

# Unmasking photogranulation in decreasing glacial albedo and net autotrophic wastewater treatment

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## ABSTRACT

In both natural and built environments, microbes on occasions manifest in spherical aggregates instead of solid-affixed biofilms. These microbial aggregates are conventionally referred to as granules. Cryoconites are mineral rich granules that appear on glacier surfaces and are linked with expanding surface darkening, thus decreasing albedo, and enhanced melt. The oxygenic photogranules (OPGs) are organic rich granules that grow in wastewater with photosynthetic aeration and present potential for net autotrophic wastewater treatment in a compact system. Despite obvious differences inherent in the two, cryoconite and OPG pose striking resemblance. In both, the order Oscillatoriales in Cyanobacteria envelope inner materials and develop dense spheroidal aggregates. We explore the mechanism of photogranulation on account of high similarity between cryoconites and OPGs. We contend that there is no universal external cause for photogranulation. However, cryoconites and OPGs, as well as their intra variations, which are all are under different stress fields, are the outcome of universal physiological processes of the Oscillatoriales interfacing goldilocks interactions of stresses, which select for their manifestation as granules. Finding the rules of photogranulation may enhance engineering of glacier and wastewater systems to manipulate their ecosystem impacts.

Cryoconites and oxygenic photogranules (OPGs) are quasi-spherical microbial aggregates that inhabit two extremely different environments on Earth (Fig. 1). The former occur on surfaces of Polar and alpine glaciers and ice sheets, and are rich in minerals (>85% by wt).<sup>1-3</sup> The latter, conversely enriched with organic matter (>85% by wt), grow in wastewater treatment systems.<sup>4,5</sup> Although the difference inherent in the two is therefore obvious, recent studies have recognized their remarkable similarities with respect to granular morphology, structural formation, and key microbial group.<sup>4,6-8</sup> In both, mat-forming filamentous cyanobacteria envelop inner materials forming a spheroidal aggregate (Fig. 2). This network of filamentous organisms and extracellular polymeric substances (EPS) stabilizes a granular habitat by interconnecting with other microorganisms and mineral particles.<sup>1,2,4,7,9</sup>

Since the first report in the 19<sup>th</sup> Century during exploration in Greenland,<sup>10</sup> granular and biogenic characteristics of cryoconites have been documented with their presence believed to promote glacial melt. However, only recently have we learned that granular structure of these glacial aggregates is formed by filamentous cyanobacteria (Fig. 2A) and the granules' EPS, especially humified organic matter, absorb significantly more sunlight than surrounding ice.<sup>1</sup> The spectral albedo of ice surfaces covered with cryoconites was 5-15%, significantly lower than 40-55% from clean bare ice surfaces.<sup>1,11</sup> Absorbing light and associated heat, cryoconites induce the formation of melt holes—the formed holes themselves extend surface albedo reduction—and grow further beneath quiescent water of the holes (Fig. 1AB).<sup>1,12</sup> Cryoconites get redistributed over the ice surface as the holes collapse during the melting season<sup>13,14</sup> while their dense granular feature helps them to avoid washout in ablation.<sup>1</sup> Cryoconites and cryoconite holes are estimated to cause up to 20% of Polar glacial runoff.<sup>15-18</sup> Climate change, especially Polar amplification,<sup>19</sup> is expected to expand cryoconite-induced surface darkening, enhancing melt in the future.<sup>14,15</sup>

The reports on OPGs followed a serendipitous discovery in 2011 that activated sludge in a closed 20 mL vial left on a lab windowsill changed to a granule (Fig. 3).<sup>4,20</sup> This “hydrostatic photogranulation” occurred with the enrichment of Oscillatoriales, often found in low abundances or undetected in activated sludge.<sup>4,6</sup> Reactors seeded with hydrostatically-formed OPGs and operated with mixing and illumination were used to treat wastewater without aeration—The aeration alone accounts for 50-60% of energy for wastewater treatment<sup>21,22</sup> or 10-16% of energy used by municipalities (the water-wastewater treatment sector in the U.S. accounts for 30-40% of energy by municipalities, and usually the two thirds of it is for wastewater treatment).<sup>22,23</sup> In reactors new OPG biomass rapidly propagated, concomitantly occurring with chemical-oxygen-demand (COD) removal and nitrification.<sup>5</sup> Proximal growth of phototrophic and heterotrophic microbes within a granule favors carbon fixation during wastewater treatment, leading to net autotrophy.<sup>4,5</sup> Granular biomass enables effective solid/liquid separation, a conventional challenge in both activated sludge and algae-based wastewater treatment, hence showing potential to make wastewater systems compact.

*How can granules with considerable similarities be formed in these barely related environments?*

Notwithstanding their differences, similarities between cryoconite and OPG suggest that a core granulation mechanism is conserved between them. In this interdisciplinary review work, we explore this granulation mechanism by conducting intra and inter comparisons of the two granule systems. Selection of Oscillatoriales, and co-occurring microbial communities, in cryoconites and OPGs was studied. We also researched on stresses prevalent in each granule niche and interrogated how they can be related in forming similar granular products conquering vast differences in macroenvironments.

## Selection of Oscillatoriales in cryoconites and OPGs

A strong converging point for cryoconites and OPGs is the ubiquity and abundance of Oscillatoriales. These cyanobacteria have been found as the most abundant group of the Cyanobacteria phylum, not infrequently among entire bacterial phyla.<sup>4,6,24</sup> Oscillatoriales make up the subsection III genre of Cyanobacteria based on Bergey's bacterial taxonomy.<sup>25</sup> Oscillatoriales are unbranched filamentous but distinguished from other groups of filamentous cyanobacteria, i.e., the subsections IV and V cyanobacteria, by being non-heterocystous and mostly motile.<sup>25,26</sup> The subsection IV cyanobacteria, Nostocales have been found in some cryoconites and OPGs, but their presence was sporadic and mostly with less population than Oscillatoriales.<sup>4,24,27</sup>

Oscillatoriales are not, however, selected at genus or family level across these granules, although cryoconites' geographical and in OPGs both source- and variable-anchored dominance is present (Table 1). Segawa et al. reported biogeographical patterns of Oscillatoriales in cryoconites from their investigation of 15 Polar and Asian alpine glaciers.<sup>27</sup> The Oscillatoriales in this study were grouped into 15 operational taxonomic units (OTU), which accounted for 87% of total number of cyanobacterial OTUs. In cryoconites from the Arctic glaciers in Alaska, Greenland, and Svalbard (total 15 sites on seven glaciers), OTU1, Oscillatoriales cyanobacterium showing 99% similarity with *Phormidesmis*, was ubiquitous and the most abundant taxon from nearly all investigated sites. In Southern and Northern Asian alpine cryoconites (21 sites on six glaciers), on the other hand, *Microcoleus* OTU4 and Oscillatoriales OTU0 (*Leptolyngbya*)<sup>24</sup> were found to be prominent cyanobacterial taxa. *Phormidesmis* was occasionally found abundant in these Asian alpine cryoconites, but its detection was intermittent, and the population was also significantly less than that of the dominant Oscillatoriales genera. These results are partly supported by Uetake et al.<sup>24</sup> and Gokul et al.<sup>28</sup> who, respectively, showed that *Phormidesmis priestleyi* was the primary bacterial species in cryoconites sampled from 38 sites on 10 glaciers in Greenland and 37 sites across an ice cap in Central Svalbard.

In OPGs, the genus *Microcoleus* has been found as a prime cyanobacterial taxon regardless of geographic origins (Table 1). Milferstedt et al. studied nine hydrostatic cultivations using activated sludge from four wastewater treatment plants in Europe and the U.S. and found that this genus was the major cyanobacterial clade, comprising up to 99% of cyanobacteria.<sup>4</sup> This was also the case for OPGs produced in both the French and the U.S. OPG reactors treating local municipal wastewater—These reactors were both seeded with hydrostatically-formed OPGs. Nevertheless, the same study also showed that other genera were occasionally dominant in OPGs (Table 1). Some European hydrostatic OPGs showed an unclassified Oscillatoriales genus being more abundant than *Microcoleus*. Stauch-White et al. who investigated five hydrostatic cultivations with activated sludge from one U.S. wastewater treatment facility showed that the genus *Tychonema* was most abundant in the formed OPGs.<sup>6</sup> *Tychonema* and *Microcoleus* belong to the same family, Microcoleaceae.

The enrichment of Oscillatoriales in hydrostatic OPGs is remarkable because their presence in activated sludge inoculums was in very low abundances, frequently undetected.<sup>4</sup> Also in reactors, the enrichment of Oscillatoriales in OPGs growing is apparent. Abouhend et al.<sup>8</sup> showed that as the size of OPGs increases, the density of cyanobacteria, analyzed by the quantity of phycobiliproteins and microscopy, increases. Similarly a study analyzing bacterial community based on various size groups of Greenlandic cryoconites found that although *Phormidesmis* were already abundant in small-size (<250 µm) granules they got further enriched as the granule size increased.<sup>24</sup> Common in cryoconites and OPGs, the granule size increase occurs with the

appearance of concentric cyanobacterial layers. Studies on OPGs and cryoconites found consistent results that outer layers of filamentous cyanobacteria were mainly present in granules greater than certain sizes.<sup>8,29</sup> In smaller granules, Oscillatoriales were found throughout the granules, indicating photic limited distribution of these cyanobacteria.

Two bacterial phyla, Proteobacteria and Bacteroidetes, are also abundant and ubiquitous in cryoconites and OPGs,<sup>4,29–34</sup> suggesting their role in sustaining these microbial ecosystems. However, unlike Cyanobacteria, their taxonomic assignments at class and suborder levels in both granule types are highly variable. Similarly, the dominant cyanobacteria *P. priestleyi* in Arctic cryoconites were not correlated with other bacterial taxa.<sup>24,28</sup> Furthermore, based on the RNA-based 16S rRNA analyses, these two and other bacterial phyla were outweighed by Cyanobacteria in Asian alpine cryoconites<sup>29,32</sup> and frequently in Arctic cryoconites.<sup>31,33</sup> Excluding cyanobacteria, Milferstedt et al. reported finding no core bacterial community from hydrostatic OPGs from European and the U.S. cultivations.<sup>4</sup> Consequently, bacterial community other than Oscillatoriales are considered general habitants in OPGs and cryoconites, reflecting local and regional effects.<sup>4,24,30,31</sup> Nevertheless, Gokul et al.<sup>28</sup> contended that Actinobacteria should be the microbial keystone taxa for cryoconites due to their high connectivity in modular community structure found in cryoconites from an ice cap on Svalbard. This actinobacterial group was also thought to be responsible for humification of organic matter, posing dark color in cryoconites.

Gliding motility is an important trait of Oscillatoriales—heterocystous cyanobacteria are usually immotile<sup>26</sup>—and therefore it may be a phenotypic characteristic required for granulation of cryoconites and OPGs,<sup>4</sup> henceforth photogranulation. Motility allows Oscillatoriales to move toward or away from the light source, which is an essential substrate but also a stressor.<sup>35,36</sup> Oscillatoriales glide over a solid surface, including other trichomes.<sup>35–37</sup> For this to occur, Oscillatoriales are known to secrete significant amounts of extracellular slime or mucilage.<sup>37</sup> Copious amounts of mucilage, often in tubes, around or left behind filamentous cyanobacteria have been reported for both cryoconites and OPGs.<sup>1,7</sup> Hence, the movement of Oscillatoriales with their exuded EPS is thought to enhance binding of minerals and also attract bacteria that use cyanobacterial metabolites, including EPS and oxygen, promoting agglomeration in both cryoconites and OPGs.<sup>1,7,9,38,39</sup>

Nevertheless, the enrichment of Oscillatoriales and their motility is insufficient to explain how they form spherical granules. In natural environments, from rivers and lakes in temperate regions to those in the Arctic and Antarctica, Oscillatoriales are often dominant in solid-anchored microbial mats.<sup>36,40–44</sup> Castenholtz also reported that in hot-spring microbial mats dominant with *Oscillatoria terebriformis*, interwoven trichomes were free moving.<sup>35</sup> Concurrently, hydrostatic cultivation of the same activated sludge inoculum occasionally produces flat mats rather than spherical OPGs,<sup>45,46</sup> although mats were enriched with Oscillatoriales (Fig. 4). The coexistence of ‘filamentous OPG’ along with spherical OPG in reactors has also been observed.<sup>47</sup> Takeuchi et al. documented that mashed cryoconites did not return granular despite the enrichment of filamentous cyanobacteria.<sup>1</sup> These examples of literature suggest other causality in addition to or other than Oscillatoriales that is required for photogranulation.

### Selection pressures for photogranulation

Microbial granulation occurs in widely varying environments and under diverse microbial metabolisms. In environmental engineering systems, granular processes have been used for methanogenic wastewater treatment,<sup>48</sup> aerobic wastewater treatment,<sup>49,50</sup> removal of nitrogen via anaerobic ammonia oxidation,<sup>51</sup> etc. Nevertheless, microbial granulation is rare—otherwise

granules would be as ubiquitous as biofilms attached to substrata—suggesting that only selected conditions allow the phenomenon to occur. Growing granular, OPGs and cryoconites may share selection pressures with other granules. Furthermore, high similarity between OPGs and cryoconites renders an expectation that types of selection pressures and stresses inducing such selective forces would be the same or highly related.

Conventional wisdom is that hydrodynamic shear<sup>52–55</sup> and hydraulic selective pressure (HSP) (i.e., selective washout)<sup>48–50,56–58</sup> are vital to form granules. The tangential shear stress exerted on microbial aggregates and enhanced aggregate-aggregate collision are known to be essential for granulation.<sup>54,55</sup> Not only for granules used in built systems but for cryoconites<sup>1</sup> and other natural granules, such as lake algal balls (also known as Marimo),<sup>59</sup> shear has been viewed as a critical component for their physical shaping. HSP—while shear can be still present in non-granulating systems—provides strong impetus for microbial cells to granulate, thereby avoiding cell washout. For example, reactors seeded with activated sludge and operated with little HSP continued to support the floccular growth but those with HSP resulted in granulation.<sup>58,60</sup> Barr et al. further showed that divergence between the two systems occurred with substantial change in microbial physiology, shown with metaproteomics analysis, unnecessarily the structure of community.<sup>60</sup> This HSP has been the basis for primary use of sequencing batch reactor (SBR) for engineered granule processes in which selection pressure can be induced by short settling and discharging unsettled biomass out of the systems.<sup>50,56</sup>

The formation of OPGs by hydrostatic batch, however, defies these dogmas since the hydrodynamic shear must be negligible and there is no real HSP established (Fig. 3). Nevertheless, like other engineered granules, OPGs are also produced in SBRs under the incidence of hydrodynamic shear as well as HSP. Typical mixing conditions in SBRs for OPGs induce shear, computed as velocity gradient, at 40–60 s<sup>-1</sup>.<sup>4,5</sup> Hydrodynamic shear also showed strong influence on OPGs' size development in SBRs (Abouhend et al., in preparation). The reactor with the lowest shear studied, 15 s<sup>-1</sup>, allowed OPG to increase up to 5.5 mm in diameter (mean size = 0.94 mm). On the other hand, the reactor with the highest mixing intensity, 140 s<sup>-1</sup>, did not allow OPG to grow beyond 2.5 mm (mean size = 0.36 mm). For HSP, settling time was 10–15 min,<sup>4,5,61</sup> in good agreement with other granular processes,<sup>50,51,56,62</sup> and substantially shorter than settling time (a few hours) employed in the activated sludge process. Hence, granulation of OPGs occurs in a wide spectrum of hydraulic conditions, from the typical spectrum of hydrodynamic shear and HSP for engineered granular processes to where these are virtually nil.

Characterizing hydraulic conditions of glacier surfaces and understanding its effect on granulation of cryoconites would obviously be more challenging. Glacier surfaces, particularly the ablating ice surface where cryoconites grow, show three prominent physical features: ice surface; cryoconite holes; and meltwater streams (Fig. 5). Each of them has distinct hydraulic conditions, which are most dynamic for meltwater stream, intermediate for ice surface, and least for cryoconite holes.<sup>30</sup> Literature shows that cryoconite holes in alpine glaciers, in which ice surface is typically steeper than that in Polar glaciers and ice sheets, seldom persist through an entire ablating season.<sup>13,63,64</sup> Cryoconites in collapsing holes are subjected to dispersal to the ice surface and may undergo subsequent rounds of hole formation and hole destruction or washout via meltwater, which should all involve certain levels of hydrodynamic shear and HSP. Nevertheless, there would be always some hydrostatic periods for cryoconites when they sit at the bottom under quiescent water inside the hole, analogous to OPGs' hydrostatic batch (Fig. 6). It is well accepted that water in the cryoconite holes is almost static,<sup>1,12</sup> although water could sweep in via the gaps of the ice crystals of the glacial ice.<sup>2</sup> Extended period for quiescent conditions can be expected for cryoconites



residing in large melt holes in flat ice surface, which is more common for Polar ice sheets. Indeed, abundant cryoconites tend to be located dispersed on ice surfaces in alpine glaciers while they are more limited inside the holes for Polar ice sheets and glaciers.<sup>14,65</sup>

Thus, some commonality between OPG and cryoconite may be seen from the hydraulic condition perspective, and this can be further extended with its effect on their physical properties. Hydrostatically-formed OPGs are more porous and permeable than OPGs generated in SBRs.<sup>66</sup> When the outer layer is removed from hydrostatically-formed OPG, the internal biomass often drips out.<sup>4</sup> On the other hand, OPGs produced in reactors are more tightly held together and more spherical, which likely results from their growth in the presence of shear and HSP. For cryoconites, there has been some consensus among researchers that alpine cryoconites are more tightly aggregated than Polar cryoconites. Hence, cryoconites experiencing more dynamic hydraulic events in alpine glacier surfaces, analogous to OPGs in SBRs, might provide explanations to this observation.

The hydraulic commonalities, however, do not provide clear explanation to the photogranulation mechanisms since they can be countered by a recurring notion: *how can OPGs, and possibly cryoconites, granulate both with and without hydrodynamic shear or hydraulic selective pressure?* Although cryoconites and OPGs are always under the influence of certain hydraulic conditions, including a hydrostatic realm, this question requires us to look at other environmental stimuli that may impact their formation.

Microbes in perennial cryoconites<sup>2,38,67</sup> live experiencing frequent freeze-thaws and long-term freeze over the winter. It is well accepted that Oscillatoriales in cryoconites and other habitats in the cryosphere are not true psychrophilic but psychrotrophic microbes,<sup>41,68</sup> and must tolerate various cold stresses, including osmotic stress.<sup>69</sup> Numerous freeze-thaw cycles can also naturally induce feast-famine conditions by limiting substrate availability to microbes beyond cold stresses. These cryoconite microbes also must be protected from high-intensity sunlight, including UV, radiating onto glacier surfaces.<sup>70</sup> The limited resource environment, including carbon, nitrogen, phosphorous, sulfur, and iron, can also induce microbial stress. For example, the level of dissolved nitrogen in cryoconite holes was an order of magnitude lower than that in precipitation or surface meltwater.<sup>32</sup> Furthermore, the mass of *Phormidesmis* in Arctic cryoconites was correlated with the amount of minerals, implying nutrient limitations.<sup>71</sup>

A preliminary study in the author's laboratory showed that OPGs maintained granular and treatment functions (oxygen production and COD removal) even after numerous freeze-thaw cycles, indicating that Oscillatoriales in OPGs can also persist in cold stresses like those in cryoconites. However, OPGs have been formed at typical room temperatures and no reports so far have indicated that cold stresses are required for granulation of OPGs. Then, a question may arise: *Is cold stress not essential for photogranulation?* In hydrostatic cultivation of OPGs, depletion of dissolved inorganic nitrogen (DIN)<sup>6,7,72</sup> and biologically available iron<sup>72</sup>—the latter includes dissolved and easily extractable iron—has been consistently documented, suggesting microbial stresses related to limitation of these essential resources. In reactor operation of OPGs, however, a significant level of DIN remains in the effluent<sup>5,39,61</sup>—the fate of iron in reactor operation has not yet been reported—thus, OPG granulation still occurred even when nitrogen was sufficiently available. *Is nitrogen limitation not a core requirement for photogranulation?* In terms of phosphorous, some sets of hydrostatic cultivation of OPGs showed depletion while other sets as well as reactor operation showed a plenty of phosphate remaining in water.<sup>6,7,72</sup> In fill-and-draw operation in SBR for OPGs, repetitive feast-famine conditions occur, and this repetitive feast-

famine cycle has been suggested as a core selection pressure for microbial granulation.<sup>73–76</sup> In hydrostatic cultivation of OPGs, in contrast, only one-time shift from feast to famine conditions is expected to occur in this single batch.

So, what seems to emerge is not a universal stressor or stressors governing granulation but various stressors under which each cryoconite and OPG system manifests. In a hydrostatic cultivation of OPGs, OPG granulates without experiencing hydrodynamic shear, HSP, and feast-famine cycles but with other stresses, including nitrogen (specifically DIN) limitation and light stress. OPGs in reactors treating wastewater, on the other hand, encounter no limitation of nitrogen but shear stress, HSP, and repetitive feast-famine pressures. Cryoconites on Arctic ice sheets and Asian alpine glaciers would experience different levels of physical and chemical stresses, including solar radiation and hydraulic events.<sup>27</sup> And of course, cold stresses are something that microbes in cryoconites must deal with but not for those in OPGs. Despite all these (and more) variations, we see that granular products form in high similarities with the enrichment of the order Oscillatoriales in Cyanobacteria.

### **Arrays of stresses and their goldilocks interactions for photogranulation**

Hydrostatic OPG, SBR OPG, cryoconites in melt holes, cryoconites on bare ice surfaces, cryoconites in alpine glaciers as well as in Polar glaciers. All are under certain stress fields but there is no universal ‘external’ cause for their granulation. The more we look the more divergence we find. This probably makes sense because environmental factors associated with the niches of cryoconites and OPGs, and their intra variations, are already vastly different. *So, how can photogranulation occur under these barely related environmental conditions?*

Recently, Gikonyo et al.<sup>77</sup> reported another new way to produce OPGs, generating OPGs directly from activated sludge with application of mixing (i.e., hydrodynamic batch). This study conducted 27 batches that varied in the level of three forms of energy, shear stress or kinetic energy, light energy, and chemical energy, to activated sludge inoculums. It found that granulation with enrichment of Oscillatoriales only occurred under certain combinations of these three stresses. For example, granulation was observed in cultivations with ×4 dilute activated sludge inoculum combined with ‘low light’ and ‘high shear’. However, increase in light intensity in the otherwise same condition led to no observable OPG granulation. Although it was for aerobic granule sludge, Wu et al.<sup>52</sup> predicted with biofilm modeling that granulation would occur under combined effects of shear and substrate levels, and theoretically it is possible to generate aerobic granule sludge also under a non-mixing condition.

Hence, the zone of photogranulation may exist for various combinations of ensembles of stresses in which a magnitude of each stress type should also matter. This goldilocks photogranulation notion,<sup>77</sup> therefore, provides a means to explain why highly similar photogranular products occur across so diverse yet limited environments, including a hydrostatic environment which, per conventional knowledge, should not support microbial granulation.

### **Conclusions and finding the rules of photogranulation**

For cryoconite and OPG granulation to occur, it is evident that Oscillatoriales are selected regardless of environmental conditions. OPGs in hydrostatic batch, hydrodynamic batch, or in SBR operations with entries of wastewater (with its microbiome), all result in selection of this microbial group. Hydrostatic batch of OPGs provides panoramic view on it. The enrichment and dominance of Oscillatoriales occurs with turnover of original microbial community,<sup>4</sup> including bloom of microalgae which also occurs before the advent of Oscillatoriales.<sup>7,72</sup> We also know that

photogranulation does not require a specific lineage of Oscillatoriales, which means that any members of the subsection III cyanobacteria should be able to form cryoconites and OPGs. Nevertheless, the geographical dominance for cryoconites and the frequent dominance of Microcoleaceae in OPGs suggest that Oscillatoriales adapted to or preselected in regional glaciers<sup>27</sup> and wastewater environments get selected and drive the granulation process.

The selection of Oscillatoriales itself, however, is not sufficient for photogranulation. In natural environments, microbial mats dominant with Oscillatoriales are ubiquitous. The OPG systems also have shown the formation of mats or streamer-based filamentous conglomerates. There should be other or additional causation for the phenomenon to occur. For this, we contend that goldilocks interactions of various stresses causes universal physiological responses from Oscillatoriales, which select for their manifestation in spheroidal aggregates (Fig. 7). The blends of external stimuli causing their granulation must be highly variable. Yet, regardless of these variations, Oscillatoriales may respond with common physiological mechanisms, leading to the highly shared observable properties. Consequently, in order to enhance our understanding of the photogranulation phenomenon, efforts should be directed toward identifying common physiology rules, including phenotypic effects, rather than common cohort of external causations—the latter of which unlikely exists for the entire photogranulation systems.

A few metagenomic studies have been done on cryoconites<sup>78–80</sup>—There is none yet for OPGs. Edwards et al.<sup>78</sup> found functional enrichment of genes in stress response, nutrient cycling, and motility, suggesting that these microbial functionalities play role in sustaining cryoconites. Nevertheless, advancing knowledge of cryoconite granulation would require us to know actual expression and regulation of the genes, since the growth of Oscillatoriales, again, could lead to different phenotypes. Christmas et al.<sup>68</sup> sequenced the genome of *Phormidesmis priestleyi* originating from Arctic cryoconites and proposed that regulation of biosynthesis of EPS could be involved for its cold tolerance as well as the formation of cryoconite granules. We expect that Oscillatoriales' physiology for dwelling in Polar environments and forming cryoconites is not necessarily coupled, since microbial mats found in these harsh environments are also often dominated by the same cyanobacterial clade.<sup>41,43,44</sup>

Metatranscriptomic and metaproteomics evaluations will be key to finding Oscillatoriales' physiological properties required for the granulation of cryoconites and OPGs. Cryoconites from different glaciers, different melt holes, or even assorted sizes within the same hole, could show diverse transcriptomic and proteomic outfits. However, convergences on genes/proteins, especially those related to stress response and cell surface properties, are expected to emerge among all granules of cryoconites. Similar prediction is also made for OPGs. Finally, finding the –omics convergences between OPGs and cryoconites may provide the opportunity to uncover the rules for photogranulation. When these core physiological traits remain on for Oscillatoriales, we hypothesize that granular products related to cryoconite and OPG would occur regardless of geophysical environments. Indeed, Oscillatoriales-dominant granular products have appeared in coastal zones of Lake Baikal<sup>81</sup> and cultivations of Oscillatoriales isolates, from North Sea microbial mats and intertidal mudflats, in seawater environments.<sup>82–84</sup>

The implications of the photogranulation phenomenon are immense. Cryoconites avoid washout and expand on glacier surfaces, causing further decreases in surface albedo and enhanced melt. OPG presents a new path toward sustainable wastewater treatment with self-aeration and net autotrophy in compact systems. Finding the rules of photogranulation may enhance engineering of glaciers and wastewater systems to manipulate their ecosystem impacts.



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**Table 1. Geographical dominance of Oscillatoriales in cryoconites and OPGs.** The table is generated based on review of phylogenetic studies reported in: Edwards et al.<sup>64</sup>; Gokul et al.<sup>28</sup>; Milferstedt et al.<sup>4</sup>; Segawa et al.<sup>27,29</sup>; Stauch-White et al.<sup>6</sup>; Uetake et al.<sup>24</sup> Operational taxonomic units (OTU) shown in the table are OTUs from Segawa et al.<sup>27</sup>

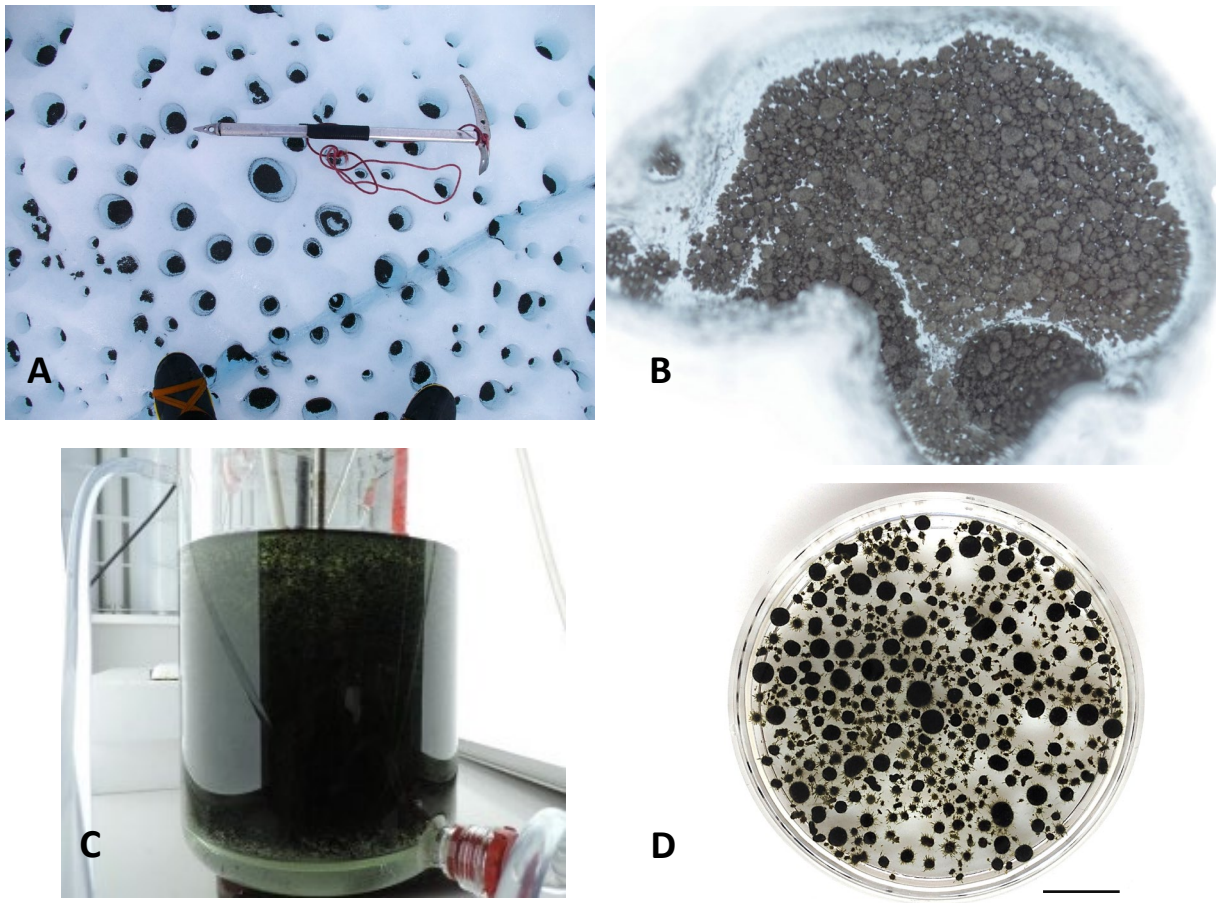
Origins of Cryoconites and OPGs	Dominant	Occasionally abundant
Arctic cryoconites	<i>Phormidesmis</i> OTU1	OTU0 ( <i>Leptolyngbya</i> )*, OTU7
Asian alpine cryoconites	<i>Microcoleus</i> OTU4 OTU0 ( <i>Leptolyngbya</i> )*	OTU8 ( <i>Leptolyngbyaceae</i> )** <i>Phormidesmis</i> OTU1
Antarctic cryoconites	OTU7 OTU8 ( <i>Leptolyngbyaceae</i> )**	
European HS-OPGs	<i>Microcoleus</i>	<i>Leptolyngbya</i> , <i>Plectonema</i>
U.S. HS-OPGs	<i>Microcoleus</i> , <i>Tychonema</i>	
French SBR-OPGs	<i>Microcoleus</i>	Unclassified Oscillatoriales
U.S. SBR-OPGs	<i>Microcoleus</i>	

HS-OPGs: hydrostatically-formed OPGs

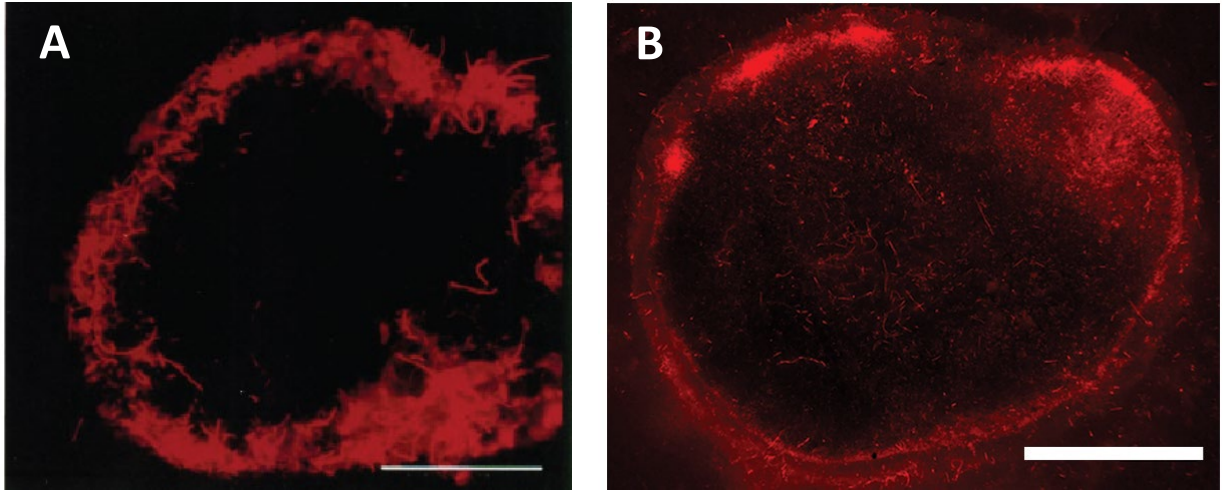
SBR-OPGs: OPGs produced in sequencing batch reactors

\*by Uetake et al.<sup>24</sup>

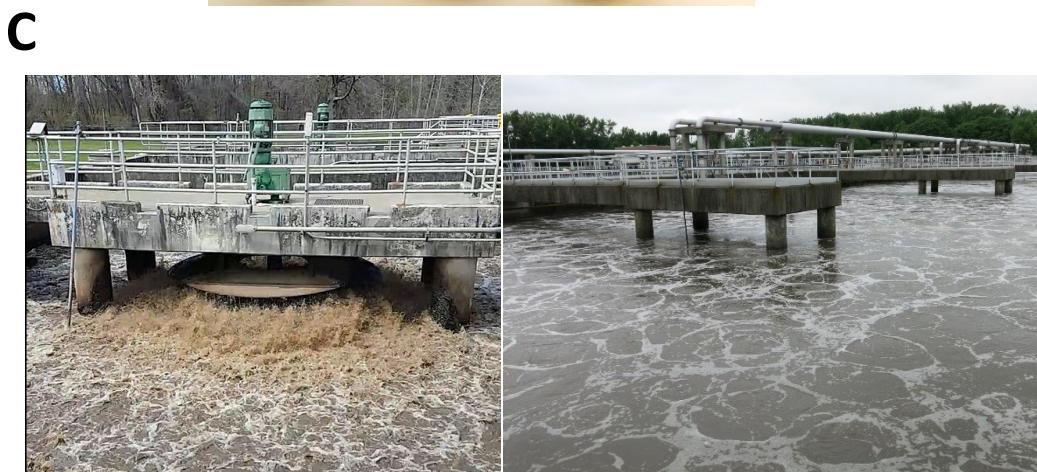
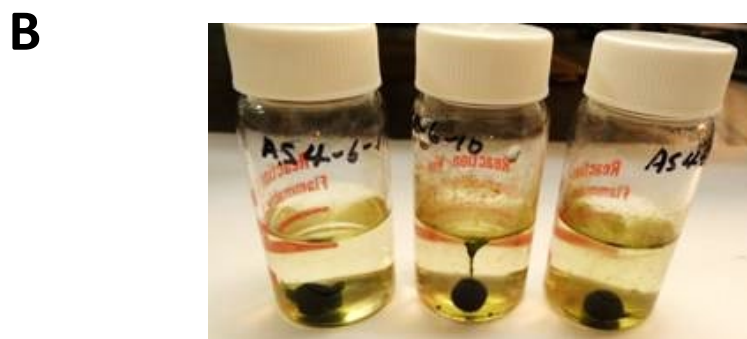
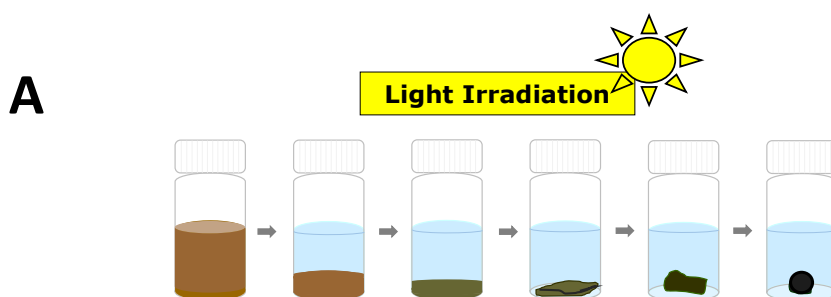
\*\*by Segawa et al.<sup>29</sup>



**Fig. 1. Cryoconites and oxygenic photogranules (OPGs).** A) Melt holes, also known as cryoconite holes, on a glacier surface on the Greenland Ice Sheet (August 2012). B) Cryoconites at the bottom of a single melt hole. C) An OPG reactor treating municipal wastewater without mechanical aeration. D) The OPG reactor's mixed biomass. Scale bar: 1 cm.

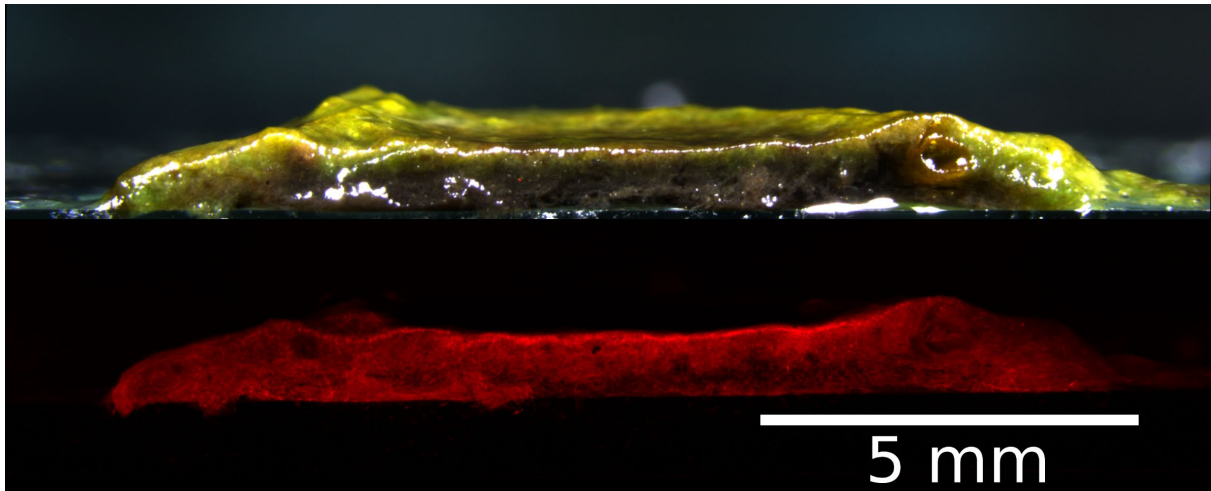


**Fig. 2. Resemblance in structural formation of cryoconite and OPG revealed by autofluorescence microscopy of cyanobacteria.** A) Cross-section of an Asian alpine cryoconite (Yala Glacier, Nepal) by Takeuchi et al.<sup>1</sup> B) Cross-section of a hydrostatically-formed OPG generated from a French activated sludge inoculum by Milferstedt et al.<sup>4</sup> Scale bars for panels are A: 0.5 mm; B: 5.5 mm.

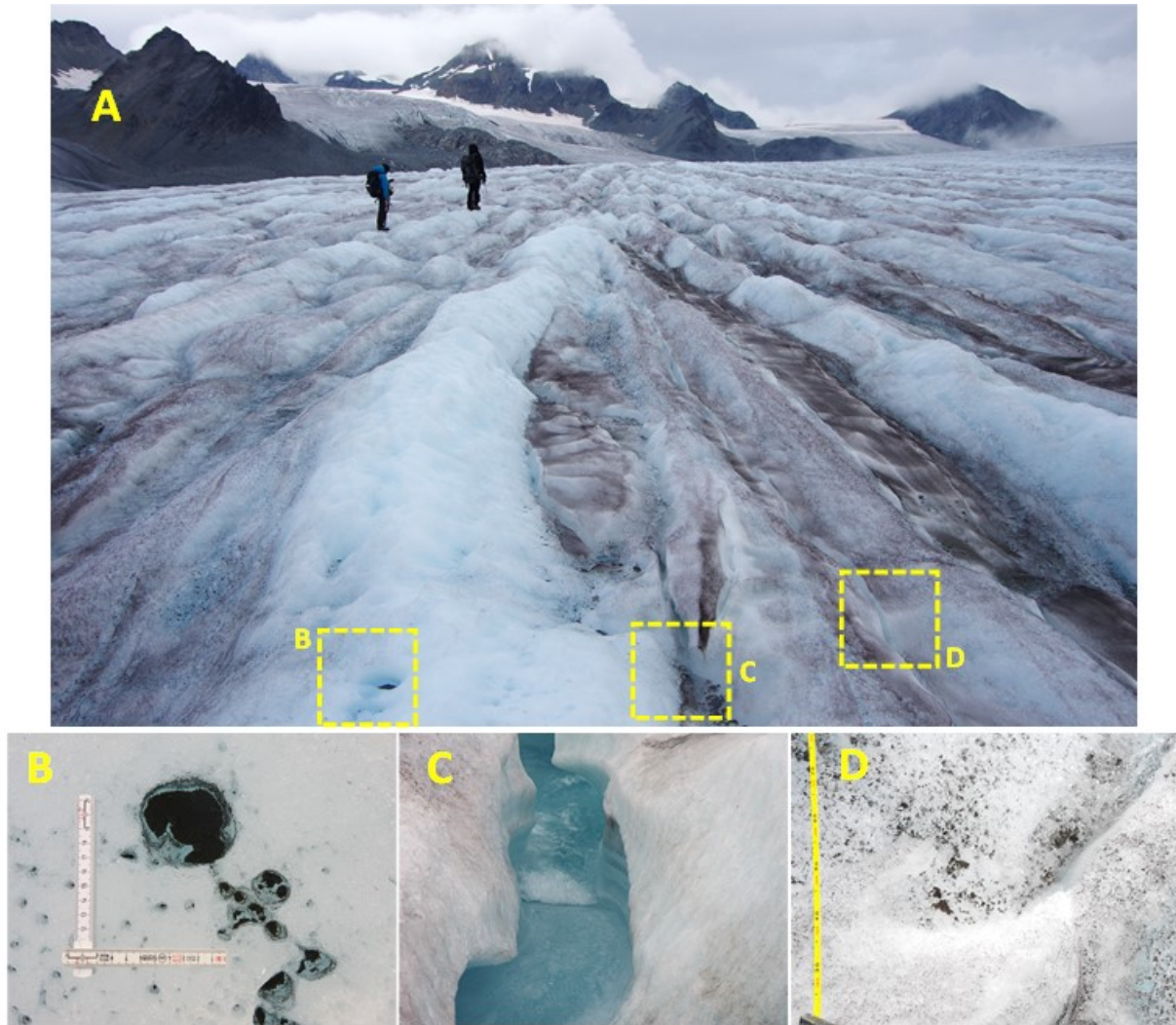


**Fig. 3. Hydrostatic granulation of OPG from activated sludge inoculum.** The mixed liquor of activated sludge obtained from the aeration basin of wastewater treatment plant, is inoculated in a closed 20 mL glass vial and placed under illumination with no agitation at room temperatures. This cultivation typically generates a single OPG in a few weeks. A) Schematic of progression in the formation of an OPG in hydrostatic cultivation. B) Hydrostatic OPGs formed from the same activated sludge inoculum. C) Aeration basins of the activated sludge process; aeration and mixing is provided by a mechanical mixing system (left) or a submersed air diffusing system (right).

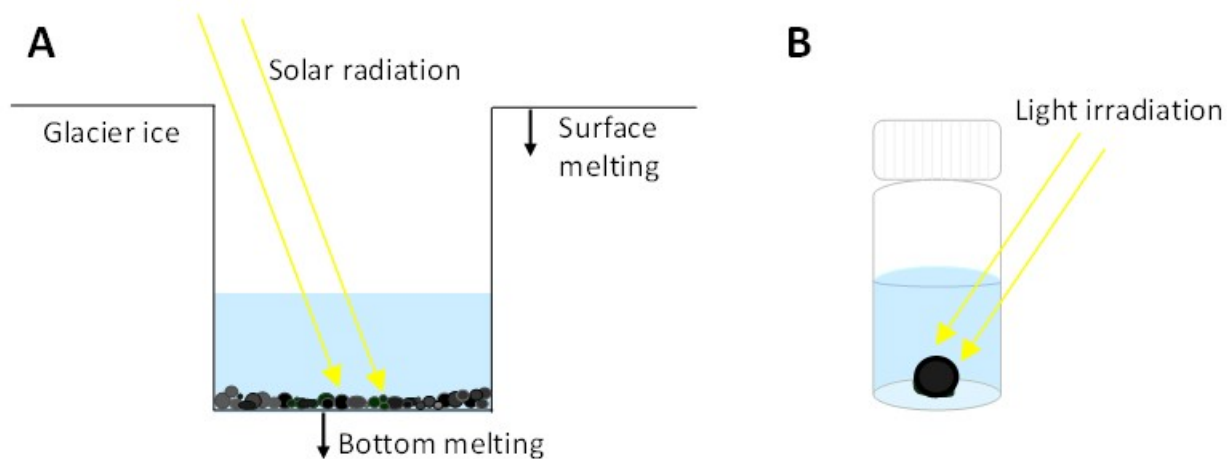




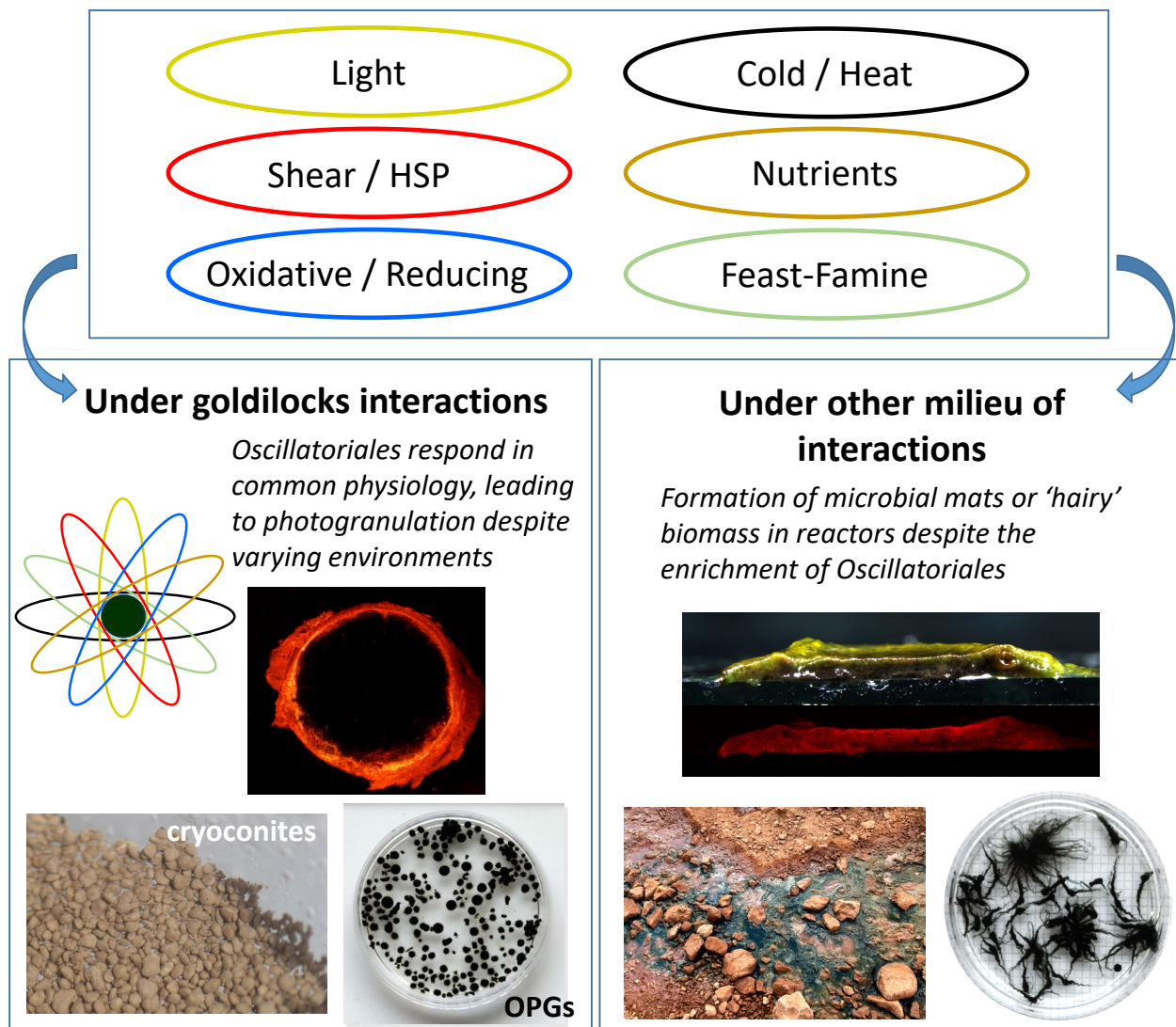
**Fig. 4. Microbial mat resulting from hydrostatic cultivation with an activated sludge inoculum.** This cultivation resulted in a flat mat instead of a spherical OPG. Top: Cross-section of the microbial mat by light microscopy. Bottom: autofluorescence of cyanobacterial phycocyanin in the same cross-section as in the top. Photo Credit: Kim Milferstedt and Jérôme Hamelin, INRAE, Laboratoire de Biotechnologie de l'Environnement (LBE).



**Fig. 5. Glacier surface with different hydraulic conditions.** A) ablating glacier ice surface (Gulkana Glacier, Alaska, August 2019), B) cryoconite holes, C) meltwater stream, and D) ice surface with distributed cryoconites.



**Fig. 6. Schematic of cryoconite hole and hydrostatic OPG system.** A) A melt hole on a glacier surface containing cryoconites at the bottom beneath quiescent water. B) A hydrostatic batch vial where a sphere-like OPG is developed from an activated sludge inoculum.



**Fig. 7. Photogranulation occurring under goldilocks interactions of stresses.** Enrichment of Oscillatoriales often occurs in both granular and non-granular morphologies. The review suggests that Oscillatoriales are granulated by universal physiological processes when they interface stresses in the goldilocks interactions. The photogranulation interaction zone may be selective or limited—otherwise cryoconite or OPG-like granules should be commonplace—yet highly variable accounting for their occurrences under barely related environmental conditions.