Contrasting snow algae communities along an elevational gradient

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*Abstract*: Snow algae grow in melting summer snowfields throughout polar and alpine regions, forming extensive blooms that can alter snow surface albedo and increase melt rate. The factors that determine when and where a snow algae bloom will form are poorly understood; fundamentally, we need to know what algae species are present in this poorly understood microbiome. We used a novel *rbc*L and 18S amplicon high-throughput sequencing (HTS) approach to assess community composition in 33 snow algae samples from the Coast Range of British Columbia, Canada. We found distinct communities at different elevations, with *Chloromonas* prevalent at lower elevations and *Sanguina* above treeline, while *Chlainomonas* was abundant across all elevations. Alpha diversity was variable; surprisingly, samples from highest elevations contained the highest diversity. We observed the snow algae *Chloromonas krienitzii* transition from green to orange over the course of the summer, and our observations suggest that different life stages of this species occupy distinct habitats above and below the snow surface. The snow algae microbiome is more diverse than previously thought, and future work must take into account variable community composition in snow algae blooms.

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# Introduction

Snow algae are cold-adapted microalgae that bloom in melting summer snow throughout alpine and polar regions1. Red snow algae blooms can cover vast areas of snowfield, which decreases snow surface albedo—by one estimate, enough to substantially increase the rate of snow melt2. Earlier snow melt exposes darker surfaces below, contributing to snow-albedo feedback and global warming3, which can threaten water supplies4 and worsen drought5. Thus, snow algae could play an important role in snow melt regulation, but little is known about how these blooms form. We don’t know how cells colonize the white snow to initiate the bloom, what triggers snow algae to divide and produce pigment, or why one patch of snow turns red while the neighboring snow remains white. Critically, we lack basic understanding of the species composition and diversity present in snow algae blooms, which is fundamental to resolving long-standing mysteries about snow algae bloom formation.

Snow algae blooms can color snow red, orange, or green, and can one or contain multiple species of algae. Most snow algae blooms are caused by green algae of class Chlorophyceae1, although blooms caused by Trebouxiophyceae6 or Chrysophyceae7,8 have been reported. Among the Chlorophyceae, both *Sanguina nivaloides*9 or *Chlainomonas*10 produce the red pigment astaxanthin responsible for the red color of some snow algae blooms. Orange snow is reported to be caused by *Sanguina aurentia*9 and some species of *Chloromonas*9,11, while algae lacking secondary pigments color the snow green, such as *Chloromonas pichinchae*12. However, some species are known from only one or two samples, and the recently described *Chlainomonas* and *Sanguina* are poorly explored, with only two species in each.

Snow algae community composition is largely unknown. Some studies batch sequencing samples, and microscopy suggests that blooms are dominated by a single species. The red cells of *Sanguina nivaloides* are widespread and cosmopolitan9,13, but 18S amplicon surveys of red snow from across the Arctic14 and Japan15 show that that snow algae blooms typically contain several secondary species; in addition to the dominant red snow algae (likely *Sanguina*, as it had not been described at the time), these studies found lower relative abundance of *Chloromonas*, as well as *Raphidonema* in Arctic sites. 33 red snow samples from across the Arctic were relatively homogeneous in community composition14, but genetic comparison of the morphologically diverse snow algae communities found in mountains from temperate latitudes16 is lacking.

Despite over a century of study, no one has successfully cultured the snow algae that produce red secondary pigments. In model organism green algae *Chlamydomonas reinhardtii* cells produce secondary pigments in just one life stage, remaining green for much of their life cycle17, thus red snow algae could originate from green cells. Green snow is frequently found in waterlogged snow, sometimes alongside red snow, leading some to hypothesize that green snow could be an earlier life stage of red snow. Two studies comparing green and red snow using amplicon high-throughput sequencing (HTS) found they were caused by different species6,15; however it remains an open question whether green snow can develop into red snow.

In this study, we surveyed snow algae communities from the mountains surrounding Vancouver, BC, Canada using light microscopy and 18S and *rbc*L amplicon HTS. The *rbc*L gene (coding for the large subunit of Rubisco) specifically targets phototrophs, and is more differentiated than the traditional 18S ribosomal marker gene, thus allowing us to specifically target snow algae. Our goal was to see what species were present, and how community composition varies over elevation.

# Results

## Field and microscopy observations of distinct snow algae bloom types

We observed pink and green patches of snow algae in forested sites beginning on May 14. Snow algae was widespread in our study area, and we collected 309 samples from 33 dates and 13 mountains over the course of the summer (S1, S2). We observed an elevational shift in snow algae throughout the season: forested sites bloomed earlier in the season and melted out sooner, while high elevation sites did not bloom until later in the season, but persisted until the first snowfall of winter 2019 (S2).

We observed some consistent patterns between cell morphologies and field site characteristics. In forest sites, orange or green snow was typically dominated by green or yellow-orange ovate cells with short spines (*Chloromonas cf. brevispina*; Fig. 1). In snow runnels in forest clearings we observed a shift over repeated site visits; on initial visits snow runnels contained green snow hidden from sight 2 - 5 cm below the surface (Fig. 1b, S4\_), on subsequent visits the surface of these runnels was orange and contained cell morphologies resembling *Chloromonas krienitzii* (Fig 1e, S3\_, S4\_). Red or pink snow was mostly found in open areas with high sunlight, and contained cells similar in appearance to *Sanguina nivaloides* or *Chlainomonas rubra*, in many sites mixed in with green cells of variable morphology (Fig 1). *Sanguina nivaloides* morphologies were primarily at high elevation sites, although we did observe several blooms dominated by this morphospecies in shaded forest sites.

## Diverse *rbc*L reveals snow algae taxonomy with high-resolution

Samples were dominated by green algae (Chlorophytes), primarily the genera *Chloromonas*, *Chlainomonas*, and *Sanguina* (Fig. 2). Trebouxiophyceae (Chlorophytes) such as *Raphidonema* were present in low abundance (Fig. 2), as well as low abundance of Ochrophytes (primarily *Hydrurus*) at several low elevation sites (S5). One *rbc*L OTU defied taxonomic classification, with a best BLAST score of 88%, and top hits in both Trebouxiophyceae and Chlorophyceae (Algae H, Fig. 2).

Our taxa-specific *rbc*L primers showed considerable variation within *Chloromonas* and the closely-related *Chlainomonas* (Fig 2, S6). We found several distinct clusters among *Chloromonas* amplicon sequence variants (ASVs) not matching any known species on GenBank (I, G, Fig. 2), but the majority of *Chloromonas* ASVs did not form distinct clusters (Other *Chloromonas*, Fig. 2).

Our three relative abundance data sets were generally consistent. 18S, *rbc*L, and cell count data were correlated for *Sanguina* (stats). *Chloromonas* and *Chlainomonas* cell counts were correlated with *rbc*L (stats, stats); however, 18S classified samples dominated by *Chlainomonas* as containing *Chloromonas* (S5), likely due to high target region similarity (S7). However, in some samples cell morphology was a poor predictor of taxonomic composition. For example, we were surprised to find sample X1.1 contained few *Sanguina* reads (Fig. 3b, S5b) despite being dominated by red cells similar of similar morphology to *Sanguina* (Fig. 1g, S4\_).

## Snow algae community composition varies with date and elevation

We found snow algae community composition was strongly influenced by elevation across all three datasets: *rbc*L, 18S, and cell count. *Sanguina* was prevalent at higher elevations, and *Chloromonas* dominant at lower elevations (Fig. 1g, Fig. 3, S5). Low elevation sites were compositionally similar, while high elevation samples showed inter-site variability (UniFrac Fig. 3a, S8). Lower elevation sites were dominated by *Chloromonas krienitzii* and *Chlainomonas rubra*, while *Sanguina nivaloides* was only abundant in samples from above treeline (Fig. 3b), along with one OTU of *Chloromonas*, and *Raphidonema* (H, I, Fig. 3b). Several sites above treeline were more similar to low elevation sites, including patches of green snow above treeline dominated by *Chloromonas* (N1.5, G1.4, Fig. 3) and red snow patches dominated by *Chlainomonas* (G1.2, P1.9, Fig. 3).

*Chlainomonas* was abundant across all elevations. Some samples were nearly completely dominated by *Chlainomonas*, but it was widespread in samples containing *Sanguina* and *Chloromonas krienitzii* as well (Fig. 3b).

## A green snow algae turning orange at the surface

Snow algae blooms dominated by *Chloromonas krienitzii* formed in low angle runnels, often in clearings at elevations near treeline. Based on our observation that the subsurface snow was green, and surface snow turned orange through the course of the season we sampled both orange and green snow to see if they were the same species (Fig. 4a). The surface samples contained primarily orange cells resembling *Chloromonas krienitzii*, while the subsurface samples contained green cells including biflagellates and polar quadriflagellates (Fig. 4b, c). Both surface and subsurface samples were dominated by *Chloromonas krienitzii* reads (Fig. 4d). Unexpectedly, we found high relative abundance of *Chlainomonas* in surface samples, and in one subsurface sample, which we did not detect in cell counts.

# Discussion

We found snow algae communities were highly variable from site to site, with communities at lower elevation dominated by *Chloromonas*, and higher elevation sites dominated by *Sanguina*. Microscopy studies of snow algae in USA and Slovakia found similar results, with *Chloromonas* is prevalent in forested areas and *Chlamydomonas cf. nivalis* above treeline16,18. Our cell counts, 18S, and *rbc*L data support this finding.

The obvious question is: what environmental factors driving those differences in community composition? Further research will be needed to determine the specific environmental factors driving community differences, but our field observations does provide some testable hypotheses. In forested areas, green cells would be shaded from damaging solar radiation, while above treeline pigmented cells of *Sanguina* and *Chloromonas* are likely protected by their astaxanthin pigment19,20. This fits with our observation that green cells of *Chloromonas krienitzii* were hidden below the snow surface, and pigmented cells were more prevalent on the surface (Fig. 4); possibly pigment production is triggered as snow level melts lower, and cells are exposed to higher light at the surface. However, this does not explain why *Chloromonas krienitzii* was not found at higher elevations: our *C. krienitzii* dominated samples were taken from sites that were ~100 m from the nearest tree, and were not notably shaded by topography in any way. Anecdotally, we observed *C. krienitzii* developed over running water, so their distribution could be linked to the presence of streams below the snowpack.

Our results show that *Chlainomonas* is an important, hitherto unrecognized component of the snow algae microbiome. Prior to this study, *Chlainomonas* were thought to only bloom in highly saturated, waterlogged snow overlying mountain lakes10,21, but we found *Chlainomonas* at most sites we sampled. Only one site high in *Chlainomonas* was near water (S9), the other *Chlainomonas* dominated sites were not near water, nor was the snow noticeably mushier than the surrounding snow. This does not rule out the possibility that these sites contained high moisture at some point; further sampling is needed to determine its habitat distribution, but the broad distribution of *Chlainomonas* in most samples suggests that this taxa may be an important component of the red snow microbiome.

In contrast to typical alpine ecosystems with lower diversity at higher elevations, we observed the highest diversity at the highest elevation sites (W1.1, W1.2, P1.5, S8). These sites were our only samples collected on glaciers, and were also collected latest in the summer. These samples contained the highest proportion of *Raphidonema* (Trebouxiophyceae I, Fig. 3), which is phylogenetically distant from Chlorophyceae, and thus could contribute heavily to phylogenetic diversity metrics, however Shannon and Simpson diversity was also highest for these three samples, which only took into account the evenness of OTU abundance, so the presence of *Raphidonema* alone cannot explain the even spread of OTUs at these sites. 22 suggest that *Raphidonema* is a slow-growing opportunistic soil algae that blows into snow fields from surrounding dirt to colonize the snow. Perhaps it’s slow growth in snow means it is only found later in the season; or sites late in the season were closer to bare ground that seeded populations of this algae.

Our use of cross-referenced 18S, *rbc*L, and cell count data uniquely allowed us to compare our findings between datasets. *Sanguina* was highly correlated between all three. One notable difference was that no reads were assigned to *Chlainomonas* from our 18S library, despite this genera being highly abundant in *rbc*L. Samples containing primarily *Chlainomonas* in our *rbc*L and cell count data were marked as containing only *Chloromonas* in 18S. At the time of publication, only two 18S *Chlainomonas* were present on GenBank (MF803743.1, MF803745.1); possibly our *Chlainomonas* sequences are different enough from the reference that they were mislabelled as *Chloromonas*, especially likely given how closely related the two genera are. Samples dominated by *Sanguina* in our 18S library contained lower abundance in *rbc*L, which is likely due to primer bias: because our custom *rbc*L primers were designed to target *Chloromonas* and *Chlainomonas* (*Sanguina* not being available at the time), this likely led to a over-representation of these taxa in our *rbc*L library.

Our findings highlight the remaining unexplored diversity in the snow algae microbiome. One abundant OTU of algae, “Algae H” is widespread and abundant, but poorly matches all *rbc*L GenBank sequences. However, out 18S sequences did not detect any such cluster. Non-specific priming on an unsequenced gene is one possibility, another is that this represents a novel clade of snow algae, or perhaps an endosymbiotic algae within the ciliates that were abundant in many of our samples. Interestingly, the sample with the highest relative abundance of this OTU (X1.1) had red cells that were visually distinct from other *Sanguina*, with thicker cell walls that were slightly less than perfectly spherical in some cells (S4); clearly, further investigation is needed.

# Conclusions

Our side by side comparison of snow algae communities shows that they are more diverse than previously thought, and suggests that some snow algae may be adapted to specific micro-habitats such as shade and moisture within the melting snowpack. As the different snow algae species may have varying habitat preferences, different taxa could have different life cycles that permit them to survive in their niche, thus future work to understand the biological mechanisms of snow algae blooms should take community composition into account. Different communities of snow algae will likely affect albedo and local snowpack chemistry in different ways6, and so different snow algae communities could impact the microbiome and greater ecosystem in unique ways.

# Methods

## Field sampling and microscopy

We collected snow algae from mountains near Vancouver, British Columbia, Canada throughout the summer of 2018. To capture the extent of snow algae diversity in our local mountains we collected as many samples as possible from different elevations, dates, mountains, and micro-habitats within the snow (S2). In total we collected 310 colored snow samples from 13 different mountains on 33 different dates from elevations between 880 m and 2150 m above sea level. In early season we sampled at lower elevations, moving uphill as the snow algae bloom progressed upwards in elevation.

We scooped samples from visibly colored snow into 50 mL centrifuge tubes using sterile technique. To prevent melting en route to the lab we stored tubes in a bag of snow. Back in the lab, we melted each sample at room temperature on the bench and removed a 1 mL aliquot for light microscopy. Immediately after, samples were stored at -20 °C for up to eight months until DNA extraction.

We used light microscopy to characterize the cell morphologies in each sample. We prepared a slide of cells fixed in 2% gluteraldehyde, and at 400x magnification starting from the center of the slide counted up to 100 cells. We classified each cell as either *Sanguina nivaloides*9, *Chloromonas cf. nivalis*23,24, *Chloromonas cf. brevispina*16,25, *Chloromonas krienitzii*25 *Chlainomonas rubra*21, or “Other” based on similarity to published photographs.

## DNA extraction and amplicon library preparation

We selected 33 out of 310 samples for rbcL and 18s high-throughput amplicon sequencing. We chose these samples to represent the variation in date, elevation, geographic location, snow color, micro-habitat, and cell morphology. We freeze-dried these samples for up to 48 hours until until samples appeared completely desiccated. To allow water vapor to escape sample tubes while freeze drying we poked holes in each lid; as a safeguard against cross-contamination from airborne snow algae powder we wrapped tubes individually with paper towels. We mini-pestled 5 mg of sample at room temperature to physically rupture cell walls. Based on our observation that samples dominated by *S. nivaloides* cell morphologies had lower DNA yields, and had many intact cells following various lysis methods, we used 20 mg of these samples to increase our DNA yield.

To extract DNA from the crushed cells we added 800 mL 1x CTAB extraction buffer26, 1% B-mercaptoethanol, 5 L each of proteinase K and RNAase A, and incubated these at 65 °C for 30 minutes. We centrifuged samples at 10,000 g for 3 minutes to pellet cell debris, then added 700 L of supernatant to an equal volume of 24:1 chloroform:isoamyl alcohol. We inverted samples to mix and centrifuged again at 12,000 g for 10 minutes. We precipitated DNA by transferring the top layer to 700 L ice-cold ethanol, which we gently mixed, and spun over Qiagen DNA columns for 30 s at 15,000 g. We washed columns twice with 70% ethanol, and finally dissolved our DNA by spinning with 50 L of sterile TE buffer. As a negative control we processed a sterile distilled water sample alongside each batch, treating it exactly the same as the other tubes; all negative controls did not contain Qubit-detectable levels of DNA.

We used rbcL as a marker gene, which offers high resolution between microalgae species27, and has reference data for snow algae available on GenBank. We also sequenced each sample with 18S primers, due to it’s coverage across a wide range of taxa, and its ubiquity in reference databases. We designed rbcL primers to target an approximately 400 bp section of this gene, based on 20 snow algae rbcL GenBank sequences from *Chloromonas* and *Chlainomonas* (GenBank accession numbers AB434272.1, LC012752.1, LC012747.1, AF517072.1, LC012738.1, LC012739.1, AB434267.1, EU030690.1, LC360494.1, AJ001878.1, AB022225.1, DQ885964.2, DQ885962.1, AJ001879.1, AB022226.1, AB022530.1, LC012751.1, AB504764.1, EU030689.1, AB101508.1). *Sanguina* sequences were not included because they were not available at the time. We designed primers using the primer design tool from the Eurofins Genomics website (www.eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/). The resultant primers were rbcL369F (5’-GAA CGT GAC AAA TTA AAC AAA-3’) and rbcL870R (5’-ACC WGA YAD ACG WAG AGC TT-3’). To target 18S we used Euk1181 (5’-TTA ATT TGA CTC AAC RCG GG-3’) and Euk1624 (5’-CGG GCG GTG TGT ACA AAG G-3’)28.

We constructed our amplicon library using a two-step PCR29. In the first PCR we amplified template DNA using our primers attached to a universal adapter, and in the second PCR we re-amplified that product to attach a 6 bp index to the universal adapter at the 3’ end. The first PCR total volume was 25 L, consisting of 1 L template, 12.5 L Q5 high-fidelity 2X MM (New England BioLabs), 1.25 L each of forward and reverse primer, and 9 L of ddH2O. The second PCR was the same except we reduced our reaction volume to 20 L by using only 5 L of ddH2O. The cycling conditions were the same for both primer pairs for the first PCR, with an initial denaturation at 98 °C for 30 s, followed by 30 cycles of 98 °C for 5 s, 58 °C for 10 s, and 72 °C for 25 s, with a final extension at 72 °C for 2 minutes. For the second indexing PCR we started with an initial denaturation at 98 °C for 30 s, then 10 cycles of 98 °C for 10 s, 65 °C for 30 s, and 72 °C for 30 s with a final denaturation of 72 °C for 5 min. After each PCR, we purified using Agencourt AMPure XP kit (Beckman Coulter). We quantified final DNA concentration with Qubit (Thermo Fisher), and standardized sample concentration for pooling. The pooled library was then loaded and run on an Illumina MiSeq V3 kit.

## Bioinformatic processing

Reads were demultiplexing using CUTADAPT30. Samples were filtered and trimmed, errors removed, dereplicated, pair end reads merged, and chimeras removed following the default pipeline of DADA231.

We assigned taxonomy for both *rbc*L and 18S ASVs using IDTaxa with threshold set to 5032. We made custom reference databases for each to include snow algae GenBank sequences. We made our rbcL database by downloading 30,865 algae rbcL sequences from GenBank with the query “rbcl[gene] AND (algae OR chlorophyta OR trebouxiophyceae) NOT (18s OR ribosomal OR psaB OR atpB) AND 300:2000[slen] NOT plasmid NOT unverified NOT mRNA NOT bacteria NOT mitochondrion”. We removed ambiguous snow algae annotations based on the most recent snow algae phylogeny23. Because entries labelled as *Chloromonas cf. brevispina* and *Chloromonas cf. nivalis* are polyphyletic we re-labeled these as “unassigned *Chloromonas*”. To assign 18S reads we initially ran our ASVs against SILVA33, and then re-ran ASVs assigned to Chlorophyta on our custom 18S snow algae database based on the following GenBank query: “(18S OR ribosomal)[gene] AND (chloromonas OR chlainomonas OR sanguina OR raphidonema OR KMY-2018) NOT (rbcL OR psaB OR atpB) AND 300:3500[slen] NOT plasmid NOT unverified NOT mRNA NOT bacteria NOT mitochondrion”. This database was edited to reflect the most recent snow algae taxonomy and remove ambiguous annotations10,23.

As most OTU clustering algorithms are optimized for 18S data, we chose to assign high level OTUs based on the results of t-SNE clustering34 (Fig. 2a). We tested this with perplexity values ranging from 1 to 100, and found comparable results with perplexity ranging from 10 to 50. We compared sample similarity using UniFrac with default settings35.

For software information, see Supplementary Table 9. All raw fastq files are freely available on the European Nucleotide Archive under the project accession PRJEB34539. All scripts are available at <https://github.com/cengstro/bc_snow_algae_amplicon>.

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# Author Contributions

CE, LQ, and KY designed this study. Samples collected by CE with assistance from KY and LQ. KY and CE prepped samples for sequencing. All analyses were completed by CE and KY. All authors discussed the results and contributed to the final manuscript. CE wrote the manuscript with major input from LQ and KY.

# Additional information

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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