Snow algae in the alpine and subalpine zones of British Columbia

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Snow algae grow in melting summer snowfields throughout polar and alpine regions, forming extensive blooms that reduce albedo and increase snow melt. Green, orange, and red patches of snow algae can harbor morphologically diverse algae, but despite their global ubiquity the algal species composition of blooms is largely unknown. 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. Predominant species were in, or closely related to, the genera *Sanguina*, *Chloromonas*, and *Chlainomonas*. The most common species above treeline were *Sanguina.* Below tree line by *Chloromonas* were dominant. *Chlainomonas* was abundant in samples across all elevations. We found that species composition was highly variable from patch to patch, even on the same mountain. Although, snow algae in the Coast Range are represented by the same relatively few genera that dominate most reports of snow algal blooms around the globe, we found a surprisingly high diversity at the species level.

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# Introduction

Reduction in cryospheric albedo has become the focus of attention in recent years as an important feedback loop of global warming (REFS). Microbial growth on snow surface can reduce albedo, notably when snow algae form extensive blooms on melting summer snow1–3. Red snow algae blooms are found on every continent, and on snow overlying sea ice4–9. Despite their global ubiquity, we are only beginning to identify the algae that are the primary producers in these complex microbiomes.

Most snow algae blooms that have been reported are dominated by are green algae of class Chlorophyceae, although other classes of algae can also cause snow algae blooms10–12. Within the Chlorophyceae, *Sanguina nivaloides* is prevalent in red snow worldwide13. Currently just two species are described for *Sanguina* although there are other closely related genera that have yet to be named (REF). Other Chlorophyceae include *Chloromonas* (fourteen species identified to date) and the closely-related but morphologically distinct *Chlainomonas* (only two species identified)14,15. Of the three genera, only [HOW MANY?] *Chloromonas* species have been successfully cultivated in the lab (REF).

Microscopy suggests that snow algae blooms are in many cases are dominated by single species. In the mountains of southwestern USA and Czech Republic red snow was prevalent above treeline, dominated by cells similar in appearance to *S. nivaloides*, while below treeline green or orange snow were prevalent dominated by cells similar to *Chloromonas*16,17. Red snow dominated by *Chlainomonas* type cells were reported from slushy snow overlying alpine lakes15,18. However, microscopic identification of snow algae is complicated by the fact that the same species may look completely different at different life stages or under different environmental conditions. Isolates of *Chloromonas krienitzii* grown in the laboratory are green, bean-shaped biflagellates, but genetically identical isolated single-cells from field samples appear as orange spheres with thick cell walls, short spines, and no flagella19. Conversely, different species can look nearly identical: recent phylogenetic work shows that four similar looking cells previously referred to as *Chloromonas cf. nivalis* actually contain multiple genetically distinct clades, likely defining distinct genera14.

Previous studies using 18S amplicon high-throughput sequencing (metabarcoding) suggest that snow algae blooms are more or less the same from one location to another. Thirty-three red snow communities across the Arctic were remarkably similar: all were dominated by an OTU known as “uncultured Chlamydomonadaceae,” with low relative abundance of *Chloromonas polyptera* and *Raphidonema nivale*2. Another study using 18S metabarcoding of snow algae in the mountains of Japan found neighboring red and green snow patches with highly similar species composition, although the red snow snow contained one OTU not found in the green bloom20. In contrast, side-by-side red and green snow in Greenland contained completely different species10.

Given how little is known about snow algal species distribution and diversity, particularly in alpine habitats, our goal was to identify the snow algae species in blooms on different mountains and from different altitudes within a single mountain range, the Coast Range of British Columbia, Canada. The sequence of ribosomal 18S is highly conserved, making it an ideal universal barcode for eukaryotes, but at lower taxonomic levels 18S is too similar to resolve closely related taxa. To complement 18S sequencing, we also targeted the more differentiated *rbcL* gene, coding for rubisco, thus only targeting the photosynthetic community. 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 blooms with high taxonomic resolution. We found blooms above treeline contained a distinct set of taxa. Elevation defined a gradient of relative abundances of different snow algae taxa. There were no significant differences between mountains. [IS THIS TRUE?]

# Results

We collected 309 snow algae samples from 13 mountains throughout the summer of 2018. We first detected snow algae on May 18, and we continued to sample our low elevation sites at Seymour (S) and Hollyburn (H) until the snow melted out in early July. We did not detect snow algae above treeline until June 20, At higher elevations, both the snow and snow algae blooms persisted throughout the entire summer.

By microscopy, we identified morphologically distinct snow algae dominating in different habitats. In shaded, forested sites we often found green or orange patches of snow, containing green or orange ovate cells with short spines resembling *Chloromonas cf. brevispina* (Fig. 1c). In forest clearings we observed low-angle linear drainage channels or runnels containing high concentrations of snow algae. In May and early June these runnels contained green snow hidden 2 to 5 cm below the white snow surface, and on subsequent visits these same sites contained orange snow at the surface, with morphologies resembling published images of *Chloromonas krienitzii* (Fig 1b; REF TO PUBLISHED IMAGES]. Red snow was prevalent above treeline, dominated by red cells similar in appearance to *Sanguina nivaloides* [FIG. 1?] although we also occasionally observed green snow patches above treeline. We found red snow dominated by morphologies similar to published images of *Chlainomonas rubra* in unshaded sites at all elevations.

Of the 309 samples studied by light microscopy, we chose 33 for 18S and *rbcL* amplicon sequencing using Illumina next generation technology. 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 ?????? Of the Chlorophytes, the top genera were *Chloromonas*, *Chlainomonas*, and *Sanguina*, but most ASVs were not assigned to genus level (Fig. 2A). We used t-SNE to generate operational taxonomic units (OTUs), and found many OTUs that did not correspond to any known taxa on GenBank (Fig. 2B): several OTUs related to *Chloromonas*, and one Chlamydomonadaceae OTU that was only assigned to the species level.

Above and below treeline samples each 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. Additionally, high elevation sites contained several taxa not found at low elevations: an unannotated OTU of *Chloromonas*, and ASVs with a best BLAST match to *Raphidonema longiseta* (KM462868.1) were found at the three highest elevation sites. 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 \_DON’T FORGET TO ADD NUMBER).

# Discussion

We found that alpine snow algae blooms can vary widely from site to site. Different taxa were dominant at different elevations, with alpine sites dominated several distinct taxa, most notably *Sanguina*. *Chloromonas krienitzii* was dominant in mid-elevation runnels appearing as green motile cells below the snow surface, and later in the season developing orange pigment at the surface. *Chloromonas* and *Chlainomonas* were abundant across all elevations, including many species [or OTUs if you prefer, but not ‘taxa’ given that you are talking about something within a genus] that are not represented in GenBank.

Our findings highlight the remaining unexplored diversity in the snow algae microbiome. Most of the diversity was only detected in *rbcL*: samples enriched in Chlamydomonadaceae E did not correspond to anything we detected in cell counts and 18S. One possibility is that this OTU represents a novel taxa of algae whose 18S is highly similar to Chloromonas. Another possibility is primer bias: perhaps this OTU was over-represented in *rbcL* and underrepresented in 18S.

Previous studies suggested that *Chlainomonas* is restricted to waterlogged snow overlying mountain lakes, but our results suggest that this genus is widespread and abundant in all types of snow algae blooms15,21. We did not detect any association with a particular habitat: only one site dominated by *Chlainomonas* was near water (S9), but other *Chlainomonas* sites were not notably wetter than the surrounding snow.

Although 18S, *rbcL*, and cell counts were generally consistent, we observed some discrepancies. Most notable was the lower relative abundance of *Sanguina* spp in the *rbcL* dataset relative to the 18S dataset (Fig. 3). Primer bias could partly explain this discrepancy, because we designed our *rbcL* primers off of *Chloromonas* sequences, as *Sanguina* reference data was not available at the time. Additionally, we observed that *Sanguina* cell morphologies were more resistant to lysis than other cell types in microscopy following lysis. *Chlainomonas* was overrepresented in *rbcL* relative to cell counts, possibly due to primer bias again, or higher rRNA gene copy number typical of larger cell volumes22. None of our 18S ASVs returned BLAST results for *Chlainomonas*, despite there being two representative GenBank sequences in our reference database (MF803743.1, MF803745.1). Possibly the *Chlainomonas* 18S sequence in outhe r target region (V7-V8) is not different enough from closely-related *Chloromonas* to be detectable in this survey.

Species composition is likely governed, at least in part, by different site habitat characteristics. The absence of *Sanguina* from low elevation sites could be indicative of habitat specificity: perhaps it is outcompeted at low elevation sites by taxa that are better adapted to the low light of the shade. Given the prevalence of airborne microalgae, it seems less likely that *Sanguina* is restricted in it’s dispersal capabilities23. Perhaps *Chloromonas krienitzii* dominates in low-angle runnels where moisture is sufficient; alternatively, we observed that many runnels overlay streams, which could relate to the dispersal mechanism of this species. We found *Raphidonema* only in high elevation sites that were sampled late in the growing season. 24 suggests that this is a slow-growing opportunistic soil algae that blows into snow fields from surrounding dirt to colonize the snow. Perhaps sites late in the season were closer to bare ground containing source populations of this algae; alternately, perhaps this algae is slower to grow in cold temperatures on the snow, and is outcompeted by the better adapted *Sanguina*.

Methods

## Field sampling and microscopy

We collected snow algae from the Coastal Mountain Range near Vancouver, British Columbia, Canada throughout the summer of 2018. To capture the extent of snow algae diversity in our local mountains we collected samples 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 morphology as either *Sanguina nivaloides***???**, *Chloromonas cf. nivalis***???**,**??**, *Chloromonas cf. brevispina***???**,**??**, *Chloromonas krienitzii***???** *Chlainomonas rubra***???**, 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 buffer**???**, 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 species**???**, 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’), targeting the V7-V8 hypervariable regions[**???**;@].

We constructed our amplicon library using a two-step PCR**???**. 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 CUTADAPT**???**. 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**???**. 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 phylogeny**???**. 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 SILVA**???**, 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 annotations**???**,**??**.

As most OTU clustering algorithms are optimized for 18S data, we chose to assign high level OTUs based on the results of t-SNE clustering**???** (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 settings**???**.

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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