https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020

Biogeosciences Discussions

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 cells from Haloarchaea. Intact cells of *Halococcus morrhuae* and *Haloferax sulfurifontis* demonstrated the ability to induce freezing as warm as –18 ˚C, while lysed cells of *Haloquadratum walsbyi* and *Natronomonas pharaonis* were unable to serve as 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, assessing their ability to become airborne, and their abilities to impact cloud formation, is necessary to improve 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

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020

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., 2020; 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 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, 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, and a 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 (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

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020

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 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 possesses 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). *Haloferax* possesses exclusively a sulphur-rich S-layer and exists as rod- or irregular-shaped cells several microns in size

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020

(Elshahed et al., 2004). *Haloquadratum* can produce halomucin in addition to its S-layer and grows in it 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).

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) 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

Saline media for all four haloarchaeal species tested, *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 alteration: one gram per litre of glycerol was supplemented to medium 1091 for growth of *H. walsbyi.* 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 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. 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)), samples were diluted to reduced salinities shown in Table 2. This reduction in salinity also inherently caused some of the haloarchaeal cells to lyse. 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;

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020

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. Cell lysis thresholds were determined by serially diluting cultures with deionized water as shown in Table 2 and microscopically assessing cellular survival. Samples of active, log-phase cultures were diluted 1:15 with deionized water. 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, 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.). From the number of wells frozen at each temperature step, the fraction frozen is calculated. Standard error was calculated for each spectrum. Deionized water and uninoculated diluted media were run as controls for all species during each experiment.

Thermal and chemical treatments were conducted to isolate heat-labile (e.g., proteinaceous) and organic INPs in the diluted samples. 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 a 1.5-mL aliquot of suspension and the mixture heated to 95 °C for 20 min while illuminated with UVB fluorescent bulbs to generate hydroxyl radicals (residual H_2O_2 is removed using catalase (McCluskey et al., 2018)), and the sample retested in the IS. Remaining INPs were possibly aggregates of cellular material that were not fully digested, inorganic INPs in the media, or other biological materials resistant to heat and peroxide digestion.

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

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020

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 (observed up to –3 ˚C but typically < –20 ˚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 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 and can thus contribute to INP populations at very relevant atmospheric freezing temperatures.

As 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 *H. walsbyi* and *N. pharaonis* demonstrated no ability to serve as INPs (i.e., the fractions frozen were not higher than the media blanks). Interestingly, the dilution of the log-phase cultures 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. 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.

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 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*. The ice nucleating activity of all three dilutions was reduced by heat to the same degree (~0.5 ˚C) and especially reduced by peroxide, indicating the samples contained some heat-labile, likely proteinaceous, INPs but a more substantial amount of some other biogenic organic INP (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 up to 1.3 ˚C and 1.9 ˚C for the heat and peroxide treatments, respectively. For the largest decrease, the 1:30 dilution dropped by up to 3.1 ˚C and 4.2 ˚C 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) 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).

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020

For the other three haloarchaeal species, only *H. sulfurifontis* exhibited a decrease in frozen fractions—fraction frozen dropped by up to 3.4 ˚C and 4.1 ˚C for heat and peroxide, respectively. This decrease was mostly observed when the fraction frozen was approximately 0.2 to 0.8, 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 peroxide, indicating this species heat-labile, probably proteinaceous, 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.

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. All species were introduced to hyposaline conditions to: (1) negate 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 members of both the domain Archaea and the class Haloarchaea have genetic motifs indicative of ice-binding surface proteins. Therefore, further work is needed to directly evaluate the surface properties to disentangle which constituents are responsible for ice formation in the *Halococcus*, and others within 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

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020

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-
220 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.

Acknowledgments: The authors would like to acknowledge Prof. Stephen Schmidt and Dr. Pacifica Sommers of the
225 University of Colorado, Boulder for assisting with preservation and culturing of the haloarchaea after arrival in Colorado. J. Ceniceros was supported by the U.S. Department of Commerce, National Oceanic and Atmospheric Administration, Educational Partnership Program under Agreement No. NA16SEC4810006. This work was funded by a NASA EPSCoR Research Infrastructure Development for the state of South Carolina.

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020

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

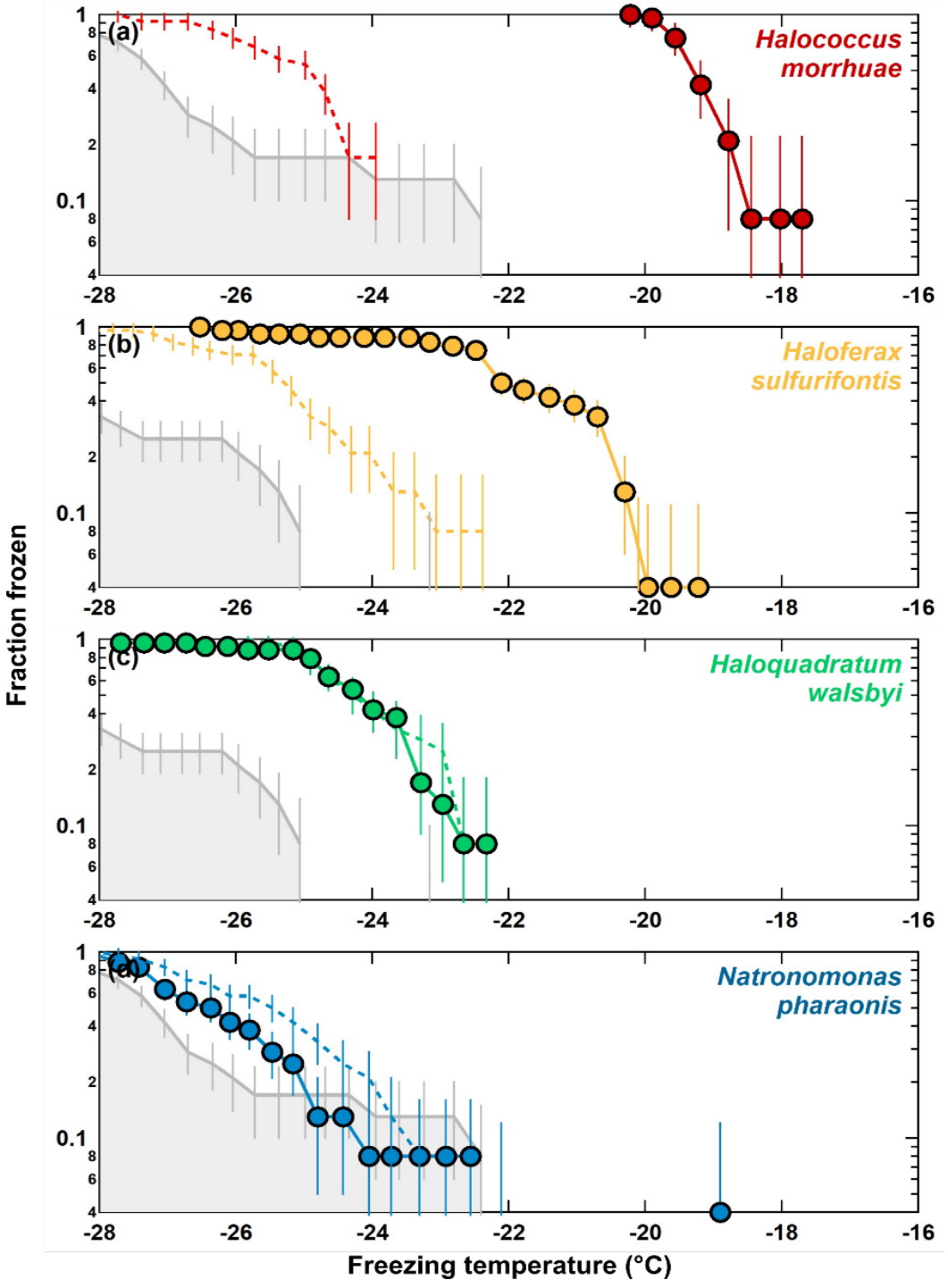
Haloarchaeal species	DSMZ medium recipe	Initial cell concentration (mL^{-1})	Salinity in ppt (%)
Halococcus morrhuae	97	2.7×10^{9} *	160 (16.0%)
Haloferax sulfurifontis	1018	3.5×10^{9} *	177 (17.7%)
Haloquadratum walsbyi	1091	2.4×10^{8}	210 (21.0%)
Natronomonas pharaonis	371	1.1×10^{9} *	207 (20.7%)

* Initial 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*.

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.

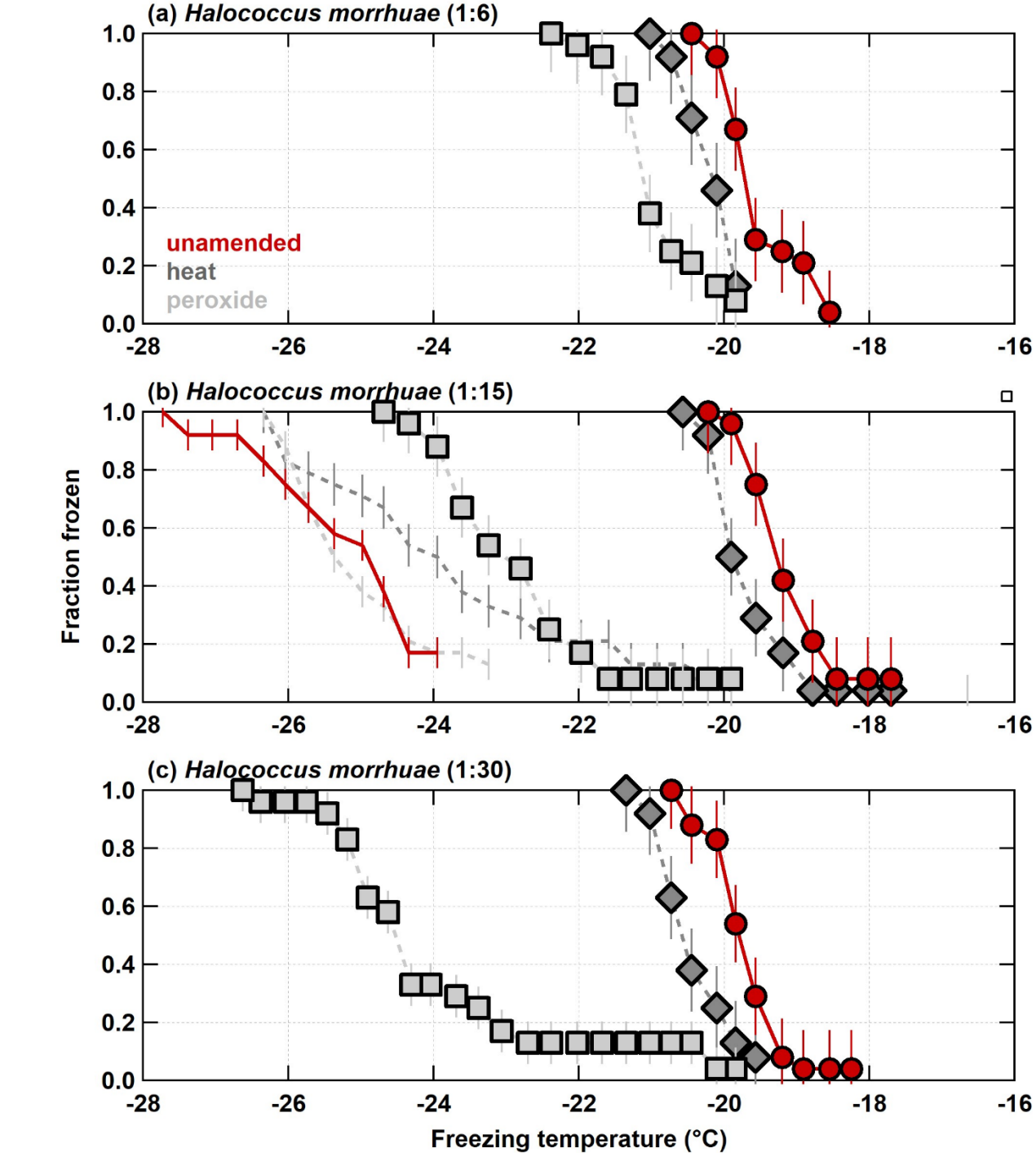
Haloarchaeal species	Cell state	Dilution factor	Diluted cell conc (mL^{-1})	Diluted salinity in ppt (%)
	intact	1:6	4.5×10^{8}	26.7 (2.7%)
Halococcus morrhuae	intact	1:15	1.8×10^{8}	10.7 (1.1%)
	intact	1:30	9.0×10^{7}	5.3 (0.5%)
Haloferax sulfurifontis	intact	1:15	5.8×10^{8}	11.8 (1.2%)
Haloquadratum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

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020



240 **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. 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.**

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020



245

Figure 2. Unamended, heat-labile (heat), and organic (H_2O_2) frozen fractions for each of the three *H. morrhuae* dilutions from the processing treatments. Plain lines in panel (b) represent spectra for the sterile media controls that also underwent the thermal and peroxide treatments.

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020

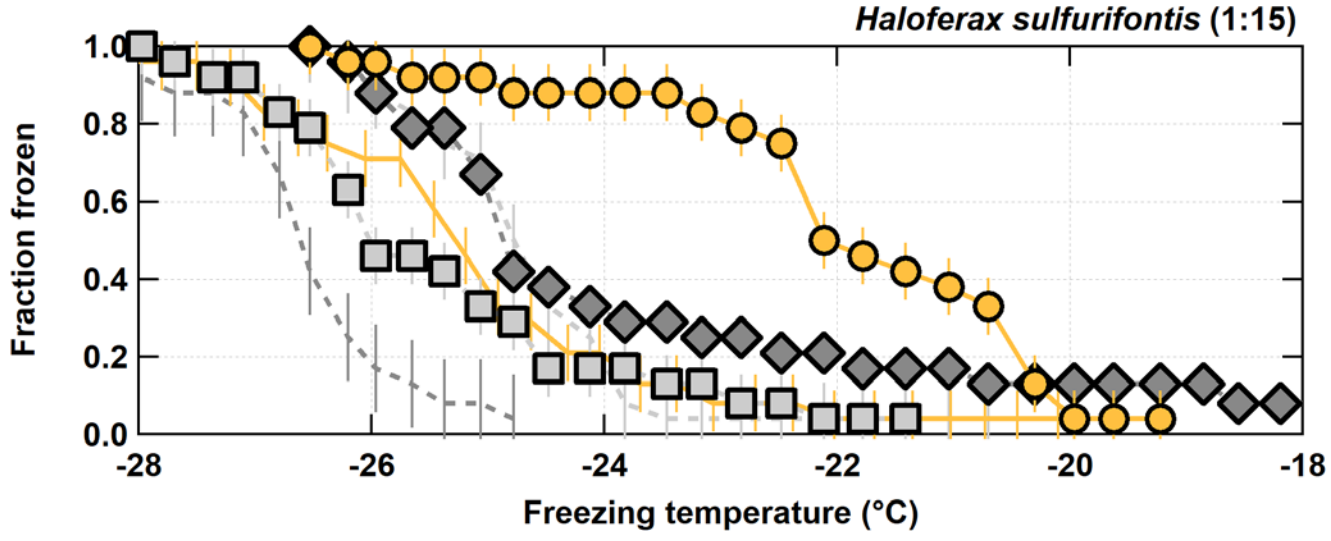


Figure 3. Unamended, heat-labile (heat), and organic (H_2O_2) frozen fractions for each of the 1:15-diluted *H. sulfurifontis* from the processing treatments. Plain lines represent spectra for the sterile media controls that also underwent the thermal and peroxide treatments. *H. walsbyi* and *N. pharaonis*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

https://doi.org/10.5194/bg-2020-367
Preprint. Discussion started: 4 November 2020

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