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A Frozen Asset: The Potential of Flow Cytometry in Constraining the Glacial Biome

To The Editor:

Today, around two thirds (~51M km^3) of Earth's freshwater is locked away in glaciers, ice caps and ice sheets (1). Yet, wholesale recognition that these frozen assets constitute Earth's largest freshwater ecosystem is only recent (2). Considering the global scale of this glacial habitat, and its important role in Earth's climate system, it should neither be neglected nor overlooked. Here, we evaluate a cytometric definition of this globally significant, icy biome.

A sceptical reader more familiar with marine and terrestrial biomes may dismiss the notion of a glacial ecosystem on the grounds that, being "as pure as driven snow," this icy world is not significantly contaminated by microbes. Yet, long-standing evidence points to microbial habitats in unlikely, cold environments including clouds, precipitation, and snow-cover (3); while cores from the Earth's two ice sheets reveal the prevalence of immured microbes in glacier ice, with concentrations in the order of 10^2-10^7 cells/mL in ice of 750 ka in age (4–6). Assuming these microbial abundances are broadly representative, a total of between 4×10^{25} and 7×10^{29} cells may lie entombed in glacial ice. However, this is a conservative assessment for the glacial biome because the elevated biomass associated with active habitats at the ice surface and glacier bed remains poorly constrained and eukaryote communities are excluded. In comparison, Whitman et al. (7), omitting glacierized environments, reported global estimates for *Bacteria* and *Archaea* abundance in all non-glacial freshwater (1.3×10^{25} cells), and rainforest, tundra, and alpine soils (Table 1). The comparable magnitude and associated uncertainties of estimates of glacial ice biomass illustrate its potential importance, and motivates continuing investigation.

Microbial ecologists have typically preferred to concentrate cells on membrane filters and enumerate cells stained for nucleic acids using epifluorescence microscopy. However, the low concentrations of cells typical of individual samples from snow, glacial ice, or meltwaters, coupled with their presence in an aqueous media, would seem to readily lend flow cytometry (FCM) as an ideal analytical tool for the quantification of cells associated with glacial samples. Nevertheless, despite a much longer history of application in other aquatic habitats (8), and its capability for multi-parameter interrogation of heterogeneous microbial populations at the single-cell level (9), FCM has yet to become an established tool within glacial ecology.

Notwithstanding infrequent application, the potential utility of FCM for probing the glacial cryosphere has been demonstrated by the small, but growing, number of glacial and snowpack studies. Initially, FCM enumerations employed Hoechst 33342 to discern microbes in ice from depths of ~3 km below the Antarctic ice sheet surface (6). Subsequently, studies applied SYTO stains to enumerate microbes and explore cell size distributions for samples drawn from the GISP2 ice core (5), while measurements of cell concentrations in Tibetan glacier snowpacks (10) have paralleled the perspective that SYBR Green is a more effective nucleic acid stain for freshwater samples. However, these snapshots of communities within glacial ice or snow are not a true reflection of the complexity of community and habitat dynamics that characterize the glacial biome.

While both water and cellular mobility in deep ice may be limited (11), the dynamic and porous nature of a glacier's surface potentially facilitates the translocation of cells and nutrients. The atmosphere–ice interface exhibits complex topography and its hydraulic conductivity evolves as

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Table 1. Quantities of cells and associated macronutrients and macromolecules for a variety of Earth's biomes, including estimations for annual riverine and glacial runoff volumes. The first-order estimate for cells in glacier ice uses a median concentration from published data.

UNITS	RAINFOREST SOIL[a]	TUNDRA & ALPINE SOIL[a]	FRESHWATER[a]	RIVERINE RUNOFF ($Year^{-1}$)[a,b]	OCEANIC PHOTIC ZONE[a]	GLACIER ICE	GLACIER RUNOFF ($Year^{-1}$)[c]	UNCERTAINTY (±%)
Cells	1.0×10^{27}	2.0×10^{28}	1.3×10^{25}	4.6×10^{25}	3.6×10^{25}	3.6×10^{29}	3.2×10^{21}	–
Gg C[d]	2.0×10^{4}	4.0×10^{5}	2.6×10^{2}	9.1×10^{2}	7.2×10^{2}	7.2×10^{6}	6.3×10^{-2}	–
Gg N[e]	4.2×10^{3}	8.4×10^{4}	5.5×10^{1}	1.9×10^{2}	1.5×10^{2}	1.5×10^{6}	1.3×10^{-2}	23
Gg P[e]	1.2×10^{3}	2.4×10^{4}	1.6×10^{1}	5.5×10^{1}	4.3×10^{1}	4.3×10^{5}	3.8×10^{-3}	30
Gg DNA[f]	3.2×10^{3}	6.4×10^{4}	4.2×10^{1}	1.5×10^{2}	1.2×10^{2}	1.2×10^{6}	1.0×10^{-2}	31
Gg RNA[f]	3.6×10^{3}	7.3×10^{4}	4.7×10^{1}	1.7×10^{2}	1.3×10^{2}	1.3×10^{6}	1.2×10^{-2}	2
Gg Protein[f]	2.3×10^{4}	4.6×10^{5}	3.0×10^{2}	1.1×10^{3}	8.3×10^{2}	8.3×10^{6}	7.3×10^{-2}	1

[a]Source: (7) Whitman et al., Proc Natl Acad Sci USA 1998;95:6578–6583.
[b]Source: (1) Oki and Kanae, Science 2006;313:1068–1072.
[c]Source: (13) Irvine-Fynn et al., Environ Microbiol 2012;14:2998–3012.
[d]Assuming 20fg C/cell: (7) Whitman et al., Proc Natl Acad Sci USA 1998;95:6578–6583; (11) Price, FEMS Microbiol Ecol 2007;59:217–231.
[e]C-ratios from: Fagerbakke et al., Aquat Microbiol Ecol 1996;10:15–27; Vrede et al., Appl Environ Microbiol 2002;68:2965–2971.
[f]C-ratios from: Simon and Azam, Marine Ecol Prog Ser 1989;51:201–213.

the crystalline structure weathers and degrades with both incident radiation and meltwater enlarging the interstitial spaces (Fig. 1). Although incident solar shortwave radiation may penetrate to 10 m, limits of this porous ice "photic zone" to < 2 m are more typical. Under ablating conditions, the meltwater influx to, and flow within, this depth-limited aquifer is highly variable, allowing storage in and release from the interstitial voids. Consequently, with concentrations of $10^2 - 10^3$ cells/mL in glacier ice but $> 10^6$ cells/mL in the interstitial meltwater (12), the redistribution of microbes is highly plausible.

One of the first FCM-based studies exploring the changing aspects of the glacier photic zone ecosystem focused on the export of cells from the surface of a High-Arctic glacier (13). Findings included (i) a temporal change in the proportion of bacterial cell-sized particles exhibiting green autofluorescence; (ii) an inverse non-linear relationship between meltwater discharge and cell abundance, with in-stream concentrations significantly lower than those of the near-surface ice; and (iii) disparity between melting glacier surface cell inputs (from airborne deposition and ice melt) and output (in meltwater runoff) implying a retention of cells and "biological darkening" of the ice surface. Combined, these observations indicate complex glacier surface transport processes mediate the delivery of cells, DNA, and organic matter to aquatic and terrestrial downstream habitats.

Since microbes are present on glacier surfaces world-wide (2), and by crudely assuming that the study by Irvine-Fynn et al. (13) is representative of the global cryosphere, it is tempting to extrapolate a first-order estimate of the export of *Bacteria* and *Archaea* from glacier surfaces worldwide. Currently, outside Antarctica, seasonally melting ice extends over $\sim 7.3 \times 10^5$ km^2, suggesting between 10^{21} and 10^{26} cells may be contained in the porous glacial photic zone. Surprisingly, this compares particularly well to the 4×10^{25} cells found in the $\sim$200 m deep photic zone of the Earth's oceans (7). Moreover, with a global area-weighted mean glacier mass balance for 1983–2003 of -0.3 m w.e. a^{-1} (14) and assuming a 70-day ice-melt season, we estimate an annual delivery of 3.15×10^{21} cells from glacier ice to downstream environments. While this number may represent only $<0.1\%$ of the Earth's annual fluvial export of *Archaea* and *Bacteria* to the oceans, and is ignorant of snowmelt contributions, the cellular delivery represented as quantities of nutrients and macromolecules may be locally important for deglaciating catchments (Table 1). Although these data are speculative, they emphasize the need for catchment- or glacier-scale studies to improve on the current lacuna in understanding of cell mobility, budgets, and potential release of bioavailable nutrients and macromolecules to the extra-glacial environments.

Clearly, ambiguities remain regarding the microbes and products that enter, reside in, and leave the glacier habitat. Yet innovative application of FCM has the potential to enable novel insights into the glacial biome, with focus in three distinct areas.

Firstly, to date, discrete studies have surveyed individual glacier surfaces or subsurfaces, explored temporal variations or compared a limited number of catchments across an environmental gradient. Consequently, our existing perceptions of Earth's glacial biome are fiercely extrapolative, as is the case for other aquatic systems: if the calculations presented above, and in Table 1, serve no other purpose, they aptly illustrate this present state-of-the-art.

Resolving the fundamental mechanisms governing heterogeneity in microbial community composition and functions across multiple spatial and temporal scales is one key challenge for understanding microbial ecosystems. Here, we contend that to catalyze such a transition for glacial ecology, intensive sampling and characterization of microbial populations, processes, and interactions on spatial and temporal scales spanning many orders of magnitude is merited. In the first instance, this might be achieved by intensive and extensive comparative studies of many catchments—a tactic which demands a standardized methodological approach. As such, considering its high-throughput and reproducibility, FCM is the model platform for quantifying inputs, throughputs, and outputs of microbial

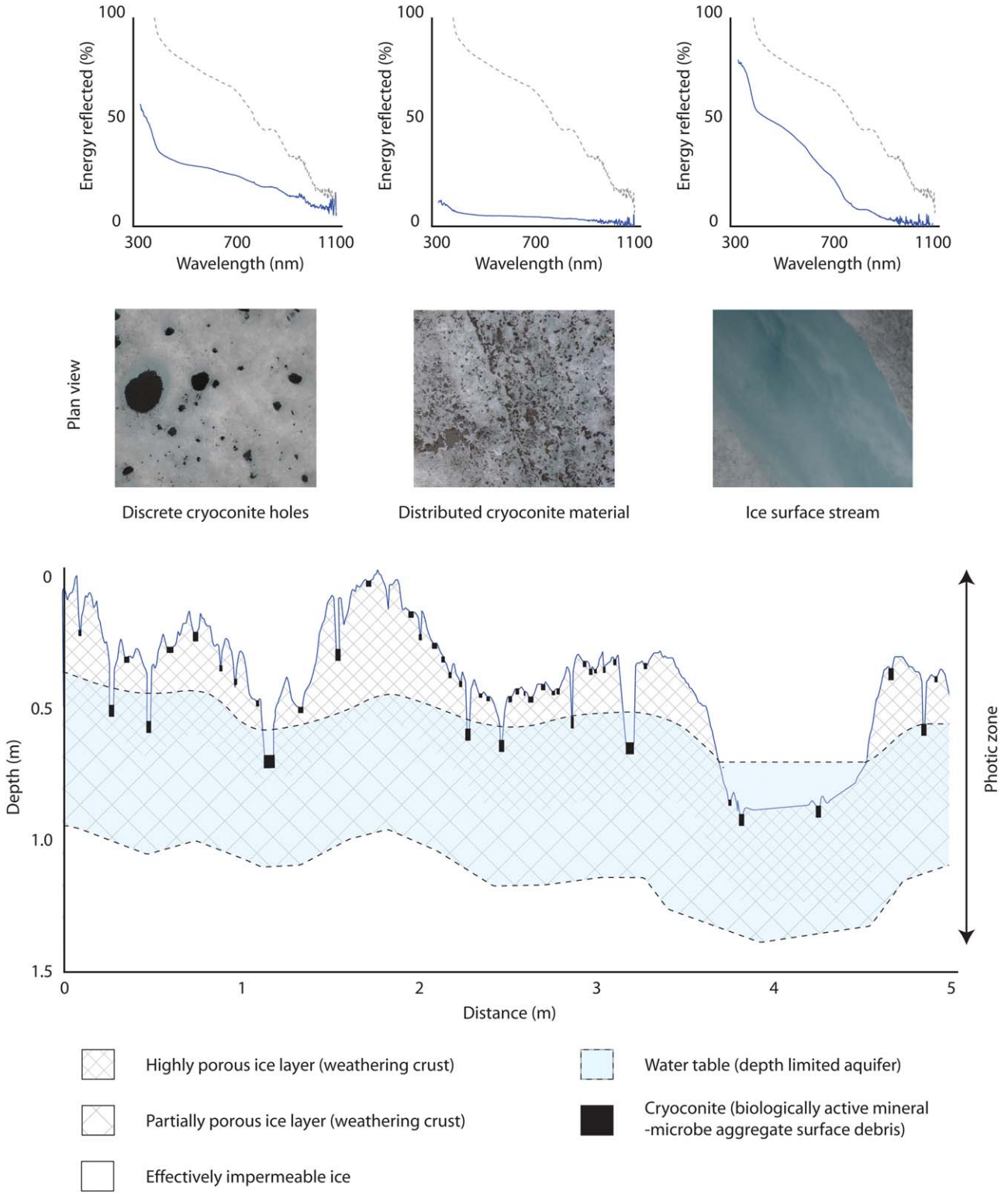

Figure 1. Conceptual model of the glacier surface habitats, highlighting the photic zone, porous ice layers and the perched water table which all represent opportunities for vertical and lateral microbial translocation, with decreasing likelihood as depth from surface increases (redrawn from Müller F, Keeler CM. Errors in short-term ablation measurements on melting ice surfaces. *J Glaciology* 1969;8(52):91–105). Cryoconite is a semi-stable accumulation of inorganic and organic dust, itself harbouring a microbial community. Planimetric images for the key habitats are shown (image area approximately 30 × 30 cm) with surface reflectance plots (solid line) contrasted to a clean glacier ice reflectance reference (dashed greyed line) for ultraviolet to infrared wavelengths. Note the influence biologically active communities have upon glacier surface reflectance.

biomass from different components of glacial systems over time and space.

Secondly, advances in FCM technology pave the way for multi-parameter interrogation of particle and cell properties, extending the utility of FCM far beyond merely counting cells. Multiple combinations of laser excitation and detector arrays may be used simultaneously to examine samples, either unstained or stained. Autofluorescence signals from unstained populations may prove informative of sub-population dynamics (13,15). As previously summarized (16), fluorescence emission is potentially the most sensitive photonic probe in the absence of sample preparation and/or destruction: polycyclic aromatic hydrocarbons (PAHs) can be observed using deep UV (<250 nm) excitation, longer wavelength UV (~375 nm) cedes fluorescence in the visible wavelengths from microorganism metabolites (e.g., flavin adenine dinucleotide), and visible spectrum excitation yields fluorescence in the red to NIR (>500 nm) range for a variety of microbial pigments (e.g., phycobiliproteins and chlorophylls). This analytical potential is aided by the diminished natural autofluorescence of inorganic, mineral particles (15). Moreover, the diverse range of molecular probes available offer an additional wealth of possibilities, but these must be subject to careful validation. For example, a fundamental question for any environmental microbiologist relates to the growth states and viability of a microbial community so the application of proprietary "Live/Dead" stains combined with FCM is not uncommon. In the glacial context, caution must be advised, as populations under stress may yield anomalous results (13,17).

In addition to physiological probes, phylogenetic stains may be useful. These necessitate fluorescent in situ hybridization (FISH) with oligonucleotides targeting ribosomal RNA prior to FCM. This is a well-established methodology in other aquatic environments, but FISH has hitherto only seen limited use in glacial environments (18). Since pyrosequencing reveals temporal shifts in the relative abundance of higher phylogenetically ranked bacterial taxa present in supraglacial habitats (19), FISH-FCM would appear a promising methodology for the high-throughput quantification of phylogenetic groups within glacial samples. Furthermore, deeper understanding of links between taxonomic composition and functionality in glacial communities is required. Flow activated cell sorting (CS) may prove particularly useful, for example, in conducting FISH-CS of experimental microcosms subjected to stable isotope probing. Sorted subpopulations may then be analyzed using metagenomics or NanoSIMS methods to yield further phylogenetic or functional information at the sub-population or individual cell level.

Using FISH-CS for selecting subpopulations or even single cells for down-stream genomics analyses faces challenges. These principally relate to the post-sorting amplification of nucleic acids by either PCR or whole genome amplification which is prone to contamination or fixative inhibition. One promising advance is the method by Yilmaz et al. (20) who reported FISH and FISH-CS of bacterial populations without fixation, thus improving the quality of nucleic acids for later analyses. It remains to be seen whether this convenient method is readily adaptable to samples from glacial environments; however, prior storage of unfixed material at −80°C did not adversely affect the quality of results, so existing protocols for glacial sample preservation (12) are likely to prove compatible. Furthermore, whole genome sequencing of cells preserved in deep ice cores (15) extracted by CS may prove feasible.

Finally, the development of portable, rugged field instruments opens the possibilities for near real-time FCM of glacial samples. This strategy minimizes artifacts from storage (or fixation) and lends itself to intensive or continuous monitoring of cell fluxes, readily coupled to environmental monitoring campaigns and further facilitating collation of comparable and collaborative suites of data related to the glacial biome.

In conclusion, the recent adoption of cytometric methods within glaciology has revealed Earth's icy reservoirs to harbor a wealth of microbial life. However, much remains unknown in terms of processes, diversity, and dynamics of the glacial ecosystem both in the surface photic zone and at depth. We have demonstrated that FCM has a currently untapped potential to advance our understanding in the emerging field of glacier microbial ecology. Continuing FCM progress, driven by the cytometry community, promises to answer critical questions on microbial abundance, community, dynamics, and function within Earth's frozen freshwater assets.

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