Species composition of snow algae blooms in the alpine and subalpine zones of British Columbia

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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 species that comprise snow algae blooms. We sequenced 18S and *rbcL* amplicons from 33 snow algae samples collected from a range of alpine and subalpine habitats in the Coast Range of British Columbia, Canada. Species composition varied from bloom to bloom: above treeline sites were dominated by *Sanguina*, while *Chloromonas* were dominant below treeline. *Chlainomonas* were abundant in samples across all elevations. The highest diversity was contained within the genus *Chloromonas*, which included OTUs that likely represent novel species of snow algae.. This work provides the first study of the regional diversity and distribution of snow algae.

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### *Keywords*: snow algae, microbiome, amplicon, *rbcL*, 18S, alpine, Illumina, British Columbia

### *Run title*: Alpine snow algae

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# Introduction

Each summer in polar and alpine snowfields worldwide, vast areas of snow surface are colored red by snow algae blooms. Such blooms are reported from every continent (Marchant, 1982; Yoshimura et al., 1997; Duval et al., 1999; Segawa et al., 2018; Vimercati et al., 2019) and Arctic sea ice (Gradinger and Nurnberg, 1996), and can be extensive: in Alaska, snow algae spectral signatures were detected in one third of a 1,900 km2 icefield (Ganey et al., 2017). In recent years snow algae have received attention for their role in reducing snow surface albedo, which could substantially impact snow melt rates and therefore glacier mass balance and spring runoff (Lutz et al., 2016; Ganey et al., 2017). Despite their global ubiquity, we are only beginning to identify the species that cause snow algae blooms.

Much of the previous work on snow algae relied on cell morphology for identification, but microscopic identification of snow algae is not reliable. One complication is that the same species can look completely different at different life stages or under different environmental conditions.. For example, cultured *Chloromonas krienitzii* are green, bean-shaped biflagellates, but single cells from field samples appear as orange spheres with thick cell walls, short spines (Matsuzaki et al., 2015). Conversely, different species can look nearly identical: recent phylogenetic work found that four distinct field samples dominated by similar-looking cells previously referred to as *Chloromonas cf. nivalis* are genetically distinct clades (Matsuzaki et al., 2019).

A diversity of algae have been reported from snow algae blooms. Chlorophyceae are predominant in many blooms, including the genera *Sanguina*, *Chloromonas*, and *Chlainomonas*. Sanger sequencing of red snow samples has shown that *Sanguina nivaloides* is predominant in red snowfields worldwide (Procházková et al., 2019). The taxonomy of this genus is only recently established; while only one other species is ascribed to this genus, many sequences from red snow samples form an unnamed sister clade to *Sanguina*. Twelve species of *Chloromonas* have been isolated from snow in the mountains of Japan????(Matsuzaki et al., 2019); other species of *Chloromonas* have been found to be the dominant species in orange and green snow algae blooms in the Alps?? (Remias et al., 2013, 2018). Distinctive large (~40 $) red-pigmented quadraflagellate *Chlainomonas* have been found in red snow overlying alpine lakes in central Europe, western USA, and New Zealand (Novis et al., 2008; Remias et al., 2016; Procházková et al., 2018). Two species are currently assigned to the genus *Chlainomonas*, but the taxonomy is not well-established. Non-Chlorophyceaen snow algae include Chrysophyceae found in Antarctica, the Alps, and Svalbard (Remias et al., 2019; Soto et al., 2020), and Trebouxiophyceae found in green snow (Lutz et al., 2015).

While many species of snow algae have been isolated or sequenced from snow, much less is known about the composition of snow algae blooms: which species predominate, which species co-occur, and how species are distributed across the landscape. Previous studies using 18S amplicon high-throughput sequencing (metabarcoding) suggest that snow algae blooms are highly similar across continents, but these studies have had poor species resolution. Thirty-three red snow communities across the Arctic were remarkably similar to one another. All were dominated by an “uncultured Chlamydomonadaceae” with low relative abundance of *Chloromonas polyptera* and *Raphidonema nivale* (Lutz et al., 2016). Similarly, another study using ITS2 metabarcoding study found 24 red snow sites across the Arctic and Antarctic contained similar assemblages, also dominated by “uncultured Chlamydomonadaceae” with secondary abundance of *Raphidonema* and *Chloromonadinia* (Segawa et al., 2018). In contrast, red and green blooms can be quite distinct (Lutz et al., 2015; Terashima et al., 2017). Other 18S surveys targeting different 18S regions were limited to genus or family level taxonomic resolution (Hamilton and Havig, 2017). As 18S is highly conserved across eukaryotes, sequences can be too similar to resolve between closely related taxa.

Virtually nothing is known about the regional variation in the species composition of snow algae blooms. Our goal was to identify the algae in blooms on different mountains and elevations in the Coast Range of British Columbia, Canada. In addition to traditional 18S metabarcoding, we designed primers to target a hypervariable region of *rbcL* (coding the large subunit of rubisco), thus targeting only photosynthetic species with high taxonomic resolution. Additionally, we used light microscopy to describe the relative abundance of different morphologies in each field sample. By using three cross-referenced metrics of relative abundance, we were able to describe the algal species assemblage with high taxonomic resolution. We discovered a much greater diversity of snow algae than expected. Although we did not find differences in bloom composition between mountains, there were distinct blooms at high versus low elevation.

# 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 within 24 hours following collection, with samples maintained on ice. We prepared a slide of cells fixed in 2% gluteraldehyde, and counted up to 100 cells at 400x. We classified each cell based on similarity to published photographs, as either *Sanguina nivaloides* (Procházková et al., 2019), *Chloromonas cf. nivalis* (Prochazkova et al., 2018), *Chloromonas cf. brevispina* [matsuzaki\_revisiting\_2015], *Chloromonas krienitzii* (Matsuzaki et al., 2015), *Chlainomonas rubra* (Novis et al., 2008), and for cells that did not fall into one of there categories as either “green cell” or “other”.

## 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. We mini-pestled between 5 to 20 mg of sample at room temperature to physically rupture cell walls before incubation in lysis buffer.

To extract DNA from the crushed cells we added 800 mL 1x CTAB extraction buffer (**???**), 1% B-mercaptoethanol, 5 L each of proteinase K and RNAase A, and incubated these at 65 °C for 30 minutes. We spun 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 designed custom primers to target a hypervariable region of *rbcL* of snow algae, along with established universal 18S V7-V8 primers Euk1181 and Euk1624 (Wang et al., 2014). We designed *rbcL* primers using the Eurofins primer design tool (**???**) to target a 400 bp section of *rbcL* based off of 20 snow algae GenBank sequences (Supplemental table 3)—*Sanguina* sequences were not included because they were not available at the time of this study. The primer sequences can be seen in Supplemental table 4.

We constructed our amplicon library using a standard two-step PCR approach (Meyer and Kircher, 2010). 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 (NEB), 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 CUTADAPT (Martin, 2011). Samples were filtered and trimmed, errors removed, dereplicated, pair end reads merged, and chimeras removed following the default pipeline of DADA2 (**???**).

We assigned taxonomy for both *rbcL* and 18S ASVs using IDTaxa with threshold set to 50 (Murali et al., 2018). 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: because entries labelled as *Chloromonas cf. brevispina* and *Chloromonas cf. nivalis* are polyphyletic (Matsuzaki et al., 2019) we re-labeled these as “unassigned *Chloromonas*”. To assign 18S reads we initially ran our ASVs against SILVA (Quast et al., 2013), 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”.

As most OTU clustering algorithms are optimized for 18S data, we visualized *rbcL* ASV clustering using t-SNE (Maaten and Hinton, 2008) (Fig. 2b). 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 settings (Lozupone and Knight, 2005).

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>.

# Results

We collected 309 snow algae samples from 13 mountains throughout the summer of 2018. At our low elevation sites (850 - 1500 m) we detected snow algae as early as May 18, but these sites were also the earliest to melt out. We did not detect snow algae at high elevation sites (1500 - 2200 m) until June 20, where both the snow and snow algae blooms persisted throughout the entire summer.

Using light microscopy we identified morphologically distinct snow algae in different habitats (Fig. 1, S4). Red snow was prevalent in areas of high solar exposure; above treeline sites were often dominated by red cells similar in appearance to *Sanguina nivaloides* (Procházková et al., 2019) (Fig. 1a,b). We found cells resembling *Chlainomonas rubra* (Novis et al., 2008) at all elevations, in highest relative abundance in areas receiving direct sunlight. We occasionally observed green snow patches in alpine meadows receiving full sun, but more frequently observed green snow in well-shaded, forested sites. At four low-angle sites in clearings near treeline we observed snow melt channels or runnels containing high concentrations of snow algae (Fig. 1c). In May and early June these sites contained green snow hidden 2 to 5 cm below the white snow surface, but upon subsequent visits the same location contained orange snow at the surface, whose morphologies resembled published images of the *Chloromonas krienitzii* lineage (Matsuzaki et al., 2015) (Fig. 1d). Forested sites at low elevations often contained green or orange patches of snow, containing green or orange ovate cells with short spines resembling *Chloromonas cf. brevispina* (Matsuzaki et al., 2015) (Fig. 1e,f), although we also occasionally observed red snow at forested sites containing morphologies similar to *Sanguina nivaloides*.

Of the 309 samples studied by light microscopy, we selected 33 snow algae samples for 18S and *rbcL* amplicon Illumina sequencing. Both libraries were dominated by amplicon sequence variants (ASVs) that were assigned to Chlorophyta. Our 18S library contained 67 ASVs that were assigned to Chlorophyta and 7 ASVs to Ochrophyta, while our *rbcL* library contained 642 ASVs, of which 603 were assigned to Chlorophyta, the remaining 41 were Trebouxiophyceae. The top genera assigned by the taxonomy assignment algorithm IDTaxa (Murali et al., 2018) were *Chloromonas*, *Chlainomonas*, and *Sanguina*; although most ASVs were not assigned to genus level by this algorithm, the majority of ASVs clustered near one of these genera in NMDS ordination (Fig. 2a). We used t-SNE to generate operational taxonomic units (OTUs), and found that many OTUs that did not correspond to any known taxa on GenBank (Fig. 2b). These included three OTUs that were closely related to *Chloromonas*, and one Chlamydomonadaceae OTU that was only assigned to the family level, meaning that it did not match any known algal genus on GenBank.

Above and below treeline samples contained some unique taxa, but most taxa did not exhibit a clear elevational trend (Fig. 3). *Sanguina* was the dominant taxa in most samples from above treeline, and was absent in sequences libraries from the samples below 1500 m. Additionally, these high elevation samples uniquely contained an unannotated OTU of *Chloromonas* (Fig. 3, “Chloromonas C”). The three highest elevation sites uniquely contained ASVs with a best BLAST match to *Raphidonema longiseta* (KM462868.1)—all three samples were taken from snow overlying glacier . Green snow from high alpine sites contained *Chloromonas* OTUs that were also found in sites below treeline. Low elevation sites contained *Chloromonas krienitzii*, but many sites were dominated by taxa that were found at all elevations, such as *Chlainomonas*.

Cell counts, 18S, and *rbcL* datasets were generally consistent, but there were some interesting discrepancies. *Chlainomonas* was more prevalent in our *rbcL* library than in cell counts, while *Sanguina* was underrepresented in *rbcL* compared to 18S and cell counts. We did not detect *Chlainomonas* with 18S sequencing.

At runnel sites on Hollyburn and Seymour we observed snow algae blooms that changed from green to orange over the course of the summer (Fig. 1). We sequenced both green subsurface and orange surface snow at three different sites, and found both were dominated by *Chloromonas krienitzii*, although surface samples also contained *Chlainomonas* (Supplementary \_\_).

# Discussion

We found that alpine snow algae bloom composition varied from site to site, with some elevational trends. *Sanguina* was dominant above treeline, while *Chloromonas krienitzii* was dominant in mid-elevation runnels, which initially appeared as green motile cells below the snow surface but developed into orange blooms at the snow surface. We found an unexpected diversity within *Chloromonas*, including many OTUs not represented in GenBank. Previous studies suggested that *Chlainomonas* is restricted to waterlogged snow overlying mountain lakes (Novis et al., 2008; Procházková et al., 2018) but our results suggest that this genus can be widespread and abundant in many alpine red snow sites. While one *Chlainomonas*-dominant sample was located in waterlogged snow near the edge of a pool (sample S9), the other *Chlainomonas*-dominant sites were not notably wetter than the surrounding snow, nor overlying ice.

Our findings highlight the remaining unexplored diversity in the snow algae microbiome. *rbcL* revealed greater diversity than 18S, and many *rbcL* OTUs did not closely match any annotated GenBank sequences (Fig. 2). Likely, the *Chloromonas* *rbcL* OTUs were lumped together in the 18S dataset due to high similarity. We found higher *rbcL* diversity in *Chloromonas* than *Sanguina*, which could be because *Chloromonas* lack a pyrenoid, and consequently has many non-synonymous mutations in the region of *rbcL* that codes for binding these proteins together to form a pyrenoid (Nozaki et al., 2002). One *rbcL* OTU, “Chlamydomonadaceae E”, was only assigned to family level—this OTU could represent a novel genus. However, we lack explanation for why this OTU did not correspond to any OTUs in 18S or cell counts. It seems unlikely that Chlamydomonadaceae E would have been lumped in with *Chloromonas* in 18S, given how distinct their *rbcL* sequences were from *Chloromonas*.

Although 18S, *rbcL*, and cell counts were generally consistent, we observed some interesting discrepancies (Fig. 3). We did not observe *Chlainomonas* in our 18S dataset, despite the fact that there were two representative sequences from this genus in our reference database (MF803743.1, MF803745.1). Likely, *Chlainomonas* 18S in our target region (V7-V8) is not sufficiently differentiated from *Chloromonas* to be distinguishable. Many samples contained high relative abundance of *Chlainomonas* *rbcL*, yet these samples contained lower concentration of *Chlainomonas* cell morphologies. Large cells such as *Chlainomonas* often have high rRNA gene copy number (Fu and Gong, 2017), which could account for this discrepancy. *Sanguina* was found in the same samples, but relative abundance was generally higher in 18S and cell counts. This could be due to primer bias: our *rbcL* primers were designed based on *Chloromonas* snow algae sequences (Supp \_) *Sanguina* reference data not being available at the time, which could have resulted in over-representation of *Chloromonas* relative to *Sanguina*.

Variation in bloom composition could be due to a wide range in habitat features. *Sanguina* and *Chloromonas C* were limited to sites above 1500 m in full sunlight, but many sites below this elevation also received full sunlight. By microscopy we observed *Sanguina* morphologies in shaded areas as well as in full light. Day length, snow saturation, or snowpack characteristics could all plausibly influence community composition. Two sites dominated by *Chloromonas krienitzii* were overlying ephemeral streams, one possibility is that this species colonizes the snow surface by swimming up from liquid water below. *Raphidonema* was found only at high-elevation glacier sites. In Svalbard *Raphidonema nivale* abundance increased on glacier surface snow following windstorms, and the authors suggest that this is a soil algae that grows sub-optimally on the snow surface following wind deposition (Stibal and Elster, 2005).

In conclusion, our study reveals substantial snow algae diversity within and between blooms. Different species were differentially distributed along an elevational gradient. Distinct blooms were dominated by *Chloromonas*, *Sanguina*, or *Chlainomonas*. This is the first study to thoroughly document snow algae diversity within a local region. Future studies will examine other taxa present in alpine snow algae blooms. These are complex microbiomes comprised of bacteria, archaea, viruses, phage, fungi, ciliates and other microscopic eukaryotes. The snow surface is an ephemeral environment and it will be interesting to discover whether complex symbiotic relationships such as those found in marine and freshwater plankton (refs) also occur on the snow.

# Acknowledgments

We thank Leah Tooman (Simon Fraser University) for assistance with sequencing, and Chris Rushton (Simon Fraser University) for assistance with bioinformatics. This project was funded with a Sector Innovation Grant from Genome BC (SIP016), and a NSERC Individual Discovery Grant, both awarded to LQ.

# 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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# Figure legends