30 dilutions for the reasons provided above.

Evaluating the potential for Archaea to serve as ice nucleating particles

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Abstract. Aerosols play a crucial role in cloud formation. Biologically-derived materials from ~~microorganisms such as~~ plant bacteria, fungi, pollen, and various vegetative detritus can serve as ice nucleating particles (INPs), some of which initiate glaciation in clouds at relatively warm freezing temperatures. However, determining the magnitude of the interactions between clouds and biologically-derived INPs remains a significant challenge due to the diversity and complexity of bioaerosols, and limited observations of such aerosols to facilitate cloud ice formation. Additionally, microorganisms from the domain Archaea have to date not been evaluated as INPs. Here, we present the first results reporting the ice nucleation activity of ~~a subset of archaeal~~selected genera of the class ~~cells from~~ Haloarchaea. Intact cells of *Halococcus morrhuae* and *Haloferax sulfurifontis* demonstrated the ability to induce immersion freezing at temperatures up to~~as warm as~~ –18 ˚C, while lysed cells of *Haloquadratum walsbyi* and *Natronomonas pharaonis* were unable to serve as immersion ~~warm temperature~~ INPs. Exposure to heat and peroxide digestion indicated that the INPs of intact cells were driven by organic (*H. morrhuae* and *H. sulfurifontis*) and possibly also heat-labile materials (*H. sulfurifontis* only). While halophiles are prominent in hypersaline environments such as the Great Salt Lake and the Dead Sea, other members of the Archaea, such as methanogens and thermophiles, are prevalent in anoxic systems in seawater, sea ice, marine sediments, glacial ice, permafrost, and other cold niches. Archaeal extremophiles are both diverse and highly abundant. Thus, it is important to assess~~assessing~~ their ability to serve as INPs ~~to become airborne, and their abilities to impact cloud formation,~~ as it may lead to an ~~is necessary to~~ improved understanding of biological impacts on clouds.

1 Introduction

Through their impact on the atmospheric energy budget and hydrological cycle, clouds play a prominent role in shaping Earth's climate at both global and regional scales (Baker and Peter, 2008; Boucher et al., 2013). However, due to the complexity of the microphysical processes associated with cloud formation and dynamics, clouds remain some of the most poorly constrained atmospheric features in climate models. In turn, while atmospheric aerosols strongly impact cloud formation, albedo, lifetime, and precipitation formation processes, disentangling the relationships and feedbacks among aerosols, clouds, and precipitation

remains a significant challenge (Sato and Suzuki, 2019; Stevens and Feingold, 2009). In particular, the role of aerosols called ice nucleating particles (INPs), that induce glaciation in mixed-phase and ice clouds, is highly uncertain relative to other aerosol-cloud processes (Kanji et al., 2017). Improving understanding of such ice formation processes is crucial given most precipitation, globally, is initiated via the ice phase (Lohmann and Feichter, 2005).

In the Earth's troposphere, pure water remains in a supercooled liquid state until below –38 ˚C. At temperatures greater than –38 ˚C, the assistance of INPs such as mineral dust, volcanic ash, and select biologically-produced macromolecules (e.g., from pollen, fungi, bacteria, and other sources) is required to initiate the heterogeneous formation of primary ice embryos, that continue to grow into larger ice crystals (Hoose and Möhler, 2012; Kanji et al., 2017). Depending on their surface properties and structural makeup, mineral dust and volcanic ash can raise the freezing point of water from –38 ˚C to as high as ~~approximately~~ –12 to –15 ˚C (Murray et al., 2012). At temperatures above –15 ˚C, and specifically in the immersion freezing regime (i.e., heterogeneous glaciation of a supercooled droplet), the majority of naturally-occurring INPs are biological in origin (Fröhlich-Nowoisky et al., 2016; Hoose and Möhler, 2012; Kanji et al., 2017; Morris et al., 2004). Intact and lysed cell wall fragments (Anderson and Ashworth, 1986; Du et al., 2017; O'Sullivan et al., 2015; Šantl-Temkiv et al., 2015), viable and nonviable cells (Anderson and Ashworth, 1986), and cellular byproduct materials such as exopolymeric substances, saccharides, and biosurfactants (Albers et al., 2017; Decho and Gutierrez, 2017; Demott et al., 2018; Dreischmeier et al., 2017; Perkins et al., 2020b; Zeppenfeld et al., 2019) have all been demonstrated to serve as biologically-derived INPs (Després et al., 2012; Fröhlich-Nowoisky et al., 2016). The most thoroughly-studied biologically-derived INPs of measurable abundance are strains of the bacterium *Pseudomonas syringae*, which is capable of forming ice at temperatures as high as –1 ˚C (Maki et al., 1974; Morris et al., 2008). The ability of ~~*Pseudomonas*~~ *P. syringae* to effectively facilitate ice formation is due to a specific ice nucleating protein (i.e., a protein that is ice nucleation active, designated Ina), that coordinates the crystallization of ice (Cochet and Widehem, 2000; Davies, 2014; Failor et al., 2017; Gurian-Sherman and Lindow, 1993; Hartmann et al., 2013; Šantl-Temkiv et al., 2015). The Ina protein is found in select lineages of bacteria all within the class Gammaproteobacteria (Warren, 1995). There is some evidence that slightly less effective, but highly abundant proteinaceous ice nucleating proteins can be found within fungi, but they remain to be fully categorized (Fröhlich-Nowoisky et al., 2015; Kunert et al., 2019). ~~However~~Further, a recent study identifying the first Gram positive ice nucleating bacterium from cloud precipitation suggests that ice nucleating proteins are far more widespread within the bacterial domain than previously thought (Failor et al., 2017).

Even though many laboratory and field-based investigations have alluded to the importance of biologically-derived INPs, the relatively limited available observational data~~,~~ ~~have~~have caused models to produce equivocal results regarding the global significance of biological ice nucleation in cloud and precipitation formation (Burrows et al., 2013; Hoose et al., 2010b; Hummel et al., 2018; Phillips et al., 2009; Sesartic et al., 2012; Twohy et al., 2016; Vergara-Temprado et al., 2017). This modelling issue is further complicated by a ~~and a v~~very limited understanding and representation of secondary ice formation processes and their links to biologically-derived INPs in clouds.~~,~~ ~~have caused models to produce equivocal results regarding~~

the global significance of biological ice nucleation in cloud and precipitation formation (!!! INVALID CITATION !!! (Burrows et al., 2013; Hoose et al., 2010b; Hummel et al., 2018; Phillips et al., 2009; Sesartic et al., 2012; Twohy et al., 2016; Vergara-Temprado et al., 2017)). Climate models of all scales require information on INP sources to accurately represent ice nucleation and thus cloud microphysics, especially considering: (1) biologically-derived INPs form ice at cloud temperatures as high as –1 ˚C while certain mineral dusts , aside some feldspars, glaciate modestly starting at –12 ˚C and (2) biologically-derived and mineral INP concentrations can vary by several orders of magnitude at any given temperature (Burrows et al., 2013; Demott et al., 2016; Hoose et al., 2010a; Hoose et al., 2010c; Kanji et al., 2017; Mccluskey et al., 2019; Petters and Wright, 2015; Vergara-Temprado et al., 2017).

While there have been ongoing efforts to identify and characterize INPs in the eukaryotic and bacterial domains (Dreischmeier et al., 2017; Failor et al., 2017; Fröhlich-Nowoisky et al., 2016; Hill et al., 2014; Kanji et al., 2017; Kunert et al., 2019; Ling et al., 2018; Morris et al., 2008; Pummer et al., 2012; Qiu et al., 2019), to date, there has been no study that attempts to identify INPs within the domain Archaea. This is in no small part due the fact that, until relatively recently, the Archaea were believed to be largely relegated to marginal existences in extreme environments on our planet. Though recent studies have begun to reveal the true ubiquity and abundance of Archaea in Earth's ecosystems—including seawater, ocean sediment, plankton, soil, marine and terrestrial biofilms, and sea ice, where they can comprise up to 40% of the microbial taxa in an ecosystem up to 40% of the microbial ecosystem (Cavicchioli, 2011; Flemming and Wuertz, 2019; Hoshino and Inagaki, 2019; Junge et al., 2004; Mondav et al., 2014; Munson et al., 1997; Ochsenreiter et al., 2002; Santoro et al., 2019)—many Archaea remain uncultured and unculturable in laboratory settings, further complicating investigations into their possible propensities to serve as INPs. At the same time, however, the archaeal domain contains both unique cell wall compositions and cell surface structures not present in the other domains (Albers and Meyer, 2011). While appearing highly similar to the bacterial domain in both size and appearance, the archaeal cell envelope is distinct in several ways. In contrast to the Bacteria, most cultured Archaea maintain a proteinaceous surface layer, or S-layer, that provides the cell with structural stability. In many Archaea, the S-layers provide the entirety of the cell envelope. Comparatively few Archaea contain additional cell envelope polymers, and the ones that do, do not produce the near ubiquitous bacterial polymer peptidoglycan. Some Archaea do produce a structurally similar polymer, pseudomurein. The archaeal S-layers are often composed of a single protein or glycoprotein arranged in symmetrical patterns, with hexagonal symmetry being the most common (Albers and Meyer, 2011). As with the Bacteria, many surface exposed proteins are modified in a variety of ways. In addition to the S-layer itself, the archaeal domain contains its own regimen of surface proteins and structures that interact with the external environment. Thus, an entire domain worth of cell surface structures remains to be assessed for potential INP activity.

Here we present a first attempt to assess the potential for members of the domain Archaea to serve as INPs. We initiated our investigation with four members of the obligate halophilic lineage, the Haloarchaea: *Halococcus morrhuae, Haloferax sulfurifontis, Haloquadratum walsbyi,* and *Natronomonas pharaonis*. Together, the four Haloarchaea represented a variety of

cell surface designs. Halococcus is one of a limited number of genera belonging to the Archaea that does not possess an S-layer. Instead they possess~~es~~ a cell envelope that includes highly sulphated heteropolysaccharides (Schleifer et al., 1982). They can be several microns in size, exhibit a cocci (i.e., spheroidal) morphology, and do not lyse in fresh water (Legat et al., 2010; Leuko et al., 2004). *Haloferax* possesses ~~exclusively~~ a exclusively sulphur-rich S-layer and exists as rod- or irregular-shaped cells several microns in size (Elshahed et al., 2004). *Haloquadratum* can produce halomucin in addition to its S-layer and grows in its~~-is~~ trademark square morphology, a couple microns in size (Burns et al., 2007). *Natronomonas* exist as rods several microns in length and prefer alkaline hypersaline environments (Falb et al., 2005). Specifically, *H. walsbyi* and *N. pharaonis* are particularly sensitive to lysis under hyposaline conditions (Boring et al., 1963)~~—~~they readily lyse in salinities of roughly 5% salt by weight and below.

We opted to initiate our investigation with members of the Haloarchaea for a variety of reasons, including: (1) the diversity of cell surface compositions, (2) they are relatively easy to culture compared to other archaeal lineages~~the comparative ease of culture~~, and (3) the regional dominance of the Haloarchaea in relatively large hypersaline environments. While few largescale geographic areas are dominated by members of the domain Archaea, one notable exception is hypersaline bodies of water such as the north basin of the Great Salt Lake and the Dead Sea. These waterbodies extend over hundreds of square kilometres, often contain upwards of 90% Archaea, and can impact the local climate and weather patterns (Carpenter, 1993). Thus, when investigating the impact of potential archaeal INPs in an environment, halophiles ~~and hypersaline~~ offer attractive starting points.

To fully understand the interaction of Earth's climate with the microbial world, it is imperative to include the impact of the archaeal domain, since even when less prevalent, the possession of ice nucleation activity will enable a species to exert an outsized effect on its environs. And to fully understand the potential impact of the archaeal domain on Earth's climate, it is important to assess the potential for members of the Archaea to serve as INPs and contribute to cloud formation.

2 Materials and methods

2.1 Cell cultures

Cell cultures ~~Saline media~~ for all four haloarchaeal species tested were purchased from the DSMZ-German Collection of Microorganisms and Cell Cultures (~~https://www.dsmz.de/~~https://www.dsmz.de/) and the associated saline medias were prepared for~~,~~ *H. morrhuae* (medium 97), *H. sulfurifontis* (medium 1018), *H. walsbyi* (medium 1091), and *N. pharaonis* (medium 371) ~~were prepared according to the recipes provided by the DSMZ-German Collection of Microorganisms and Cell Cultures (https://www.dsmz.de/)~~ with the following alterations: (1) for medium 97 only 150 g of NaCl was added instead of 250 g NaCl for growth of *H. morrhuae* and (2) one gram per litre of glycerol was supplemented to medium 1091 for growth of *H. walsbyi*. The salinities of the media were confirmed using a handheld refractometer. All cultures were grown at 37 °C

and 100 rpm until mid-log phase (i.e., midway through the period of exponential cell growth). at the College of Charleston and tThe purity and cell density were monitored optically at using a Leica DM750 microscope (https://us.leica-camera.com/https://us.leica-camera.com/) at 1000x magnification with a 100x oil immersion objective lens. Cells were counted on a Petroff-Hausser 3900 counting chamber (http://hausserscientific.com/). 1000× magnification throughout. Table 1 provides the cell concentrations and salinities of all four prepared cultures. Cultures were measured for their ice nucleation abilities within 1 – 2 days upon Upon achieving log-phase growth (i.e., the period characterized by cell doubling), cultures were shipped overnight to the Colorado State University (CSU) on ice.

2.2 Preparation of samples for ice nucleation measurements

Due to the high salinities of the media —which would have caused significant freezing point depression— (for reference, seawater is typically 30 – 35 ppt (Bodnar, 1993) and can depress the freezing point of water by ≥ 2 °C (Irish et al., 2019; Schnell and Vali, 1975)—), samples were diluted as to reduced salinities shown in Table 2. This reductionReductions in salinity also can inherently caused cause some the cells of certain of the haloarchaeal speciescells to lyse (Boring et al., 1963; Legat et al., 2010; Leuko et al., 2004). Testing ice nucleation responses from cell lysis is relevant given: (1) cell fragments from fungi and bacteria have been previously observed to serve as INPs (Anderson and Ashworth, 1986; Du et al., 2017; O′Sullivan et al., 2015; Šantl-Temkiv et al., 2015) and (2) a less saline environment is more atmospherically relevant for how archaea might behave once incorporated in a relatively dilute cloud drop prior to immersion freezingexposed to atmospheric water vapor in the aerosol phase. Cell lysis thresholds were determined by serially diluting cultures with deionized water as shown in Table 2 and microscopically assessing cellular survival. Samples of activemetabolically active, log phase cultures were diluted 1:15 with deionized water to result in 1.1 – 1.4% salinity. This dilution was chosen based on tests using saline solution controls (made with Instant Ocean® Sea Salt) and solutions with *H. walsbyi* and *N. pharaonis* whereby, for example, freezing would not be observed until < –23 °C and then only reach ~ 0.2 fraction frozen at the instrument lower limit for a 10% salt solution. Thus, a 1:15 dilution was found to be a good compromise for preventing significant freezing point depression while still obtaining full spectra (i.e., reaching a fraction frozen of 1). Additional tests were conducted on *H. morrhuae* cultures diluted to 1:6 and 1:30 with cells remaining intact at all dilutions tested. Cultures were checked microscopically after dilution for cell density and to ascertain if cell lysis had occurred.

2.3 Immersion freezing ice nucleation experiments

INP concentrations were measured using the CSU Ice Spectrometer (IS) (Hiranuma et al., 2015; Mccluskey et al., 2018; Suski et al., 2018), which is an immersion freezing measurement device suited to testing aliquots of liquid culture. The IS is constructed using two 96-well aluminium incubation blocks, designed for incubating polymerase chain reaction (PCR) plates, placed end-to-end and encased on their sides and base by cold plates. Immersion freezing temperature spectra were obtained by dispensing 50-µL aliquots of cell suspensions into sterile, 96-well PCR trays in a laminar flow cabinet. Each sample consisted of 24 aliquots. PCR plates were then placed into the blocks of the IS, after which the device was cooled using

Sytherm XLT in a recirculating low temperature bath. ~~the device cooled at 0.33°C min^{-1} to approximately –29 °C using a recirculating low temperature bath, and the freezing of wells was recorded every 0.5 °C.~~ Frozen aliquots were detected with a CCD camera system controlled with LabVIEW (LabVIEW; NI, Inc.) as temperature was lowered at approximately 0.33 °C min^{-1} to down to –29 °C.~~.~~ The temperature uncertainty of the IS is less than ±0.2 C, which is a combination of the uncertainty in the thermocouples and the temperature variation across the blocks due to gradients in cooling. From the number of wells frozen at each temperature step, the fraction frozen is calculated. Confidence intervals (95%) were calculated based on the methodology of Agresti and Coull (1998). ~~Standard error was calculated for each spectrum.~~ Deionized water and uninoculated diluted media were run as controls for all species ~~during each experiment~~.

~~Thermal~~ Heat and ~~chemical~~ peroxide treatments were conducted to isolate heat-labile (e.g., proteinaceous) and organic INPs in the diluted samples (Barry et al., 2021; Creamean et al., 2020; Hill et al., 2016; Mccluskey et al., 2018; Perkins et al., 2020a; Suski et al., 2018; Tobo et al., 2014). The stability (or lack thereof) of INPs to these treatments provides an indication of composition. To assess the contribution of heat-labile entities, a 1.5-mL aliquot of suspension was tested after heating to 95 °C for 20 min. To remove all organic INPs, 0.75 mL of 30% H_2O_2 was added to another 1.5-mL aliquot of suspension and the mixture heated to 95 °C for 20 min while illuminated with UVA/UVB fluorescent bulbs (Exo Terra Reptile UVB, 2 × 26 W providing ~2,000 µW cm^{-2} UVA and ~300 µW cm^{-2} UVB at the distance used) to generate hydroxyl radicals (residual H_2O_2 ~~is~~was removed using catalase ~~(Mccluskey et al., 2018)~~), ~~and the sample retested~~ prior to testing~~in the IS~~. Both heat and peroxide treated samples were tested simultaneous to the unamended samples in the IS for each species, since the CSU IS can house up to four 96-well plates at one time. Remaining INPs ~~were~~ are possibly aggregates of cellular material that ~~were~~ are not fully digested, inorganic INPs in the media, or other biological materials resistant to heat and peroxide digestion (Conen et al., 2011; Perkins et al., 2020a).

3 Results and discussion

3.1 Interspecies comparison of haloarchaeal ice nucleation abilities

Figure 1 shows the results of the 1:15 dilutions of each of the haloarchaeal species. The media for all four species contributed modestly to background freezing as compared to deionized water controls. *H. morrhuae* performed best as an INP, initiating the freezing of water at temperatures as high as –17.6 ˚C. *H. sulfurifontis* also demonstrated enhanced capability to serve as an INP, freezing water at temperatures above that of deionized water and sterile media, with a freezing onset of –19.2 ˚C. These haloarchaea are not as proficient ice nucleators as other more commonly-studied biologically-derived entities such as certain bacteria (up to –1.3 ˚C) (Kim et al., 1987; Lindow et al., 1989; Maki et al., 1974; Vali et al., 1976), fungi (up to –1 ˚C) (Kunert et al., 2019; Richard et al., 1996), lichen (up to –2 ˚C) (Kieft, 1988; Kieft and Ruscetti, 1990), and pollen (up to –8 to –12 ˚C) (Hader et al., 2014; Pummer et al., 2011). The haloarchaeal species fall closer in line with less effective biologically-derived INPs such as fungal spores (onset freezing temperatures reported up to –10 ˚C, but typically initiate freezing < –25 ˚C) (Iannone

et al., 2011; Jayaweera and Flanagan, 1982) and ~~phytoplankton~~ diatoms (observed up to ~~–3 °C but typically <~~ –2~~0~~ °C) (Knopf et al., 2011). *H. morrhuae* cells were also more effective at nucleating ice even though total cell concentrations were three times higher for *H. sulfurifontis* (Table 2). One possible explanation is that *H. morrhuae* is unusual among the Archaea in that it has a cell envelop composed of polysaccharides, which have been shown to serve as ~~markers~~ tracers for ice nucleating activity (Zeppenfeld et al., 2019). In general, although not "warm temperature" INPs (i.e., that glaciate > –15 °C), these haloarchaea are comparable in ice nucleation activity to fungal spores and ~~phytoplankton~~ diatoms and can thus contribute to INP populations at very relevant atmospheric freezing temperatures.

As ~~expected~~expected, ice nucleation activity did not occur in all tested haloarchaeal species, just as with bacteria (Karimi et al., 2020; Szyrmer and Zawadzki, 1997). ~~Both~~ Lysed cells of both *H. walsbyi* and *N. pharaonis* did not exhibit ice nucleation activity ~~demonstrated no ability to serve as INPs (i.e.,~~as the fractions frozen were not higher than the media blanks~~)~~. Interestingly, the dilution of the log-phase cultures to 1:15 with deionized water resulted in the complete lysis of *H. walsbyi* and ~~the partial lysis of~~ *N. pharaonis* as opposed to *H. morrhuae* and *H. sulfurifontis,* which both remained intact. Testing ice nucleation responses from cell lysis is relevant given: (1) cell fragments from fungi and bacteria have been previously observed to serve as INPs (Anderson and Ashworth, 1986; Du et al., 2017; O'Sullivan et al., 2015; Šantl-Temkiv et al., 2015) and (2) a less saline environment is more atmospherically relevant for how haloarchaea might behave once aerosolized and exposed to atmospheric water. However, the results presented here ~~These results~~ indicate that of the species studied, lysed haloarchaeal cells (i.e., cell fragments) do not enhance ice nucleation abilities, and possibly even suppress it. This is analogous to previous work on bacteria, whereby it is well known that lysing of ice nucleation active bacterial cells decreases the efficiency at which they are INPs (e.g., Lindow et al., 1989).

3.2 Response of haloarchaeal species to heat and peroxide treatments

Each sample (i.e., all dilutions listed in Table 2) was subject to ~~thermal~~ heat and peroxide treatments to obtain the heat-labile (proteinaceous) and organic frozen fractions in addition to the total (unamended) frozen fractions. Figure 2 shows these spectra for the 1:6, 1:15, and 1:30 dilutions of *H. morrhuae*. Dilutions were only applied to *H. morrhuae* given its relatively high ice nucleating ability compared to the controls and the other haloarchaea tested. The ice nucleating activity of all three dilutions was reduced by heat by 0.3 – 0.9 °C on average (i.e., when calculating the average freezing temperature per spectrum and subtracting from average freezing temperature of the unamended INP spectrum, per dilution), ~~to the same degree (~0.5 °C) and~~but especially reduced by peroxide (i.e., by 1.5 – 4.2 °C on average). These differences are statistically significant for decreases in fraction frozen of 0.25 or more at similar temperatures when applying Fisher's Exact test ($p < 0.0479$), which was the case for all data except for the first three and last data points for the 1:15 *H. morrhuae* unamended and heat spectra (Figure 2b). These results indicate ~~, indicating~~ the samples contained some heat-labile, likely proteinaceous, INPs, but contained a relatively larger contribution from~~a more substantial amount of some~~ other biogenic organic INPs (Conen et al., 2011; Hill et al., 2016; Mccluskey et al., 2018). Interestingly, increasing the dilution, such that the sample contained less culture to deionized

water, led to a larger decrease in fraction frozen from the peroxide treatments. For the 1:6 dilution, frozen fractions dropped 0.9 ˚C and 1.5 ~~up to 1.3 ˚C and 1.9~~ ˚C on average for the heat and peroxide treatments, respectively. For the largest decrease, the 1:30 dilution dropped by ~~up to 3.1~~0.7 ˚C and 4.2~~4.2~~ ˚C on average for heat and peroxide treatments, respectively. One conceivable explanation for the increased efficacy with increasing dilution is that the peroxide~~—(~~the same volume was used in each sample, based on successful peroxide sample degradation reported in previous studies (e.g., Barry et al., 2021; Creamean et al., 2020; Suski et al., 2018)~~—)~~ remained in higher concentration to digest a lower concentration of cells; thus, less residual organic material was available to serve as INPs. It is possible that higher volumes of peroxide would be needed for higher cell concentrations to eliminate all organic material, depending on the location of the organic material (i.e., if it is extra- or inter-cellular). A recent study demonstrated excess peroxide was required to effectively reduce INP concentrations to background levels for the lignin biopolymer (Bogler and Borduas-Dedekind, 2020). We recommend future work evaluating archaeal INPs should involve a more rigorous peroxide treatment regimen to test this hypothesis.

For the other three haloarchaeal species, only *H. sulfurifontis* exhibited a decrease in frozen fractions—fraction frozen dropped by ~~up to 3.4~~2.7 ˚C and 1.8~~4.1~~ ˚C on average for heat and peroxide, respectively (Figure 3). *H. walsbyi* and *N. pharaonis* showed no response to the treatments because there were essentially no INPs to begin with, thus these species are not discussed herein. For comparison between the *H. sulfurifontis* unamended and heat spectra, data > –22 °C and < –25 °C are the only data not statistically significant. For comparison between the unamended and peroxide spectra, only data < –26 °C are not statistically significant. These~~is~~ decrease~~s was~~ were mostly observed and statistically significant when the fraction frozen was approximately 0.2 to 0.8 (average decrease in freezing temperatures within this range was 3.8 ˚C and 2.3 ˚C, respectively), as opposed to *H. morrhuae*, where the difference in temperature from the unamended to the treated frozen fractions were roughly equivalent throughout the spectra. Interestingly, *H. sulfurifontis* ~~INP~~ spectra were more responsive to the heat treatment ~~as opposed to~~than to peroxide (i.e., exhibited a larger decrease in average freezing temperatures for heat than for peroxide), indicating this species contained more heat-labile, probably proteinaceous, INPs versus organic INPs~~—,~~ the opposite of *H. morrhuae*. ~~*H. walsbyi* (lysed) and *N. pharaonis* (partially lysed) showed no response to the treatments (not shown), because there were essentially no INPs in the original dilutions above the background INPs in the media.~~Collectively, these results indicate that *H. morrhuae* contained more organic relative to heat-labile INPs, while *H. sulfurifontis* contained more heat-labile as opposed to organic INPs. These haloarchaea have very different cellular envelop compositions: *H. sulfurifontis* contains a proteinaceous S-layer while *H. morrhuae* is devoid of such an S-layer but instead possessed a cell envelope that is composed of highly sulphated heteropolysaccharides. Thus, it would make sense that *H. sulfurifontis* is more sensitive to heat than peroxide given its proteinaceous cell envelope (assuming those proteins are ice nucleation active) and *H. morrhuae* is more sensitive to peroxide than heat given its polysaccharide-rich cell envelope.

4 Conclusions

Here, we present the first reported results on the ice nucleation activity of the domain Archaea. Specifically, we focus on four species within the Haloarchaea due to their diversity of cell surface compositions, ease of culturability, and regional presence within relatively large hypersaline environments. The freezing temperature ranges measured for the haloarchaea species involved in this study are put into broader context by comparing to reported ranges for other known biologically-derived INPs (Figure 4). While not the most proficient of biologically-derived INPs such as lichen, bacteria, fungi, viruses, algae, pollen, or pollen wash water, which nucleate ice at warmer temperatures, haloarchaea fall within moderate freezing temperature ranges above phytoplankton exudates, diatoms, and fungal spores. However, this work is based on a limited subset of species and future work should focus on other members of the Archaea.

~~Specifically, we focus on four species within the Haloarchaea due to their diversity of cell surface compositions, ease of culturability, and regional presence within relatively large hypersaline environments.~~ All haloarchaea species were introduced to hyposaline conditions to ~~: (1) negate~~ reduce freezing point depression ~~due to salinity and (2) more accurately reflect the expected environmental conditions of ice nucleation in clouds, as, in theory, aerosolized haloarchaeal cells followed by immersion in cloud drops would yield similar, low salinities~~. While intact cells of *H. morrhuae* and *H. sulfurifontis* demonstrated ability as INPs, inducing freezing up to –18 ˚C, lysed cells of *H. walsbyi* and *N. pharaonis* did not exhibit ice nucleation activity over the temperature range accessible in the tests. The intact cells that demonstrated ice nucleation activity contained both heat-stable organic ice nucleating entities (*H. morrhuae*) and heat-labile material (*H. sulfurifontis*). This may be negated at cloud level salinities where *H. sulfurifontis* would be expected to lyse as well. *H. morrhuae* on the other hand remained intact at all salinities. It is also rare among both the Haloarchaea, in particular, and the Archaea, as whole, in that its surface is completely devoid of a proteinaceous S-layer. ~~Other~~ We identified additional members of both the domain Archaea and the class Haloarchaea that have proteins with the ~~genetic~~ motif DUF3494 that is commonly associated ~~s~~ with~~indicative of~~ ice-binding surface proteins (Vance et al., 2019). Ice-binding ability is a characteristic of both ice nucleating and antifreeze proteins and is influenced primarily by their size (Eickhoff et al., 2019; Qiu et al., 2019).

Therefore, further work is needed to directly evaluate the surface properties to both disentangle which constituents are responsible for ice formation in the *Halococcus*, and ~~others~~ assess the ice nucleating potential within other members of the Haloarchaea and Archaea, in general.

While halophilic archaea are prominent in hypersaline environments throughout the globe, such as the Great Salt Lake and the Dead Sea, other members of the domain Archaea such as methanogens and thermophiles are prevalent in anoxic systems in seawater, sea ice, marine sediments, glacial ice, permafrost, hot springs, submarine hydrothermal vents, and hot, dry deserts (Amend and Shock, 2001; Collins et al., 2010; Oremland and Taylor, 1978; Price, 2007; Thauer et al., 2008; Thummes et al.,

2007; Van Der Maarel et al., 1999). However, some studies allude to the fact that these extremophiles are not confined to extreme living conditions, which qualifies them as one of the most abundant prokaryotes on Earth (Delong, 1998). Thus, these microorganisms are more ubiquitous than one might think, are present in the atmosphere (Fröhlich-Nowoisky et al., 2014), and may affect cloud formation (Amato et al., 2017). Indeed, the order Halobacteriales, which contains *H. morrhuae*, has been found to be present in continental air and was relatively abundant among the Archaea found in marine air (Fröhlich-Nowoisky et al., 2014). Further, Archaea accounted for several percent of all sequences in boundary layer air sampled from 45 – 50 °S over the Southern Ocean (Uetake et al., 2020). Future work should focus on characterizing a wide range of environmentally-relevant Archaea, such as methanogens (see Creamean et al., 2020) and ammonia oxidizers (see Fröhlich-Nowoisky et al., 2014), for their ice nucleating properties and address how they may be important for regional cloud formation, and hence weather and climate, as the impacts of the Archaea on climate processes is currently unknown.

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Table 1. Medium recipes, ~~initial~~ cell concentrations, and salinities for the four haloarchaeal species after culturing.

Haloarchaeal species	DSMZ medium recipe	Initial cell concentration (mL^{-1})	Salinity in ppt (%) **
Halococcus morrhuae	97	2.7×10^{9} *	160 (16.0%)
Haloferax sulfurifontis	1018	3.5×10^{9} *	177 (17.7%)
Haloquadratum walsbyi	1091	2.4×10^{8}	210 (21.0%)
Natronomonas pharaonis	371	1.1×10^{9} *	207 (20.7%)

* ~~Initial c~~Cell concentrations were too many to count (TMTC) optically, thus were estimated based on diluted samples, which were 1:15 in deionized water dilution for *H. morrhuae* and 1:6 for *H. sulfurifontis* and *N. pharaonic*.
** For salinity, "ppt" represents parts per thousand.

Table 2. Samples produced for all four haloarchaeal species. Diluted cell concentrations and salinities were calculated based on the volume of culture mixed with media and dilution factor. ~~For the cell concentrations, "n/a" = not applicable due to lysing.~~

Haloarchaeal species	Cell state	Dilution factor	Diluted cell conc (mL^{-1})	Diluted salinity in ppt (%)**
	intact	1:6	4.5×10^{8}	26.7 (2.7%)
Halococcus morrhuae	intact	1:15	1.8×10^{8}	10.7 (1.1%)
	intact	1:30	9.0×10^{7}	5.3 (0.5%)
Haloferax sulfurifontis	intact	1:15	5.8×10^{8}	11.8 (1.2%)
Haloquadratum walsbyi	lysed	1:15	n/a*	14.0 (1.4%)
Natronomonas pharaonis	lysed	1:15	n/a*	13.8 (1.4%)

* For the cell concentrations, "n/a" = not applicable due to lysing
** For salinity, "ppt" represents parts per thousand.

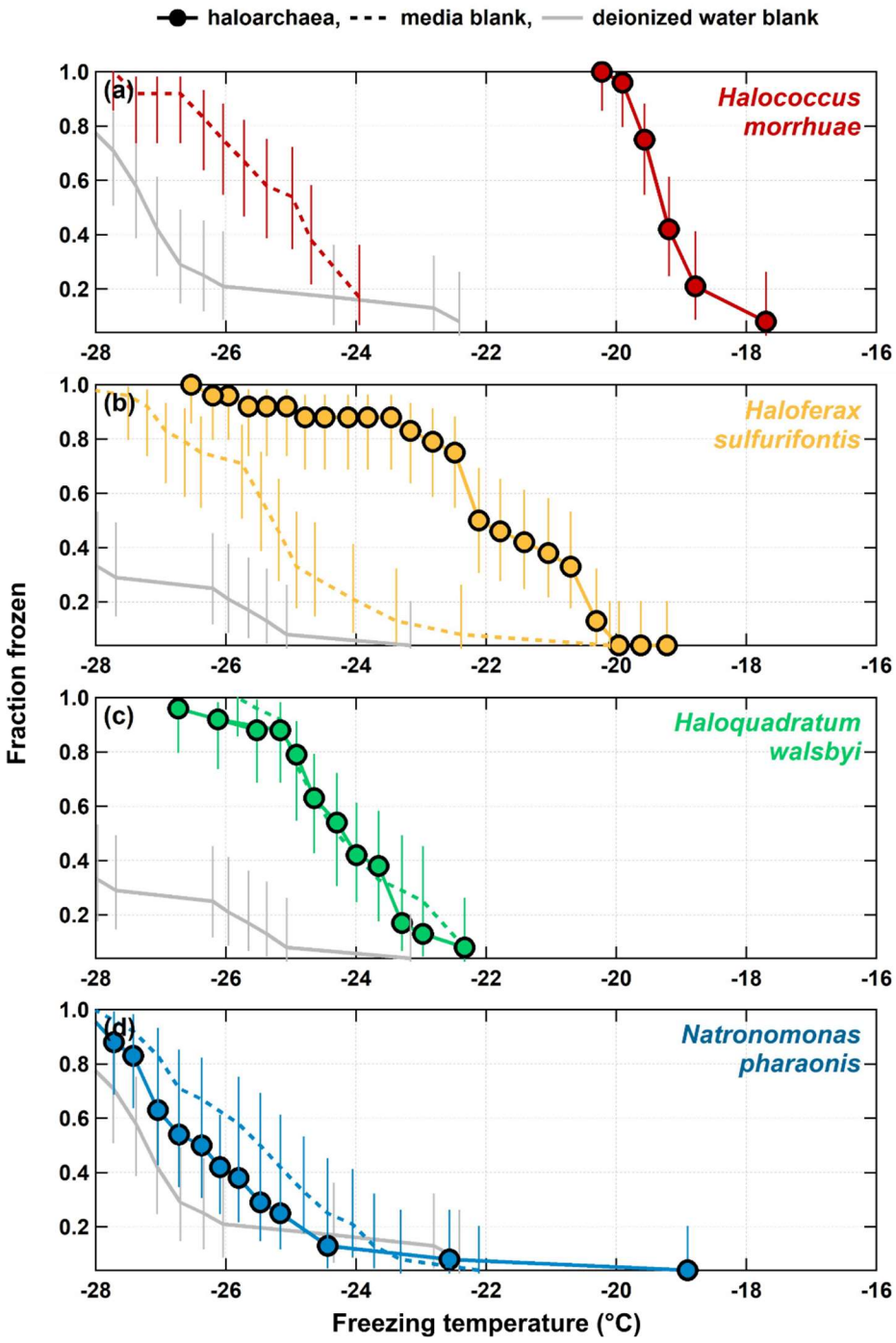


Figure 1. Freezing spectra for each of the haloarchaeal species diluted 1:15 in deionized water: (a) *H. morrhuae*, (b) *H. sulfurifontis*, (c) *H. walsbyi*, and (d) *N. pharaonis*. *H. morrhuae* and *H. sulfurifontis* did not lyse, *H. walsbyi* lysed, and *N. pharaonis* partially lysed. Note that *H. walsbyi* and *N. pharaonis* do not reach a frozen fraction of 1 because not all drops were frozen at the lower limit of the IS tests. ~~Dashed lines indicate the diluted sterile media controls and grey shading represents the deionized water blanks processed for each species.~~ Error bars indicate ~~standard error~~95% confidence intervals.

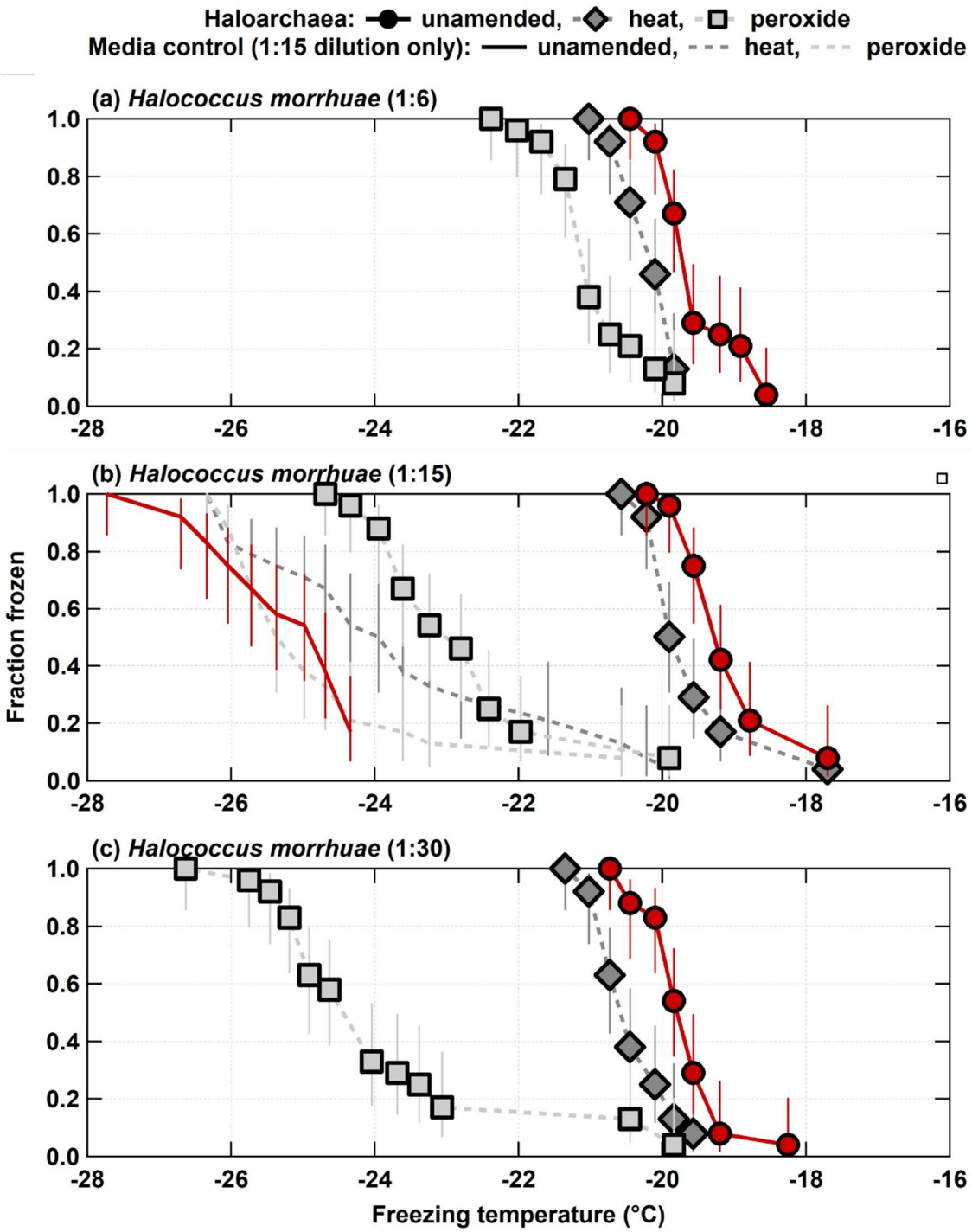
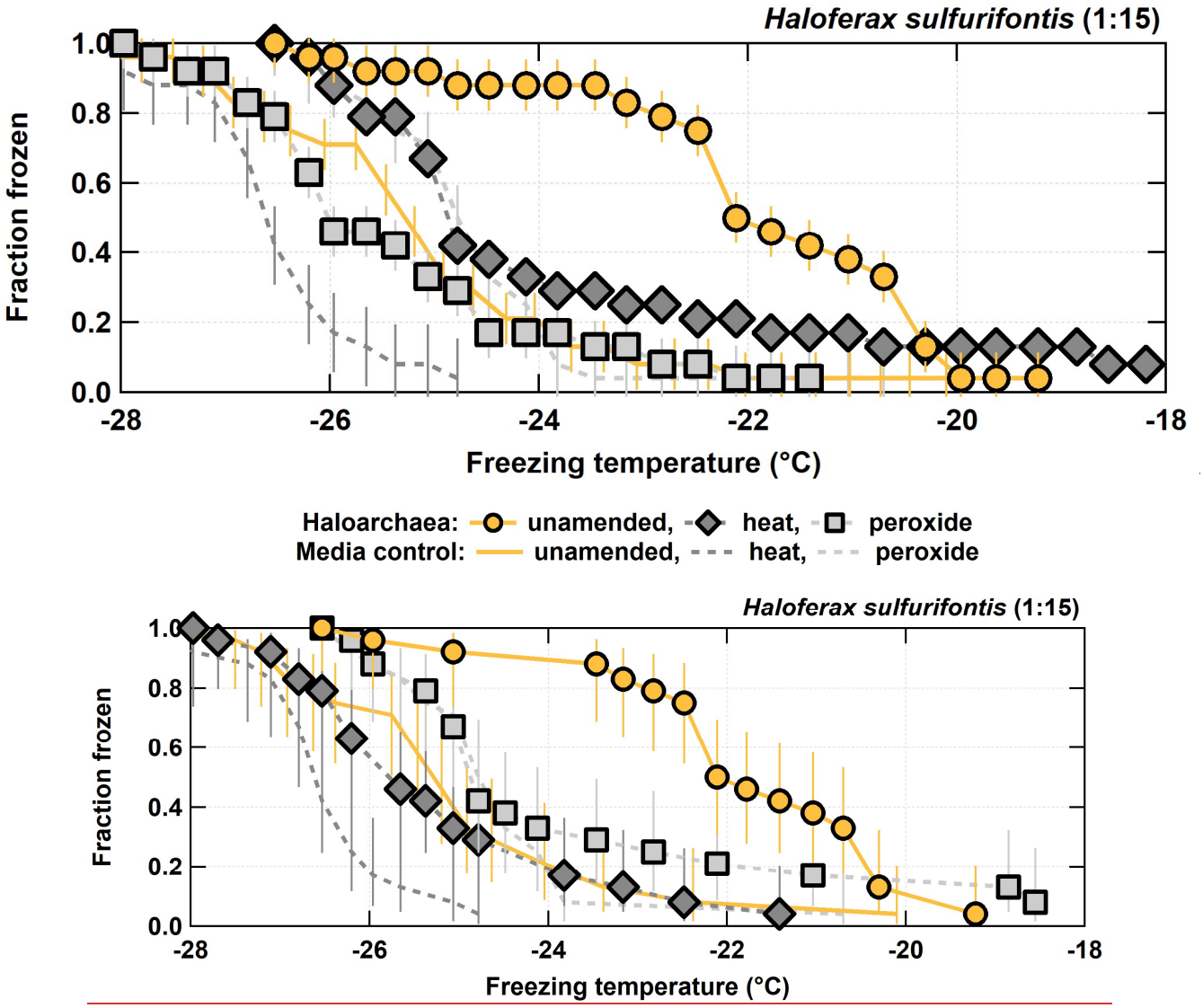


Figure 2. Unamended, heat-labile (heat), and organic (~~H_2O_2~~peroxide) frozen fractions for each of the three *H. morrhuae* dilutions from the processing treatments. ~~Plain l~~Note media controls were only conducted for the 1:15 dilution. ~~ines in panel (b) represent~~

~~spectra for the sterile media controls that also underwent the thermal and peroxide treatments.~~Error bars indicate 95% confidence intervals.



325 **Figure 3. Unamended, heat-labile (heat), and organic (~~H_2O_2~~peroxide) frozen fractions for each of the 1:15-diluted *H. sulfurifontis* from the processing treatments. ~~Plain lines represent spectra for the sterile media controls that also underwent the thermal and peroxide treatments.~~ *H. walsbyi* and *N. pharaonis* are not shown since they exhibited no INP activity in their unamended spectra. Error bars indicate 95% confidence intervals.**

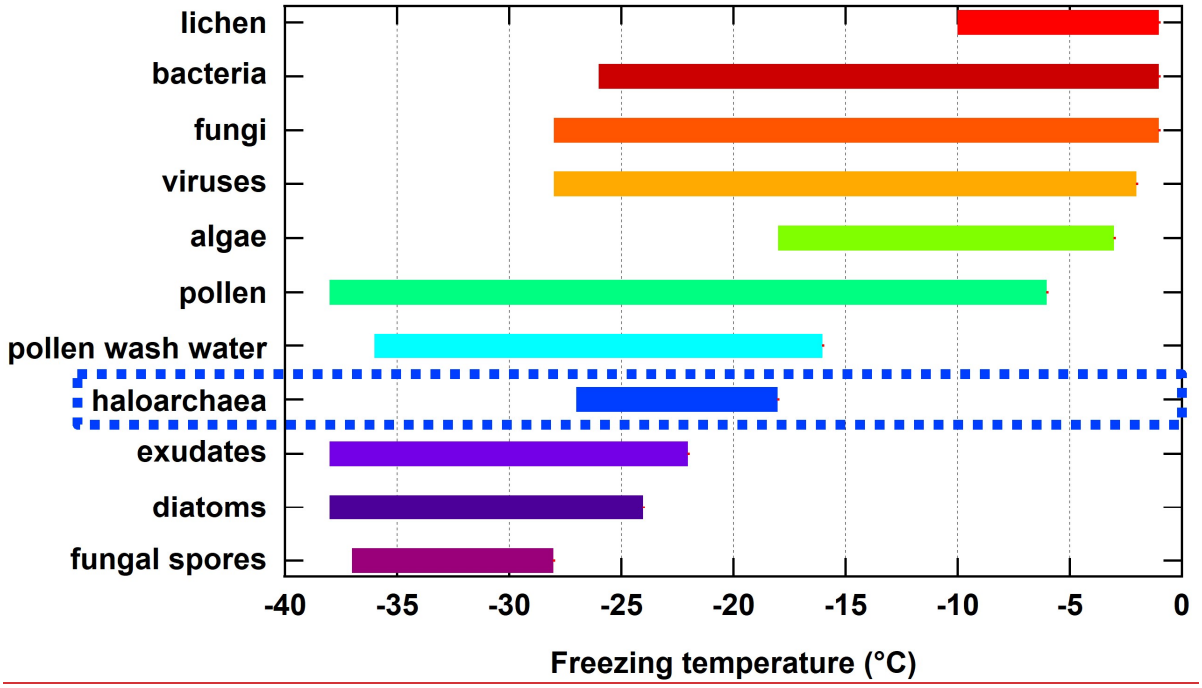


Figure 4. Summary of approximate freezing temperature ranges reported for known biologically-derived INPs. Haloarchaea data are those from *H. morrhuae* and *H. sulfurifontis* from the current work. Data for lichen, bacteria, fungi, algae, pollen, pollen wash water, phytoplankton exudates, diatoms, and fungal spores were obtained and compiled from reviews by Després et al. (2012), Huang et al. (2021)~~(Boucher et al., 2013)~~, Kanji et al. (2017), and Murray et al. (2012), and references therein. Data for viruses are more limited and were obtained from Adams et al. (2021) and Orser et al. (1985). Note that freezing temperature ranges for each result from one to multiple studies and may be dependent on the technique used (e.g., instrumental freezing limits and drop size).~~(Adams et al., 2021; Orser et al., 1985)~~

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