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, with high elevation sites containing distinct species assemblages from forested sites. In contrast to previous work, the snow algae genus *Chlainomonas* was abundant and widespread in snow algae blooms. Although we found few taxa using traditional 18S metabarcoding, the taxonomic resolution of *rbcL* revealed substantial diversity, including OTUs that likely represent previously unidentified species of snow algae. These three cross-referenced datasets reveal that alpine snow algae blooms are more diverse than previously thought. Additionally, the composition of blooms varies from site to site along an elevational gradient.

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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 species involved.

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, in particular 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 and *rbcL* sequence shows that *Chlainomonas* is closely related to *Chloromonas,* although they have many distinctive traits. While Chlorophyceaen snow algae predominate in many snow algae blooms, other classes of snow algae include Chrysophyceae found in Antarctica, the Alps, and Svalbard [24, 25], and Trebouxiophyceae found 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.

Virtually nothing is known about the regional variation in species composition of snow algae blooms, particularly in alpine regions. We set out to answer the following questions: what species of snow algae are found in our region? What patterns of co-occurrence are there between species? Which species are the most abundant? What elevational patterns, if any, might there be in species distribution? We compared snow algae species composition of 33 samples from the Coast Range of British Columbia using light microscopy and 18S and *rbcL* metabarcoding. We found *rbcL* (coding for the large subunit of Rubisco) to be an effective marker, targeting photosynthetic species with higher taxonomic resolution than 18S, revealing previously unknown diversity. By using three cross-referenced metrics, we were able to account for 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 throughout the summer of 2018 from alpine and subalpine sites in the Coast Range near Vancouver, British Columbia, Canada (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). Over the summer, 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.

Within the 24 hours following collection we fixed microscopy aliquots in 2% gluteraldehyde, and observed the 100 cells closest to the center of the slide at 400x. To avoid double counting, we We characterized morphological species composition in 122 samples using 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 to physically rupture cell walls before incubation in CTAB lysis buffer [27]. We extracted DNA in batches of 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 gene*,* coding for the large subunit of Rubisco, is an established barcode marker gene for green algae [40], and comparison of GenBank sequences showed that this marker is more differentiated between snow algae species than 18S (Supplementary Figure S2). Our primers target a 400 bp section of *rbcL*, based on the consensus of 20 GenBank snow algae sequences (Supplementary Figure 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]. Primer sequences are available in Supplementary Table S2.

We constructed our 18S and *rbcL* amplicon libraries using a standard two-step PCR approach [30] (detailed PCR 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 using GenBank snow algae sequences (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 nivaloides*. Cells resembling *Chlainomonas rubra* were common in sites at all elevations, as the dominant cell type or mixed in with other species. 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. We infrequently observed green or orange snow patches above treeline; conversely, red snow was infrequent in shaded forest sites.

Both 18S and *rbcL* amplicon libraries were dominated by reads assigned to Chlorophyta (Figure 2). The 18S library assigned with SILVA 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 nine ASV clusters using *rbcL,* which we define here as our 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 with low *rbcL* UniFrac distances [38], distinct from most high elevation samples (Figure 3). We found the highest diversity in samples that were collected latest in the season. 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 sites above 1500 m, but was absent below this elevation (Figure 4). Additionally, 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). We collected two green snow samples from above treeline (N1.5, G1.4), and both were dominated by *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 (samples 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 snow algae blooms caused by 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 *Chloromonas* and *Chlainomonas* *rbcL* that we did not detect using 18S, including many potentially novel OTUs. 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 previously reported [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. In contrast to our findings, *Chlainomonas* was conspicuously absent in other 18S metabarcoding studies [8, 15, 24]. It is possible that *Chlainomonas* was present in previous studies, but was not detected; we could not differentiate between *Chlainomonas* and *Chloromonas* in our 18S data set. Interestingly, we may have had the opposite problem in our *rbcL* dataset: *Chlainomonas* was consistantly 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 plastid genome.

Our findings highlight the remaining unexplored diversity in the snow algae microbiome. Several *rbcL* OTUs did not closely match any GenBank nucleotide archive sequences, most notably OTU “E” (Figure 2). Given the high genetic distance between this OTU and other ASVs, we would expect to see a corresponding distinct OTU in 18S, but no 18S ASVs or taxa correlated with OTU “E”. One possibility is that it was lumped in with *Chloromonas*, given that this genus was a top BLAST match, potentially the *rbcL* is highly divergent relative to 18S. Other OTUs dominated by ASVs not assigned to species were closely related to *Chloromonas*, possibly representing novel species of this genus (Figure 2). The majority of *Chloromonas* ASVs did not form distinct clusters, perhaps due to overlapping intraspecific variation between species. *rbcL* diversity is likely higher within *Chloromonas* than other genera: 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* the 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 amplicon marker barcode [40].

Variation in bloom composition could be due to a wide range of habitat features. *Sanguina* and *Chloromonas* “C” were limited to sites above 1500 m in full sunlight, but many low elevation sites also received full sunlight and did not contain these OTUs (Figure 3). Light intensity, snow moisture, or snow chemistry could all plausibly influence community composition. 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 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, our study reveals substantial diversity within and between snow algae blooms, in contrast to previous studies which found red snow algae assemblages were homogeneous across geographically distant polar sites [1, 8]. We found greater diversity using *rbcL* than 18S, and snow algae diversity at the species level may have been previously overlooked in 18S metabarcoding studies. Species were differentially distributed along an elevational gradient, with distinct blooms dominated by *Chloromonas*, *Sanguina*, or *Chlainomonas*. Future studies will examine other taxa present in snow algae blooms, and their interactions with these globally important microbiomes.

# 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 XXX for coordinates). **B.** Microphotograph 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.** Microphotograph 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.** Microphotograph 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.