VARIATION IN SNOW ALGAE BLOOMS WITH ELEVATION

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# Abstract

Snow algae blooms cover vast areas of summer snowfields worldwide, and can reduce albedo and increase snow melt. Despite their global prevalence, little is known about the algae species that comprise snow algae blooms. We used 18S and *rbcL* metabarcoding and light microscopy to characterize species composition of samples from 31 snow algae blooms from alpine and subalpine habitats in the Coast Range of British Columbia, Canada. This study is the first to thoroughly document regional variation between blooms. We found all blooms in the region were dominated by the genera *Sanguina*, *Chloromonas*, and *Chlainomonas*. There was considerable variation between blooms, most notably species assemblages above treeline were distinct from forested sites. In contrast to previous studies, the snow algae genus *Chlainomonas* was abundant and widespread in snow algae blooms. We found few taxa using traditional 18S metabarcoding, but the high taxonomic resolution of *rbcL* revealed substantial diversity, including OTUs that likely represent undiscovered species of snow algae. These three cross-referenced datasets reveal that alpine snow algae blooms are more diverse than previously thought with different species dominating at different elevations.

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# Introduction

Each summer, vast areas of snow surface are coloured red by snow algae blooms in polar and alpine snowfields worldwide. Red snowfields have been found on every continent [1–5] and overlying Arctic sea ice [6]. Snow algae blooms can be quite extensive: in Alaska, remote sensing detected snow algae in one third of a 1,900 km2 icefield [7]. In recent years snow algae have received attention for their role in reducing snow surface albedo, which could substantially increase snow melt [7, 8]. Thus, snow algae could impact summer water supplies held in mountain snowpack, and reduce glacier mass balance. Snow algae blooms have been recorded throughout history since the time of the ancient Romans [9, 10]; we do not know whether the extent and duration of blooms are increasing with extended melt seasons due to global warming. Despite their potential importance in accelerating the consequences of global warming, we are only beginning to identify the algae species that comprise snow algae blooms.

Microscopy reveals a diversity of cell morphologies in snow algae blooms, but different species can look nearly identical [11] and the same species can look completely different depending on environmental conditions. The snow algae *Chloromonas krienitzii* are small green biflagellates in culture, but cells in field samples are nearly twice the diameter, with orange pigment and short spines on a thick cell wall [12]. The environmental cues that trigger this transformation are not known, but in the freshwater green algae *Haematococcus pluvialis* red secondary pigments are produced in response to high light [13]. Green blooms of snow algae are less frequently described in the literature than red blooms, and some researchers have suggested that green snow develops into red snow [14]. However, metabarcoding studies to date have found that green and red snow are caused by different species [15, 16].

Green algae of class Chlorophyceae are predominant in many snow algae blooms, including the genera *Sanguina*, *Chloromonas*, and *Chlainomonas*. The genus *Sanguina* was only recently established and contains just two species; however, many sequences from red snow form an as-yet unnamed sister clade to *Sanguina* [17]. Previous work suggests that *Sanguina nivaloides* predominates in red blooms worldwide, while *Sanguina aurantia* is less common, having been found in just three samples from Svalbard [17]. Many species of snow algae have been assigned to *Chloromonas*, including 12 species that have been cultured [11]. Various *Chloromonas* species can form green, orange, or brown coloured blooms on the snow surface, and are found worldwide [18–20]. Less is known about *Chlainomonas,* which has been found in central Europe, western USA, and New Zealand [21–23]. The distinctive red-pigmented cells of this genus (nearly twice the diameter of *Sanguina nivaloides*) have only been reported from waterlogged snow overlying alpine lakes. 18S rDNA and *rbcL* (coding for the large subunit of Rubisco) sequence shows that *Chlainomonas* is closely related to *Chloromonas*. While Chlorophyceae predominate in many snow algae blooms, other classes of snow algae have been reported, including Chrysophyceae in Antarctica, the Alps, and Svalbard [24, 25], and Trebouxiophyceae in green snow in Greenland [16].

While many species of snow algae have been described on the basis of morphology and Sanger sequencing, metabarcoding studies have found red snow algae blooms are dominated by relatively few OTUs and are highly homogeneous between geographically distant sites. OTU composition was similar between geographically distant sites in a study of 33 Arctic red snow samples: all were dominated by “uncultured Chlamydomonadaceae 1 and 2” OTUs with low relative abundance of *Chloromonas polyptera* and *Raphidonema nivale* [8]. Another study using ITS2 metabarcoding found 24 polar red snow sites contained similar algae assemblages, dominated by “uncultured Chlamydomonadaceae A and B” with secondary abundance of *Raphidonema* and *Chloromonadinia* [1]. Other studies using 18S metabarcoding were limited to class level taxonomic assignments of algae [26]—being highly conserved, short 18S reads cannot distinguish between closely related species or genera.

Based on how little is known about the regional variation in species composition of snow algae blooms, we set out to answer the following questions: what species of snow algae are found in our region? What patterns of co-occurrence exist between species? Which species are the most abundant? Are there distinct bloom types dominated by different species? We compared snow algae species composition of 33 samples from the Coast Range of British Columbia using light microscopy and 18S and *rbcL* metabarcoding. *rbcL (*coding for Rubisco large subunit) targeted photosynthetic species with higher taxonomic resolution than 18S, revealing previously unknown diversity. By using three cross-referenced metrics, we were able to account for some of the biases inherent in morphology-based identification and PCR-based metabarcoding. We found snow algae bloom species composition was highly variable from site to site, and blooms were dominated by different species at different elevations.

# Materials and Methods

## Field sampling and microscopy

We collected 309 snow algae samples from alpine and subalpine sites in the Coast Range near Vancouver, British Columbia, Canada throughout the summer of 2018 (Supplementary Figure S1). We collected red, orange, and green snow samples from 13 different mountains from sites ranging from 880 to 2150 m above sea level (Supplementary Table S1). As snow melted at lower elevations, we collected samples from progressively higher elevations; we did not detect snow algae at higher elevations until later in the summer. We scooped samples from visibly coloured snow into sterile 50 mL centrifuge tubes, and packed tubes in snow to keep samples cold during transport back to the lab. We melted each sample at room temperature on the bench, removed a 1 mL aliquot for light microscopy, and then stored the remaining sample at -20 °C for up to eight months until DNA extraction.

We immediately fixed microscopy aliquots in 2% gluteraldehyde, and stored at 4 °C for up to 72 hours. We quantified morphospecies relative abundance in 122 samples by identifying 100 cells on a haemocytometer under 400x light microscopy. We classified cell morphology based on similarity to published photographs of *Sanguina nivaloides* [17], *Chloromonas cf. nivalis* [20], *Chloromonas cf. brevispina* [12], *Chloromonas krienitzii* [12], *Chlainomonas rubra* [21], and for cells that did not fall into one of these categories as either “green cell” or “other”.

## DNA extraction and amplicon library preparation

We selected 33 samples for *rbcL* and 18S metabarcoding. We chose this subset to include samples from different mountains, elevations, and dates, including samples containing distinct or unfamiliar cell morphologies. To lyse the cells, we freeze-dried samples and mini-pestled 5-20 mg at room temperature before incubation in CTAB lysis buffer [27]. We extracted DNA in small batches (5-6 samples) using chloroform:isoamyl alcohol [28], and purified DNA using ethanol and spin columns (Qiagen, Hilden) (Supplementary Protocol S1). As a negative control against cross-contamination we processed a tube of sterile distilled water alongside each batch, and tested this for DNA with Qubit fluorometric quantification (Thermo Fisher, Waltham, MA).

We designed custom primers to target a hypervariable region of snow algae *rbcL*.This geneis an established barcode for green algae [40], and is highly differentiated between snow algae species (Supplementary Figure S2). We designed primers to target a 400 bp section of *rbcL*, based on the consensus of 20 GenBank snow algae sequences (Supplementary Table S2). *Sanguina* sequences were not included because they were not available at the time. We designed our primers with the Eurofins primer design tool (<https://www.eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/>). To target 18S, we used the universal primers Euk1181 and Euk1624 targeting the V7-V8 hypervariable regions [29] (Supplementary Table S2).

We constructed our 18S and *rbcL* amplicon libraries using a standard two-step PCR approach [30] (detailed conditions in Supplementary Protocol S2). We purified PCR product using Agencourt AMPure XP kit (Beckman Coulter, Brea, CA). We standardized sample DNA concentration with Qubit, pooled our library, and sequenced using an Illumina V3 kit on a MiSeq (Illumina, San Diego, CA).

## Bioinformatic processing

We demultiplexed reads with CUTADAPT [31], and filtered, trimmed, denoised, dereplicated, merged paired-end reads, and removed chimeras following the default pipeline of DADA2 [32]. We assigned taxonomy to amplicon sequence variants (ASVs) using IDTaxa [33] using a high confidence parameter setting (threshold=50). Because snow algae are not well represented on databases such as SILVA, we made custom databases for both 18S and *rbcL* using all available snow algae sequences on GenBank (scripts and databases available on github). Additionally, we classified 18S ASV taxonomy with SILVA [34].

We clustered ASVs into OTUs using t-SNE [35] and DBSCAN [36]. To ensure t-SNE clusters were not an artifact of parameter selection, we ran t-SNE with different “perplexity” parameters and found the same clusters were generated from 10 to 50. To validate this unconventional OTU clustering method we overlaid these OTU clusters on phylogenetic trees using IQTree [37] (Supplementary Figure S3).

# Results

We observed morphologically distinct snow algae blooms at different elevations (Figure 1; Supplementary Figure S4). Red snow was prevalent in areas of high solar exposure above treeline, and most of these sites were dominated by cell morphologies we classified as *Sanguina cf. nivaloides*. Cells resembling *Chlainomonas rubra* were common in sites at all elevations, often as the dominant cell type. Below treeline, the dominant cells morphologies were classified as *Chloromonas cf. brevispina,* *Chloromonas cf. nivalis,* and green cells that we did not attempt to classify.

Both 18S and *rbcL* amplicon libraries were dominated by reads assigned to Chlorophyta (Figure 2). The 18S SILVA taxonomy contained 50 ASVs assigned to Chlorophyceae, 11 Trebouxiophyceae, and 7 Chrysophyceae ASVs, while the *rbcL* library contained 603 Chlorophyta and 41 Trebouxiophyceae ASVs. We were able to distinguish seven ASV clusters using *rbcL,* which we define here as OTUs, compared with just three 18S-defined algal OTUs (Supplementary Figure S5). The most frequent *rbcL* genus-level assignments were *Chloromonas*, *Chlainomonas*, and *Sanguina*. Although the majority of ASVs were not assigned to a known genus, most ASVs were genetically similar to one of these three genera. Two OTUs ,“D” and “F”, were closely related to *Chloromonas* but did not closely match any known species (Figure 2). OTU “E” was not assigned to genus level, and the ten best BLAST matches included six different genera within Chlamydomonadaceae (86-87% sequence match), two of which were *Chloromonas* snow algae (LC012735).

18S and *rbcL* taxonomic composition varied with elevation. Low elevation samples were genetically similar, distinct from most high elevation samples in NMDS of *rbcL* UniFrac distances [38] (Figure 3). Samples that were collected latest in the season had the highest diversity. Although there was no statistically significant trend between Shannon diversity and date, there was a weak correlation between Faith’s phylogenetic diversity and date (Pearson’s *r*=0.36, p=0.04) (Supplementary Figure S6). *Sanguina* predominated above 1500 m, but was absent below this elevation (Figure 4). High elevation samples contained one OTU of *Chloromonas* that was absent from low elevation sites (Figure 3 OTU “F”). *Raphidonema* was restricted to three samples from high-elevation snow overlying glaciers (best BLAST match to *Raphidonema longiseta*, KM462868.1). Most snow algae blooms above treeline were red, but we did collect two green snow samples from above treeline (N1.5, G1.4). These were dominated by reads assigned to *Chloromonas.* Across all elevations *Chlainomonas* was highly abundant, found in red snow samples in high relative abundance as well as alongside other genera as a secondary component. *Chloromonas krienitzii* was predominant around 1200 m in clearings or sparse trees.

A green to orange transition occurred from May to June at one site dominated by *Chloromonas krienitzii*. In May we observed green snow hidden 2 to 5 cm below the snow surface of a runnel (sample S2.2), but on subsequent visits in June (S4.6, S6.1, S9.2, S11.2) the surface of the runnel was orange. Microscopy revealed orange spherical cells resembling *Chloromonas krienitzii* [12], while the green snow beneath contained motile green flagellates. *rbcL* comparison of green and orange snow showed both were dominated by reads assigned to *Chloromonas krienitzii*, with the orange snow containing higher abundance of *Chlainomonas* (Supplementary Figure S7).

# Discussion

Snow algae blooms are a widespread and globally important phenomenon, yet until now the distinctions between different types of snow algae blooms containing different species were poorly defined. We present multiple data sets demonstrating elevational patterns in alpine snow algae bloom species composition. Most dramatically, *Sanguina* was dominant in red snow above treeline, while green and orange blooms of *Chloromonas krienitzii* were dominant in runnels at lower elevations. We found unexpected diversity within *rbcL* that we did not detect using 18S, including potentially unnamed species. Although *Chlainomonas* was previously only reported from red snow overlying lakes [23] we found it was widespread and abundant in a variety of alpine habitats.

Our *rbcL* data suggest *Chlainomonas* may be more widely distributed than previously thought. We did not find any evidence that *Chlainomonas* was restricted to waterlogged snow overlying lakes, as has been previously suggested [21,23]. Only one *Chlainomonas*-dominant sample was located in waterlogged snow at the edge of a melt pool (sample S8.11); the other *Chlainomonas-*dominant sample sites were not notably wetter than the surrounding snow nor located over frozen lakes. *Chlainomonas* was conspicuously absent in our 18S dataset, supporting the idea that *Chlainomonas* may have been missed in previous 18S metabarcoding studies. However, *rbcL could have overestimated abundance of this genus*: *Chlainomonas* was found in consistently higher relative abundance in our *rbcL* dataset than in cell counts (Figure 3). One possible explanation is high *rbcL* copy number: the large cells of *Chlainomonas rubra* have multiple parietal chloroplasts per cell [23], and *rbcL* is located in the chloroplast genome.

Our findings highlight the remaining unexplored diversity in the snow algae microbiome. *Chloromonas* contained the highest diversity; two OTUs related to this genus did not match any known species, and could represent unnamed species (Figure 2 “D” and “F”). However, the majority of *Chloromonas* ASVs did not form distinct clusters, perhaps due to overlapping intraspecific *rbcL* variation between species. Given the genetic distance between OTU “E” and other *rbcL* ASVs, we would expect to see a corresponding OTU in 18S, but we did not. *rbcL* diversity is likely higher within *Chloromonas* than other genera because, unlike *Sanguina* [17], most species of *Chloromonas* lack a pyrenoid [39], which houses high concentrations of cross-linked Rubisco. Lacking the pyrenoid, *Chloromonas* species have many non-synonymous mutations in the region of *rbcL* that codes for binding Rubisco together [39]. While this means that *rbcL* is a poor indicator of phylogeny within this genus [39], it nonetheless is highly differentiated and therefore an effective barcode [40].

Variation in bloom composition could be due to a wide range of habitat features. Day length, snow moisture, nutrient availability, or shade could all plausibly influence community composition. *Sanguina* and *Chloromonas* “C” were only found in late summer samples from high alpine sites above 1500 m (Figure 3). Differences in canopy cover fail to explain this pattern, as many low elevation sites also received full sunlight but did not contain these OTUs. Liquid water and garden fertilizer can stimulate snow algae growth [7], but whether different nutrients select for different species is unknown. Day length could explain some of the seasonal variation we observed: at higher elevations, the timing of melting snow coincides with longer days around the solstice, while snowmelt occurs earlier at lower elevations. Intriguingly, we only observed *Raphidonema* at high-elevation glacier sites. In Svalbard, *Raphidonema nivale* abundance increased on glacier surface snow following wind storms, and the authors concluded that *Raphidonema* was a soil algae that opportunistically colonizes snow following wind deposition, but being sub-optimally adapted for snow such blooms are short lived [41]. Given the aerial dispersal capabilities of microalgae [42] and genetic overlap between distant snow algae populations [1] it seems unlikely that geographic distance is a barrier to snow algae distribution. Underlying topography could also potentially influence species distribution: two sites dominated by *Chloromonas krienitzii* were in runnels overlying ephemeral streams, which could influence nutrient availability where surface debris is deposited in the runnel.

Previous work has shown that *Chloromonas krienitzii* undergoes distinct green and orange morphologies [12], but our study is the first to document this transition in the field. The transition over weeks suggests that this process is mediated by seasonal changes. Secondary pigments likely protect snow algae from the damaging effects of intense solar irradiation at the snow surface [43], which could be why green cells were most concentrated a few centimeters below the snow surface. *Chlainomonas* was more abundant in surface samples as measured by *rbcL*, but microscopy suggests that *Chlainomonas* was only present in low relative abundance.

In conclusion, snow algae blooms on the same mountain can contain distinct species assemblages, with different species occurring at different elevations. Blooms were dominated by *Chloromonas*, *Chlainomonas*, and *Sanguina*, with substantial diversity that was previously undetected using 18S. It will be interesting to see whether this variation extends to the bacteria, fungi, and other species in this microbiome. By identifying the primary producers that form different types of snow algae blooms, we can begin to understand the ecological interactions that underlie propogation of this globally important microbiome..

# Data availability

All raw sequence data are available under European Nucleotide Archive accession PRJEB34539. All scripts used in this study are available at <https://github.com/cengstro/bc_snow_algae_amplicon>. Supplementary materials are available at <https://www.nature.com/ismej/>.

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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.

# Conflict of interest

The authors declare this research was conducted in the absence of any conflict of interest.

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# Figure legends

**Figure 1.** Representative photographs of snow algae. **A.** Red snow bloom above treeline at sample site G1.1 (see Supplementary Table S1 for coordinates). **B.** Photomicrograph of red snow containing *Sanguina nivaloides* (Sn) and *Chlainomonas rubra* (Cr) cell morphologies. All scale bars 30 m, all microphotos taken using 63x objectives with DIC. **C.** Snow runnels in a forest clearing containing snow algae (inset). **D.** Photomicrograph of orange snow from the surface of runnel containing *Chloromonas krienitzii* (Ck) and *Chloromonas cf. nivalis* (Cn). **E.** Bronze coloured snow algae blooms below conifer canopy. **F.** Photomicrograph of bronze snow containing *Chloromonas cf. brevispina* (Cb) and *Chloromonas cf. nivalis* (Cn).

**Figure 2.** Ordination plots showing genetic distances between *rbcL* ASVs. **A.** Multidimensional scaling (MDS) plot. Taxonomy (assigned by IDTaxa) indicated by colour, and size of point is proportional to cumulative relative abundance. Dotted ellipses show OTU clusters. Stress = 0.13. **B.** t-Distributed Stochastic Neighbor Embedding (t-SNE) dimensionality reduction of snow algae *rbcL* ASVs. Dotted lines indicate OTUs. Perplexity = 30.

**Figure 3.** Non-metric multidimensional scaling (NMDS) showing *rbcL* UniFrac distances between samples. Each point represents a sample, which is labelled by sample ID and elevation (colour).

**Figure 4.** Stacked bar plots of snow algae relative abundance. Each sample is arranged on the y axis in order of elevation, with each compositional bar plot representing the (left to right) cell morphology, 18S, and *rbcL* snow algae assemblages of the same sample. colours below each bar plot indicate taxonomic categories. Morphospecies were identified by light microscopy, using similarity to published photographs as a guide. 18S OTUs are reference-based, while *rbcL* OTUs were clustered *de novo*, and named using GenBank reference data.