

Biological Sciences

Characterizing the variability of Chlorophyta from McMurdo Dry Valley streams

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Abstract

The numerous ephemeral glacial meltwater streams that flow during the summer in the McMurdo Dry Valleys of South Victoria Land, Antarctica, provide habitats for microbial mats. One of the common mat types is composed of Chlorophyta (colloquially known as a ‘green mat’ due to its colour). While the presence of these mats is regularly monitored, their taxonomic makeup is still under investigation. Using 18S rRNA gene sequencing, the composition of the chlorophyte-dense mats from between rocks and in the main channel from several streams across two valleys was examined. Samples were maintained in native stream water, and select samples from representative locations were transferred to Bristol Medium. The appearance of other eukaryotic species – diatoms and tardigrades – in these green mats completed this integrated study. The results show that the relative abundance of Chlorophyta was significantly increased with the introduction of inorganic nitrogen from Bristol Medium. Chlorophyte taxa in the *Hazen* and *Pleurastrum* genera dominated the samples across both sample types (rock or exposed) and treatments (Antarctic water or Bristol Medium). Furthermore, a reduction in overall sample diversity was observed in samples in Bristol Medium, suggesting preferential nitrogen utilization by these chlorophytes.

Keywords: 18S rRNA gene sequencing; chlorophytes; eukaryotes; Miers Valley; Taylor Valley

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Introduction

The McMurdo Dry Valleys region (MDV; Fig. 1) is the largest ice-free area on the coast of Antarctica and is a mosaic of alpine and terminal glaciers, large expanses of bare patterned ground, permanently ice-covered lakes and glacial meltwater streams (Levy 2013). Depending on the weather conditions, the streams flow for 4–9 weeks each summer (Wlostowski *et al.* 2016). Many of the streams support perennial microbial mats that are freeze-dried through the winter and resume growth when the stream flow arrives (Howard-Williams *et al.* 1989, Hawes *et al.* 1992, McKnight *et al.* 2007). The streams are underlain by a hyporheic zone that thaws as summer progresses, reaching a depth of ~50 cm, forming a layer of active mineral weathering and microbial activity (Gooseff *et al.* 2002).

Where water is present, microbial life thrives in the MDVs, particularly as the microbial mats in the freshwater streams are fed by glacial melt. The MDV streams support four types of microbial mats that can be readily distinguished in the field by their associated colour (for brevity: black, orange, red and green; McKnight *et al.* 1998). Beginning in the 1993–1994 season, the McMurdo Dry Valleys Long Term Ecological Research (MCMLTER) project has been monitoring the abundance of these four microbial mat types at a network of monitored stream reaches in two major valleys. Three of these mat types (black, orange and

red) are composed predominantly of filamentous cyanobacteria (McKnight *et al.* 1998). The fourth commonly found mat type (green) is mostly composed of eukaryotic species (Van Horn *et al.* 2016). All of these mat types contain a diverse community of primarily endemic pennate diatoms, with dominant genera typically being *Hantzschia* and *Luticola* (Stanish *et al.* 2012). The green mats are present as small patches or streamers attached to the sides or bottoms of rocks (McKnight *et al.* 1998). They are often found in the central stream channel, elevated from the benthos, as an epiphytic attachment on orange mats or as an epilithic growth on rocks. They require a substrate to anchor to for growth, and they are generally not found on stream margins and seep areas, where black mats are abundant (McKnight *et al.* 1998). Monitoring of this green mat abundance has shown that, due to their location in the stream bed, these mats are vulnerable to scour during high flows and do not persist during the extended periods of low flow in summer (Kohler *et al.* 2015a).

The genus *Prasiola* (C. Agardh) Meneghini has often been reported to be dominant or abundant in Antarctica (Seaburg *et al.* 1979, Izaguirre & Pizarro 1998, McKnight *et al.* 1998). *Prasiola* presents as tough filaments or fronds and has two distinct life stages. The first, the ‘Hormidium’ stage, is composed of tough, unbranched, rope-like filaments (West & West 1911, West & Fritsch 1927, Seaburg *et al.* 1979, John *et al.* 2002, John & Rindi 2003). These filaments loosely attach to substrata and asexually reproduce through fragmentation. Hormidium filaments eventually transform into the pluriseriate ‘Schizogonium’ stage, characterized by short, thick, multiserial fronds that form small

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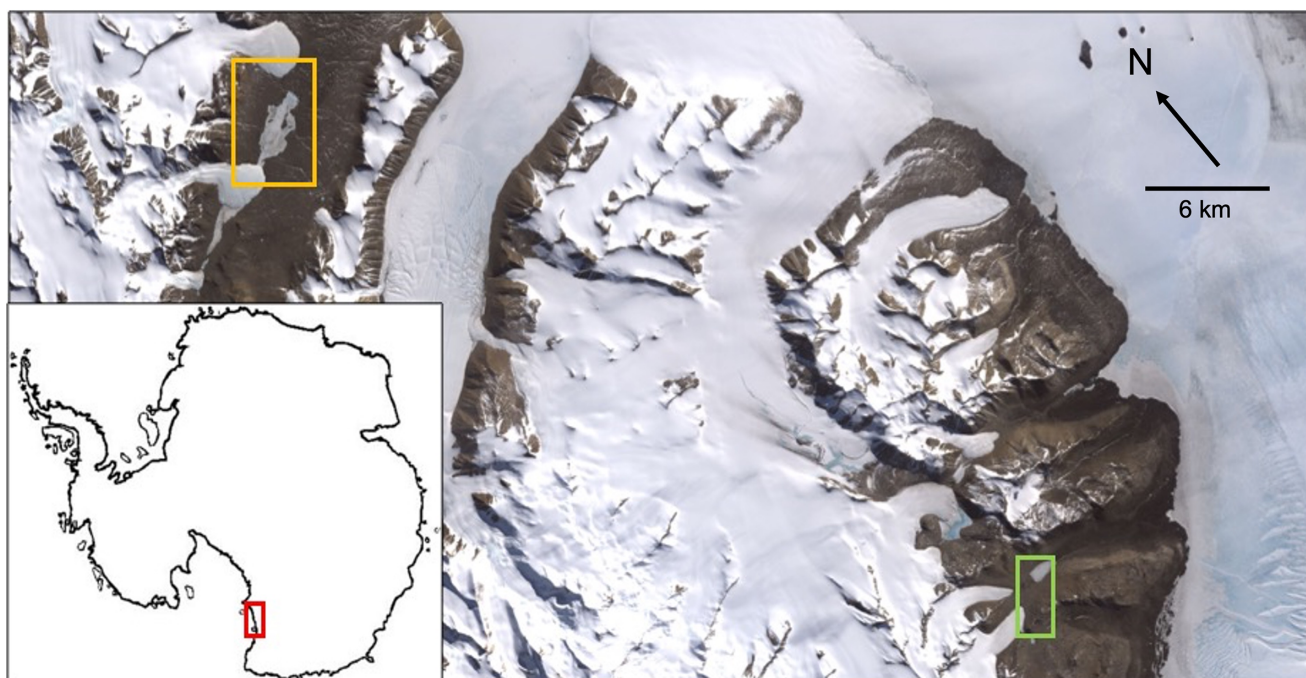


Figure 1. Reference map of the sampled valleys - Taylor Valley (yellow box) and Miers Valley (green box) - of the McMurdo Dry Valleys, Antarctica (red box).

tufts beneath rocks. There is considerable taxonomic uncertainty within *Prasiola* (Broady 1996, Kováčik & Pereira 2001). Molecular work by Moniz *et al.* (2012) suggests the presence of at least three morphologically indistinguishable Antarctic species. Previous surveys of freshwater algae, including green mats, across Antarctica (Fritsch 1912, Broady 1996, Vincent & James 1996, McKnight *et al.* 1998) report two *Prasiola* species from the McMurdo Sound Region (*Prasiola crispa* (Lightfoot) Kützing and *Prasiola calophylla* (Carmichael ex Greville) Kützing), although there is considerable variation and overlap in their descriptions (Kováčik & Pereira 2001). In addition to *Prasiola*, the identification of *Hazenella* and *Pleurastrum* in littoral zones and cryoconite holes, respectively, across Antarctica raises the question of the limited reported green algae diversity in MDV streams and lakes vs their diversity as reported in other Dry Valley features (Broady 1996, Škaloud *et al.* 2013, Millar *et al.* 2021). Advancing the understanding of the composition of these green mats in the MDVs was one purpose of initial genomic sequencing (Van Horn *et al.* 2016). The results indicated that *Prasiola* did not comprise more than 4% of the samples, and that the mats were assigned as *Chlorella* and *Chlorococcum*. Van Horn *et al.* (2016) did not confirm genera abundance using morphological methods, but it was hypothesized that this finding of low abundance was a result of a lack of representation of *Prasiola* in genetic databases and a polymerase chain reaction (PCR) primer bias. Microscopically, these genera cannot be easily confused (Ohtani *et al.* 2000). In contrast to *Prasiola*, the genera *Chlorella* and *Chlorococcum* are both described as coccoid algae and can be unicellular or mucilaginous colonies and have been previously reported at trace levels in Fryxell Basin samples (McKnight *et al.* 1998). It is possible that the composition of these green mats changed over the > 15 years from when the early morphological observations were reported. Stream hydrology may also influence species abundance, as these mats are slow to return (years) after being scoured off rocks during high-flow seasons (Kohler *et al.* 2015a).

Isolating species is essential for building genetic databases (Perterra *et al.* 2025). Studies have sustained cultures for further isolation using collected Antarctic water or developed synthetic media, reflecting sites' nutrient compositions, with mixed results (Holm-Hansen 1964, Buffan-Dubau *et al.* 2001, Pocock *et al.* 2004). Extended culturing for taxonomic investigation can be more easily sustained with freshwater medium formulated for large-scale use, without the risk of exhausting Antarctic water samples (Hu *et al.* 2008, Inan *et al.* 2023). Limited studies have sustained algal mat samples from the MDVs, but none have done so for the green mats from the streams (Van Horn *et al.* 2016, Perterra *et al.* 2025).

The purpose of this study was to better understand the composition of green mats collected from the MDV stream ecosystems and later maintained in the laboratory and used in further experimentation, including those mats used in the Matula *et al.* (2021) spaceflight life support study. In an effort to increase mat composition fidelity for long-term monitoring, we examined the variation in the Chlorophyta taxa forming these green mats from several streams across two valleys, variation in location within the stream and variation in the presence of multiple eukaryotic species such as diatoms and tardigrades in the cultured material. Additionally, to simulate a mass culturing environment, or an increase in nutrient availability due to the patchy availability of nitrifying algae (black mats), different samples of mat were transferred to Bristol Medium. The molecular characterization of the green mats and its comparison to microscopic observations are important to improving understanding of species dominance and community diversity across the MDVs, but they may also help us to identify potential gaps or biases in sequencing databases.

Materials and methods

Site description

This protocol used stream transects established for MCMLTER annual mat measurements as reference locations. Referencing the

microbial mat survey records of the MDVs, all streams selected for this sampling protocol showed sustained presence of green microbial mats over the past decade (2010–2020; mcm.lternet.edu), which was used as a selection basis. Mats were collected from six streams across two valleys (Taylor and Miers; Fig. 1). Selection preference was given to those streams included in the biodiversity study by Van Horn *et al.* (2016). Sampling was conducted over 16–21 January 2019, during the afternoon to ensure peak stream flow, in Taylor Valley (77° 26'38.717"S, 163° 9'33.321"E; Fig. 2) and Miers Valley (78° 5'52.000"S, 163° 50'47.990"E; Fig. 3) of the MDVs, South Victoria Land, Antarctica. Daily discharge rates and nitrate and phosphorus concentrations for each sampled stream are presented in Fig. 4. No discharge measurements were recorded for Huey Creek (Taylor Valley) during the 2018–2019 season. Days when green mat sampling was conducted are indicated by the black arrows in Fig. 4. The highest discharge rate for most streams occurred around the summer solstice (21 December). The culture studied for bioregenerative spaceflight use by Matula *et al.* (2021) was obtained from Von Guerard Stream. The range of streams allows for the study of diversity across valleys, lakes and stream

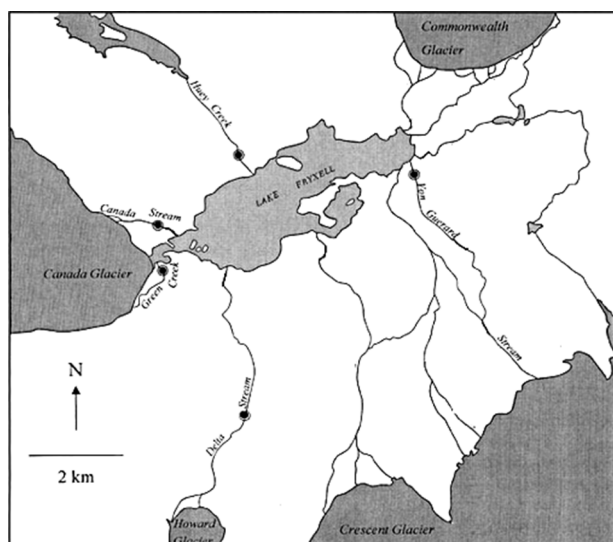


Figure 2. Areas sampled in Taylor Valley. Sample locations are marked with black circles.

lengths. Interannual stream flow regimes were considered, as Adams Stream (Miers), Green Creek (Taylor), Huey Creek (Taylor) and Canada Stream (Taylor) are documented to have steady flow and Delta Stream (Taylor) and Von Guerard Stream (Taylor) may experience summers with no appreciable flow (Wlostowski *et al.* 2016).

This study also includes long and short stream classifications (Table 1). Within the Fryxell Basin, Delta Stream is the longest and is fed by Howard Glacier (Cozzetto *et al.* 2006). The Delta Stream upper transect was dominated by intermediate to large cobbles, forming narrow channels for green mats to attach to rocks. Von Guerard Stream drains off Von Guerard Glacier (Cozzetto *et al.* 2006). While sampling was concentrated near the gauge and delta region of the transect, the area provided three distinct streambed substrata, including stable stone pavement (upstream of a flow gauge used by MCMLTER), small cobble (downstream of the same flow gauge used by MCMLTER) and coarse sediment (delta; McKnight *et al.* 1999). Canada Stream is fed by Canada Glacier and has the longest periods of flow of the sampled streams (Table 1) and the most reliable presence of algal mats, regardless of scouring events (Kohler *et al.* 2015b). The Canada Stream transect was a stable pavement made up of small and intermediate cobble. Green Creek is also fed by Canada Glacier and feeds into the southern shore of Lake Fryxell (Cozzetto *et al.* 2006). The area sampled for Green Creek was composed of intermediate cobble on top of a stable pavement made of small cobble. Huey Creek drains off a snowfield in the Asgard Range, and its sampled transect was made up of small rocks loosely cemented with small to fine sediment (Cozzetto *et al.* 2006). Adams Stream was the only stream sampled from Miers Valley and had the highest flow rate of the sampled streams (reported as a maximum with a discharge rate three times faster than the others; Gooseff & McKnight 2021a,b,c,d,e). The sampled transect was made up of intermediate cobble creating a deltaic pattern with coarse sediment cementing the cobble (McKnight *et al.* 1998).

Sampling methods

Samples were collected from the main thalweg of the stream. Both streamers (Hormidium stage) and tufts (Schizogonium stage) of green mats were gathered from between rocks and on rock surfaces below the water surface but above the benthic zone. Care was taken

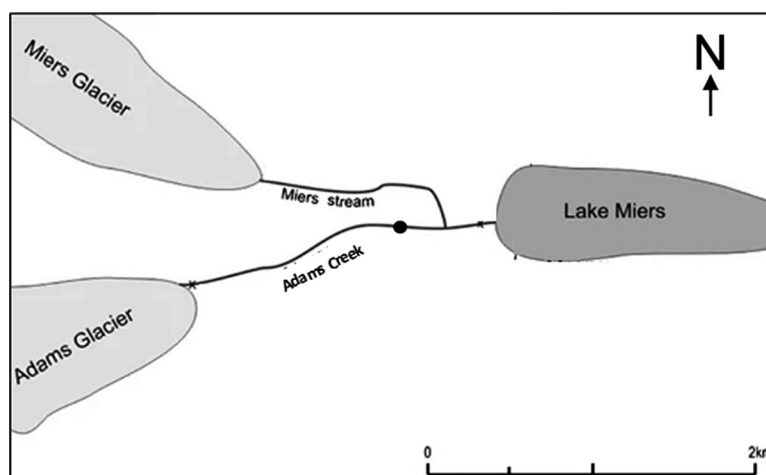


Figure 3. Sample location in Miers Valley. Sample location is marked with a black circle.

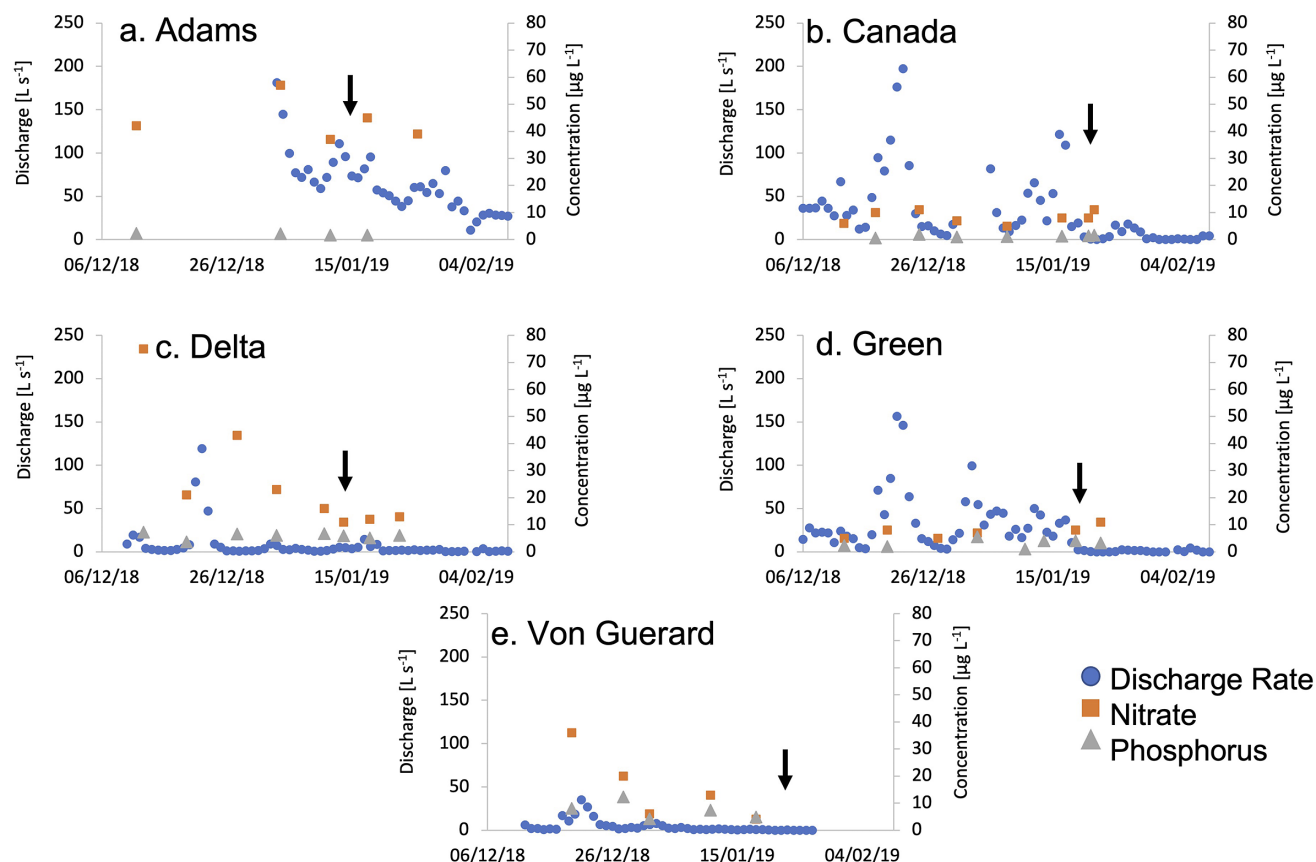


Figure 4. Hydrographic data, nitrate and phosphorus concentrations and sampled days (black arrows) for each sampled stream: **a.** Adams, **b.** Canada, **c.** Delta, **d.** Green and **e.** Von Guerard.

Table 1. The stream characteristics considered in this study. Green algal mat coverage was qualitatively observed at the transects for the 2018–2019 season.

Stream	Stream length (km, category) ^{a,b}	Average season length (days) ^{a,b}	Average annual discharge (m ³ year ⁻¹) ^{a,b,c}	Algal coverage (green mat)
Adams	2.7 (short)	52 ^b	6.83×10^5 ^c	High
Canada	1.5 (short)	71	2.06×10^5	High
Delta	11.2 (long)	55	1.05×10^5	Low
Green	1.2 (short)	57	1.42×10^5	High
Huey	2.1 (short)	41 ^b	5.79×10^4 ^b	Low
Von Guerard	4.9 (long)	44	5.97×10^4	High

^a Values from Cozzetto *et al.* (2006).

^b Values estimated from data from mcm.lternet.edu.

^c Values from Howard-Williams *et al.* (1986), reported as maxima.

to isolate green mats from the sometimes-surrounding orange mats (McKnight *et al.* 1999). Forceps and a spatula, triple-rinsed in stream water, were used to gather 3 cm × 6 cm sections of mat. A total of 48 samples were collected; at least six samples of mat were collected at each stream. Sampling Von Guerard Stream at three distinct locations allowed for testing of intra-stream species variation. Mat sections were each placed in a sterile 200 ml Nalgene screw-top sample bottle, triple-rinsed in stream water before sample deposit, and filled with ~100 ml of associated stream water, with the tops screwed on tightly. Bottles were wrapped in tinfoil and placed in a +7°C refrigerator at the field campsite within 4 h

of collection. Samples were hand-carried back, over 3 days, to the University of Colorado-Boulder in a cooler maintained at +4°C. When the bottles arrived at the Sustainability, Energy, and Environmental Community (SEEC) Phycology Lab at the University of Colorado-Boulder, the lids to the bottles were loosened to allow for ventilation, and the foil-wrapped bottles were placed on a dark shelf in a +7°C refrigerator. Cultures were checked every 3 days for any changes in colouration, potentially indicating culture death.

Culturing and microscopic methods

Subsamples of mats from the Adams, Canada and Von Guerard (pavement, cobble, sediment) collections were transferred from the sample bottles to 250 ml Erlenmeyer flasks containing 125 ml sterile Bristol Medium and lightly capped with foil. The medium contained 2.94 mM NaNO₃, 0.17 mM CaCl₂·H₂O, 0.3 mM mgSO₂·7H₂O, 0.43 mM K₂HPO₄, 1.29 mM KH₂PO₄ and 0.43 mM NaCl (pH 6.4). Over the incubation period, sterile Bristol Medium was added to the flasks to return the total volume to 125 ml. The flasks were placed on an orbital shaker table (100 RPM, 2" diameter path) in the +7°C refrigerator under a cool white fluorescent lamp (4000K) with a 12:12 h light:dark cycle. The refrigerator temperature is reflective of Canada Stream's average summer temperature (Cozzetto *et al.* 2006). While the Antarctic summer has 24 h sunlight, the light:dark cycles reflect the relative motion of the sun across the MDVs, which produces diurnal cycles in direct solar radiation on the microbial mats (Darling *et al.* 2017). Growth in the flasks was monitored at 3 day intervals over the course of

6 months through photographic comparison as a coarse quantitative method. Samples from the Von Guerard Pavement Exposed collection, cultured in Bristol Medium, were used 3 months after collecting for the Matula *et al.* (2021) study of green mat responses to spacecraft thermal environments.

Analysis by microscopy and flow cytometry provided relative abundances of species, and the flow cytometry resulted in particle counts. For microscopy, mat samples were mounted on glass microscope slides with raw stream water. An inverted microscope (Nikon Eclipse TS 100) and compound light microscope (Olympus Vanox), both with a magnification of 200 $\times$, also identified any additional eukaryotic species (including tardigrades and nematodes). The flow cytometer (FlowCAM VS-IV), with a flow rate of 0.10 ml min⁻¹ and sample volume of 0.35 ml, provided the particle density. The FlowCAM also captured magnified images (10 $\times$ magnification setting) of the counted cells for further photographic assessment of species composition.

DNA extraction and sequencing

Using a laminar flow hood and sterile equipment, ~0.5 ml sub-samples of mats from the transported sample bottle, with enough associated stream water (these samples are referred to as 'Antarctic Water') or Bristol Medium to total 1.0 ml, were placed in sterile cryovials and frozen at -20°C before shipping on dry ice to the University of New Mexico. These samples were transferred and transported after being maintained in the laboratory for 4 months.

DNA was extracted from samples using a variation of the cetyltrimethylammonium bromide (CTAB) method described in Mitchell & Takacs-Vesbach (2008). An equal volume of sucrose lysis buffer (SLB; 20 mM ethylenediaminetetraacetic acid (EDTA), 200 mM NaCl, 0.75 M sucrose, 50 mM Tris-HCl, pH 9.0; Giovannoni *et al.* 1990) was added to the sample (200 ml) and then two volumes of CTAB buffer (1% CTAB, 0.75 M NaCl, 50 mM Tris pH 8, 10 mM EDTA) and proteinase K (final concentration 100 mg ml⁻¹) were added to samples and incubated for 1 h at 60°C on a continuous rotator. Sodium dodecyl sulphate was added to a final concentration of 2%, and samples were incubated for another 30 min on the rotator. DNA was extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (50:49:1) followed by two extractions with an equal volume of chloroform. DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and two volumes of 95% ethanol and incubated for 12 h at -20°C. The samples were then centrifuged for 45 min (~21k $\times$ g), washed in 70% ethanol and resuspended in 10 mM filter-sterilized Tris buffer pH 8.0.

Samples were prepared for dual-index paired-end amplicon sequencing of the V9 region of the small subunit (SSU) of eukaryotic rRNA genes (18S rRNA genes) using 1391F 5'-GTA CAC ACC GCC CGTC-3' and EukBR 5'-GTA CAC ACC GCC CGTC-3' (Amaral-Zettler *et al.* 2009, Stoeck *et al.* 2010, Caporaso *et al.* 2012). Primers included overhang adapter sequences for compatibility with Illumina index and sequencing adapters. PCR was performed in 25 μ l triplicates using 5Prime Hot Master Mix Thermocycler conditions recommended by the Earth Microbiome Project (EMP). EMP eukaryotic primers, without blocking primers, were used during PCR: 95°C for 45 s $\times$ 35, 57°C annealing temperature for 60 s $\times$ 35, 72°C for 90 s $\times$ 35 and 72°C for 90 s $\times$ 35. Amplicons were cleaned and normalized using the Sequelprep kit (Fisher Science, Cat No. A1051001) and indexed using the Nextera XT index kit following the manufacturer's instructions. Indexed amplicons were combined and cleaned using the AMPure

XP bead-based reagent. The library was run on an Illumina MiSeq using the v3 reagent kit with 18% PhiX sequencing control DNA. Eukaryotic DNA sequences were filtered and trimmed using truncation lengths of 140 (forward) and 100 (reverse) for a Phred quality score of above 20 e using DADA2 (1.30.0, R version 4.3.1; Callahan *et al.* 2016). In total, 40% of the fragments were in the 145 bp range after trimming. Sequence length ranged from 140 to 170 bp. Sequences were interleaved, and resulting chimeric sequences, which accounted for 0.01% of merged reads, were identified and removed. Exact amplicon sequence variants (ASVs) were used to identify unique 18S rRNA gene sequences, and taxonomic assignments were made using the pre-trained naïve Bayesian classifier and the Silva database training set (v132). The National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) was used to blast sequences against the NCBI database to further refine taxonomic assignments (Sayers *et al.* 2022). The DNA sequence data are available from NCBI as Bioproject PRJNA1066885.

Statistical analysis

The statistical significance of differences in community composition was determined using a Bray-Curtis dissimilarity index while a permutational multivariate analysis of variance (PERMANOVA) was performed using the R package *vegan* (2.6-8) (Oksanen *et al.* 2017). Alpha diversity and relative abundances were evaluated using the R packages *vegan* and *phyloseq* (1.46.0) (McMurdie & Holmes 2013). A non-parametric pairwise Wilcoxon test was performed to determine the statistical significance of similarities in species richness between treatments using the R package *stats* (4.3.1). Hierarchical clustering was done by first calculating dissimilarities using a Bray-Curtis dissimilarity index and then clustering using the R packages *NbClust* (3.0.1) and *cobclust* (0.1.2) (Charrad *et al.* 2014, Aubert *et al.* 2021).

Two approaches were used to predict the most important Chlorophyta ASVs for classifying the microbial mat cultures into medium type (Antarctic Water vs Bristol Medium). First, Random Forest, a robust machine learning algorithm that used a combination of multiple decision trees and their predictors to identify the most important predictor variables, was implemented using the R package *randomForest* (4.7-1.1) (Breiman 2001). A mean decrease Gini score was generated as a proxy for permutational importance. Secondly, Indicator Species Analysis (ISA) was performed using the R package *indicspecies* (1.7.15) to verify Random Forest results (De Cáceres & Legendre 2009).

Results

Morphological observations

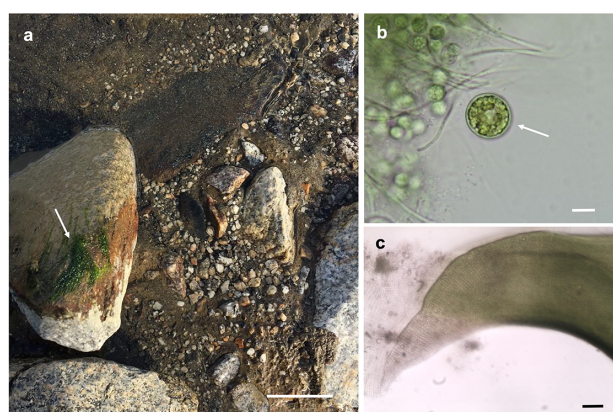
Table II includes the ash-free dry mass and chlorophyll-*a* (Chl-*a*) measurements for the 2018–2019 season. Von Guerard Stream had the highest ratio of Chl-*a* to dry mass for both the sediment and pavement regions (1.2 and 0.6 μ g mg⁻¹, respectively). While all mat samples were collected from the main thalweg of each stream, major distinctions were noted in the substrate and attachment of the mats from the two valleys. Adams Stream (Miers Valley) samples were thin (0.5 mm width), long (4 cm length) individual streamers arranged in a thick mat of epilithic growth but originating above the sand substrate (Fig. 5a). These streamers were not

Table II. The ash-free dry mass (AFDM) and chlorophyll-*a* (Chl-*a*) measurements of green mats for the studied streams in the 2018–2019 season.

Stream	AFDM (mg cm ⁻²) ^a	Chl- <i>a</i> (μg cm ⁻²) ^a	Chl- <i>a</i> :AFDM (μg mg ⁻¹)
Adams	3.9 ± 2.6	1.9 ± 0.5	0.48
Canada	10.1 ± 3.3	2.9 ± 0.4	0.29
Delta	5.8 ± 1.5	3.3 ± 1.3	0.57
Green	7.2 ± 1.7	3.4 ± 0.3	0.47
Huey	NA	NA	NA
Von Guerard (sediment)	4.5 ± 1.1	5.3 ± 1.2	1.2
Von Guerard (pavement)	5.0 ± 2.1	3.0 ± 1.2	0.6

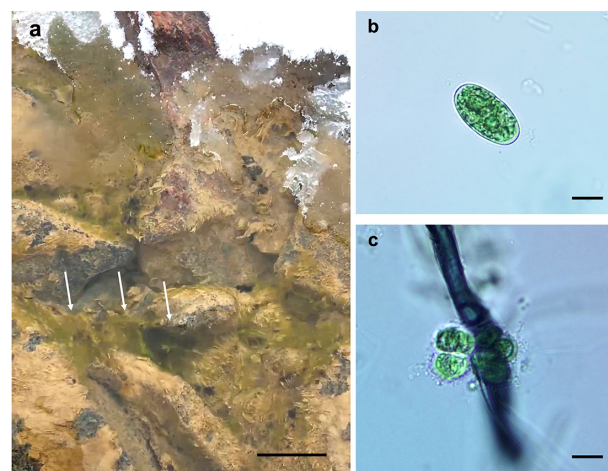
^a Values from the 2018–2019 database at mcm.lternet.edu.

NA = not applicable.

**Figure 5.** Green algae found in the main thalweg of Adams Stream in Miers Valley. **a.** Thin, filamentous mat attached to the base of larger rocks; **b.** coccoid *Pleurastrum* colonies in the filamentous matrix and among cyanobacteria; **c.** multiseriate *Prasiola* fronds. White arrows indicate described items. Scale bars: **a.** 5 cm, **b.** 10 μm, **c.** 5 μm.

supported by any other mat type, and the surrounding substrate was clear of any other mats. Colonies of coccoid *Pleurastrum* cells ranging between 5 and 15 μm in diameter were observed via microscopy. In larger, vegetative cells, the pyrenoid was visible (Fig. 5a). Larger, longitudinal arrangements of cells building multiseriate *Prasiola* fronds with wavy edges (100 μm across at base) were also isolated from these samples when cultured in Bristol Medium (Fig. 5c).

Samples from Taylor Valley were supported on a matrix of orange mat, regardless of the location among the stream bed. The morphology of sampled green material was similar across all Taylor Valley samples. Distinctly different than the Adams Stream samples, the Taylor Valley green mat was short (1.5 cm in length), tufted and originating from a central frond (Fig. 6a). Thalli were fine and short (0.25 mm width, 2 mm length), extending from midway to the end of the central frond. From these samples, there were two major Chlorophytes observed. First were ovoid, unicellular Chlorococcaceae cells with a very discernible pyrenoid and a thin cell membrane (< 1 μm); these cells were 20–25 μm in length and 10 μm across and primarily found in Canada Stream and Von Guerard Stream (all) samples (Fig. 6b). The second commonly recorded cell structure was globular, readily rounded *Hazenia* cells (7–10 μm wide and 5 μm long), typically present in pairs, with a parietal chloroplast and indiscernible pyrenoid (Fig. 6c).

**Figure 6.** Green and orange algae found in Canada Stream in Taylor Valley. **a.** Branched filamentous green algae growing on top of an orange mat in the interstitial spaces attached to larger rocks; **b.** ovoid Chlorococcaceae; **c.** globular *Hazenia* cells. White arrows indicate described items. Scale bars: **a.** 5 cm, **b.** 10 μm, **c.** 5 μm.

Other organisms were recorded in the green mat samples across both valleys (Fig. 7). Nematodes (Fig. 7a), rotifers (Fig. 7b) and tardigrades (Fig. 7f) were documented in the majority of the samples, whereas diatoms were most prevalent in the Von Guerard Stream samples, including in the culture for the Matula *et al.* (2021) study. Most diatoms were *Hantzschia* spp. (40–70 μm length and 7–9 μm width) and integrated into colonies of coccoid algae. The remaining green mat matrix was composed of cyanobacteria.

The diatom community composition of green mat samples collected as part of the monitoring programme of the MCMLTER for the studied streams is summarized in Table III. For Von Guerard Stream, the dominant diatom genera in the green mat samples were *Hantzschia* and *Luticola*, with *Stauroneis* and *Mulleria* also being common. However, in previous samples from 2012 and 2010, *Luticola* was more abundant than *Hantzschia*. Delta Stream had a similar dominance pattern to Von Guerard Stream. In contrast, for Canada Stream, *Luticola* was consistently the dominant genus, with *Hantzschia* typically rare. For Green Creek, *Luticola* was consistently either abundant or common, whereas the relative abundance of *Hantzschia* varied from most abundant to rare. The diatom communities in green mats from Adams Stream and Huey Creek have not been characterized.

18S rRNA gene sequencing analysis

Next-generation sequencing of the 18S rRNA gene for all of the green mat samples resulted in 359 695 reads. Total reads for each sample ranged from 241 to 34 858 after filtering and removing chimeras. Samples from the Matula *et al.* (2021) study are indicated in all resulting figures (Von Guerard Pavement Exposed, cultivated in Bristol Medium).

Shannon and Simpson alpha diversity metrics estimated species richness, determining that there was no statistical difference in the estimated species richness across all species when comparing cultivation medium and substrate on which the mat was grown ($P = 0.77$). There was, however, a significant difference in cultures grown in Bristol Medium vs Antarctic Water (Bray-Curtis R -statistic = 0.12, $P = 0.002$; Fig. 8a). Samples grown in Bristol Medium tended to group together separately in a principal coordinate analysis

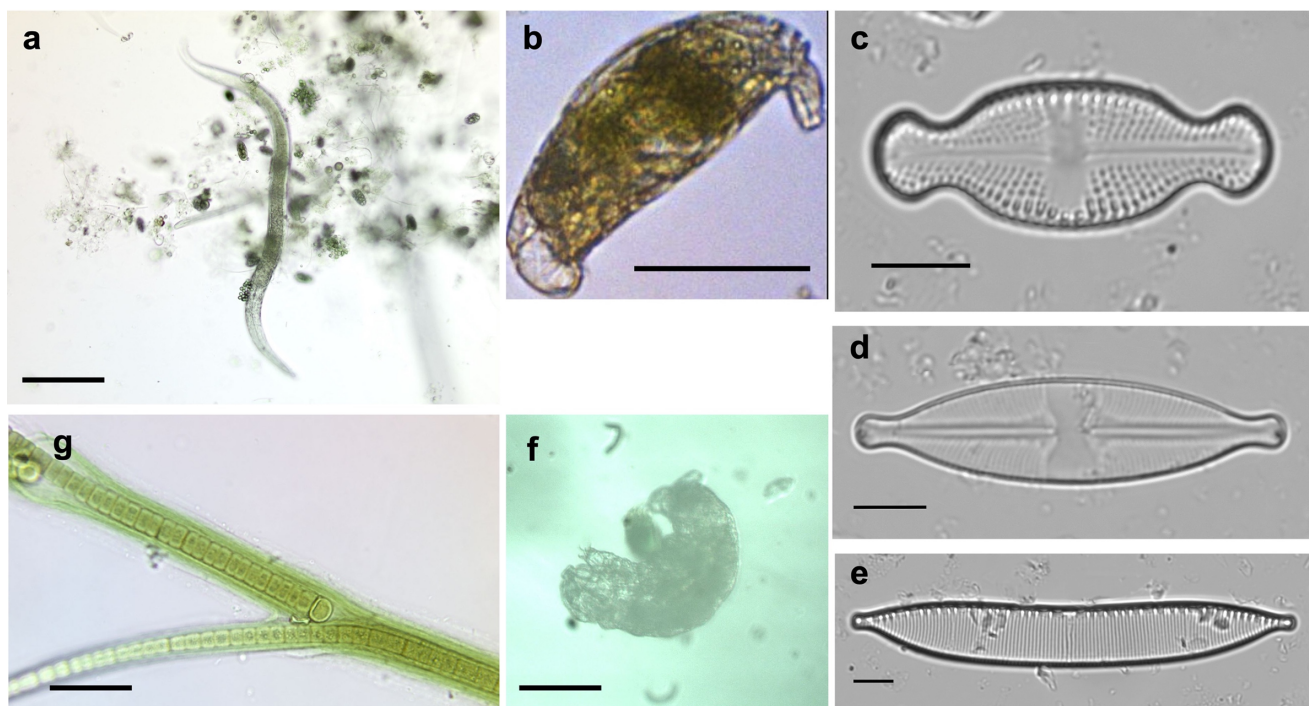


Figure 7. Microscopic organisms also found in the green mat samples: **a.** Nematode (Adams Stream), **b.** rotifer (Canada Stream), **c.** *Luticola permuricopsis* (Von Guerard Pavement), **d.** *Stauroneis latistauros* (Von Guerard Pavement), **e.** *Hantzschia abundans* (Von Guerard Pavement), **f.** tardigrade (Von Guerard Sediment) and **g.** cyanobacteria (Delta Stream). Scale bars: **a.** 100 μ m, **b.** 50 μ m, **c.** 10 μ m, **d.** 10 μ m, **e.** 10 μ m, **f.** 50 μ m, **g.** 10 μ m.

(PCoA) ordination from Antarctic Water samples. There was also a significant difference in Chlorophyta communities between cultures grown in Bristol Medium vs Antarctic water ($P = 0.003$; Fig. 8b).

There were 1030 unique ASVs identified across 31 phyla. Samples obtained in Antarctic Water were dominated by Cercozoa (21%), Rotifera (19%) and Ciliophora (14%). The remaining percentage was composed of Ochrophyta, Chlorophyta, Nucleariidae and Tardigrada (Fig. 9 & Table IV). The samples grown in Bristol Medium were most notably dominated by Chlorophyta (52%) and Rotifera (17%). The remaining percentage was composed of Chytridiomycota, Ochrophyta, Tardigrada and Cercozoa (Fig. 9 & Table V). Reflecting the microscopy observations, tardigrades were present across all treatments, whereas rotifers were notably reduced in Bristol Medium samples. Cercozoa were identified in all Antarctic Water samples but reduced in the Bristol Medium samples. Calculating the Bray-Curtis distance coefficient across the identified phyla suggested that the greatest similarity was between the Adams and Von Guerard Pavement samples cultured in Antarctic Water (Fig. 10a). After subsetting Chlorophyta to analyse the dominant phototrophic phylum, there were a total of 48 ASVs identified across three classes: Ulvophyceae, Trebouxiophyceae and Chlorophyceae.

Samples grown in Antarctic Water had a majority of *Pleurastrum* (34%), *Hazenella* (24%) and *Scenedesmus* (8%), whereas samples grown in Bristol Medium contained *Planophila* (38%), *Pleurastrum* (22%), *Chlorella vulgaris* (16%) and *Chlorococcum* (8%; Fig. 11 & Tables VI & VII). The remaining 34% of the organisms grown in Antarctic Water included Chlorophyceae, *Urospora* and *Rhysamphichloris*. The remaining 16% of organisms in samples grown in Bristol Medium included *Scenedesmus*,

Hazenella and *Muriella*. The Bray-Curtis (BC) distance index indicated a major dissimilarity between Delta, Green and Canada Antarctic Water samples and the rest of the sample set (BC = 0.8; Fig. 10b). However, Delta, Von Guerard Cobble and Von Guerard Pavement Antarctic Water samples (exposed) were extremely similar (BC = 0.0) to each other and to Miers Valley Adams Stream Bristol Medium (rock; BC = 0.05).

After blasting sequences against the NCBI database, identifications of ASV2, ASV37, ASV19, ASV17 and ASV31 were further refined with a 100% match (DNA-DNA identity) for *Planophila*, a 99.4% match for *Endolithella mcmurdensis*, a 98.81% match for *C. vulgaris*, a 100% match for *Scenedesmus* spp. and a 98.8% match for *Chlorococcum sphacosum* (Fig. 12). The machine learning algorithm Random Forest determined the presence of which taxa were most associated with growth medium used. The model error rate was estimated to be 18.75%. Class error was 0% for Antarctic Water and 75% for Bristol Medium, probably due to the small sample sizes of the latter. Due to this high error rate in the Random Forest model, ISA was performed and yielded comparable results. ASV2, identified as *Planophila* in class Ulvophyceae, was the most important predictor of medium type (Bristol Medium), with a mean decrease Gini value of 1.0, indicating its high permutation importance in this dataset (ISA $P = 0.0002$). The other most important identified taxa included *E. mcmurdensis* (ISA $P = 0.0002$), *Scenedesmus* (ISA $P = 0.0007$), *Pleurastrum* (ISA $P = 0.0019$), *Chlorella*, *Muriella*, *C. sphacosum* and *Coccomyxa*.

The machine learning algorithm Random Forest determined the presence of which taxa were most associated with the identification of the source stream (Fig. 13). The model error rate was estimated to be 93.75%. Class error was 75% for Adams Stream and 100% for the Canada, Delta, Green, Huey and Von Guerard sites.

Table III. Summary of long-term relative abundance data for major diatom genera found in green mats for the streams studied, including Von Guerard and Delta streams for 2019 samples. No green mat data are available for Adams Stream and Huey Creek. Abundant (A) is > 20%, common (C) is 5–20%, rare (R) is < 5%, not found (N/F) is 0%.

	Stream																						
	Von Guerard						Canada						Green						Delta		Adams		
	2019		2012		2010	2019	2018	2012	2001	1995	2019	2018	2013	2012	2011	1998	2019	2010	2019				
Sample no.	1	2	3	4	1	1	1	1	1	1	2	3	1	1	2	1	2	1	1	1	1	3	
<i>Hantzschia</i> spp.	A	A	A	C	C	C	R	C	R	R	R	R	C	N/F	R	R	A	A	A	C	A	C	R
<i>Luticola</i> spp.	A	A	A	A	A	A	C	A	A	C	A	A	A	A	A	A	C	A	A	A	A	A	A
<i>Stauroneis</i> spp.	C	N/F	R	R	R	R	R	R	R	R	C	C	C	R	C	A	N/F	R	N/F	C	R	R	R
<i>Muelleria</i> spp.	C	N/F	N/F	N/F	R	N/F	R	R	C	R	C	C	R	N/F	R	R	R	C	R	C	C	R	R
<i>Humidiphilia</i> spp.	N/F	N/F	R	R	C	R	C	C	A	C	C	R	C	R	R	R	A	A	N/F	C	C	R	C

Values are from the Antarctic Freshwater Diatoms online database (huey.colorado.edu/diatoms).

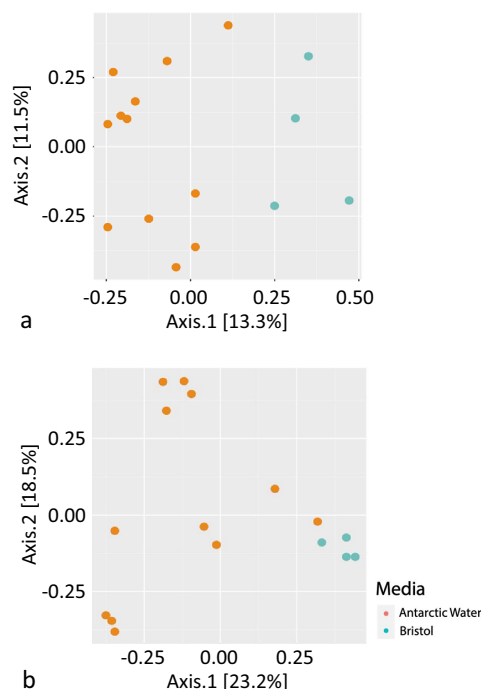


Figure 8. Alpha diversity metrics detecting differences in species richness for **a.** all included species and **b.** Chlorophyta across sampled site, location in stream and treatment (Antarctic Water or Bristol Medium).

Discussion

Identifying the phototrophic genera that comprise MDV green algal mats and their relative abundance across two valleys allows for their better monitoring. Understanding the ecological characteristics of each dominant genus may help tailor future mat collection activities.

These phototrophic genera were collected in the MDVs, and the natural environment is very specific to this area (high ultraviolet radiation, seasonal streams and cryodesiccation), but similar mats have been documented in other areas across Antarctica, including King George Island, the South Orkney Islands, James Ross Island and Terra Nova Bay (Friedl & O'Kelly 2002, Škaloud *et al.* 2013, Rybalka *et al.* 2023, Sciuto *et al.* 2023). Reports included *Pleurastrum*, *Hazenian* and *Planophila*, as these studies included morphological and genetic assessment sequencing. These mats are thriving across the continent. Due to their widespread abundance across the continent, the conducted Random Forest model suggested that a stream could not be identified solely by the species present. The high model error rate in conjunction with the genera relative abundance values corroborate the published accounts of widespread populations of *Pleurastrum*, *Hazenian* and *Planophila*. Researchers could spread their sampling demand across multiple sites, using these areas as bioprospecting units for extremophilic algae. Furthermore, these sites could be thought of as a biorepository, sustaining the unique composition of mats in native water before sampling and utilization.

Dominance within mats significantly shifted to Chlorophyta when culturing select mats in Bristol Medium. Blasting the samples revealed the dominance of *Pleurastrum* across both Antarctic Water and Bristol Medium types, regardless of them being fully exposed (exposed) or protected (rock) from the flow within the stream bed. This presence across all samples and experimental

conditions suggests the ability of Chlorophyta to acclimatize to a range of environments. The experiments executed by Matula *et al.* (2021) used a monoculture (*C. vulgaris*) as the control, as this is an extensively researched species, and a sample of Von Guerard Pavement (exposed) grown in Bristol Medium. The experiment tested the physiological response of each culture to dynamic environmental temperature cycles reflective of an extreme space-flight environment. The Antarctic mat had consistently higher chlorophyll fluorescence measurements, indicating minimal cellular stress, and consistently increasing oxygen production over time. However, the Antarctic mat had a slower biomass production rate than that of *Chlorella*, reflective of the regrowth rates documented by Kohler (2015) after high-flow seasons. Sequencing results of Antarctic green mats indicate that these dynamic temperature experiments were conducted using a majority of *Planophila*. However, it is unknown whether the other present genera (*Hazenian*, *Scenedesmus*, *Muriella*) contributed to the performance of these mats.

The presence of diatoms in all green mats from the MDVs and in the Bristol Medium culture raises questions regarding the potential relationship between diatoms and green mats. The potential symbiotic relationships between the chlorophyte, cyanobacteria and diatom communities may be a key aspect in the persistence of these green mats under the harsh local conditions, especially the rapid revival of the photosynthetic capability of the mats upon rewetting after persisting in a freeze-dried state through the winter (Stanish *et al.* 2011). Nematodes are also consistently found in green mat samples and were morphologically observed here (Dalto *et al.* 2010, Zoumplis *et al.* 2023). These taxa from the native mat community may limit the vulnerability of the culture to being overtaken by other non-native organisms while cultured in native water, but both nematodes and diatoms were reduced in the Bristol Medium (Fig. 9). It is unknown whether this was in relation to the suppression of *Pleurastrum* or the dominance of *Planophila* or a response to higher nutrient loading in the medium. This variation in the diatom community in green mats among streams and over time may be associated with the overall variation in the chlorophyte communities of the mats (Van Horn *et al.* 2016).

Due to similar rotifer and tardigrade densities, the closest of all pairs at the phylum level were mats collected from Adams Stream (exposed) and Von Guerard Pavement (exposed), both maintained in Antarctic Water. Even when cultured in Bristol Medium, this pair kept its relative distance due to similar Chlorophyta and eukaryote density (Fig. 10). Stream length (long) and level of rock protection (exposed) may suggest the presence of rotifers and tardigrades and could be used to target other stream investigations across the continent. However, while the Von Guerard Stream samples were all selected from the same stream, the various locations along the stream were significantly distant from each other when comparing all species. This may be a result of the changing nutrient content or flow rate of the stream along its entire length.

Understanding the relationship of flow, nutrients and species present may help estimate mat type density for the years before mat monitoring was established. Focusing on the genera within Chlorophyta, Adams Stream samples (rock and exposed) in Antarctic Water are relatively close to each other but isolated from the rest of the samples (Chlorophyceae, *Chlamydomonas* and *Rhysamphichloris*). Von Guerard Stream samples (pavement/cobble, exposed) all had the same genera and distribution as Delta Stream (Antarctic Water, exposed) and Adams Stream (Bristol Medium, rock). Both observations (isolation of Adams Stream and distribution of Von Guerard and Delta streams) may

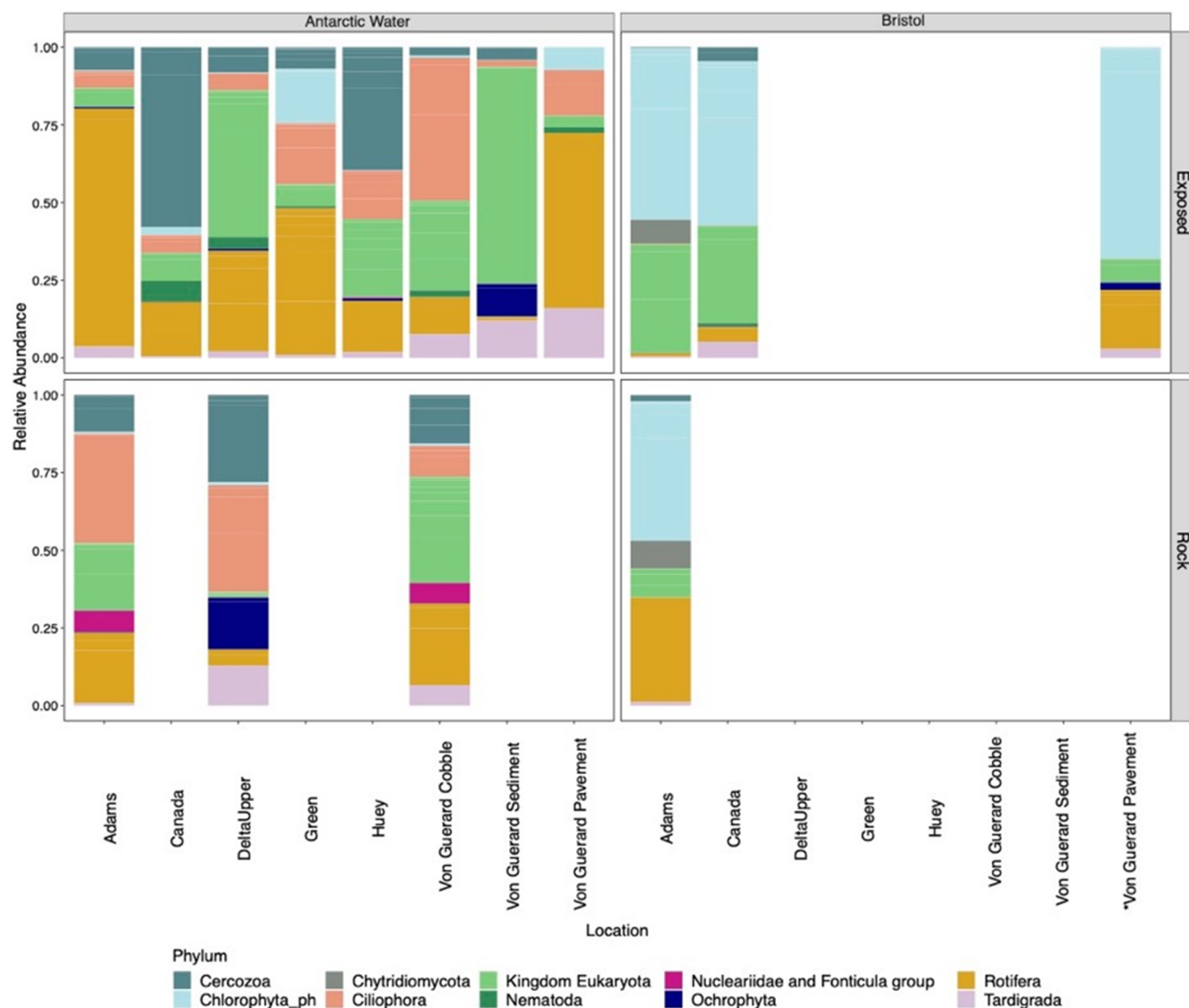


Figure 9. Relative abundance within the phyla for each sampled site, location in stream and treatment (Antarctic Water or Bristol Medium). Only phyla representing 67.5% of total abundance are shown; the remaining 32.5% of phyla were unidentifiable. DeltaUpper here was a site signifier from the McMurdo Dry Valleys Long Term Ecological Research (MCMILTER) project. *Von Guerard Pavement Exposed in Bristol Medium was used in Matula *et al.* (2021).

suggest the significance of the location of streams across the MDVs at the genus level.

The Random Forest classification of included ASVs indicated that the presence of *Planophila* and *E. mcmurdensis* distinguished whether mats were maintained in Antarctic Water or Bristol Medium. Proliferation of these algae may be a direct result of the increase of available nitrogen and phosphorous in Bristol Medium. Understanding the mat response to increased availability of nutrients may provide insights into potential shifts in mat composition with changes in flow patterns. More rapid glacial melting would potentially result in higher flow, increasing mineral weathering, expanding the hyporheic zone and providing more nutrients to the mats. Recent studies based on the ^{15}N signature of suspended particulate material in streams have demonstrated that a major pathway for N cycling in these streams is N fixation by black mats (diazotrophic *Nostoc*) followed by downstream transport of mat material, entrainment into the hyporheic zone and then release of inorganic N to the stream through hyporheic exchange (Kohler

et al. 2016). A study by Kohler *et al.* (2018) confirmed that the green mats in Von Guerard Stream acquired the ^{15}N signature of the back mat material at downstream sites. The N-cycling model developed by Kohler *et al.* (2023) helps to explain how the abundance of orange and potentially green mats may be dependent on the presence of the black mats providing an N subsidy to the stream. Nitrogen concentration across the hyporheic zone is not uniform (Niyogi *et al.* 1997, McKnight *et al.* 2004). It is hypothesized that green mat samples taken from the surface of rocks are representative of the green mats in contact with black mat-derived N, as sloughed black mats accumulate in rock pavements and increase local concentrations of nitrogen (Heindel *et al.* 2021). In terms of the potential increase in streamflow associated with a warming climate, an increase in streamflow may accelerate this N-cycling pathway if the growth of the black and/or green mats does not become limited by scouring during high-flow events (Cullis *et al.* 2014).

The results of the molecular analysis did not identify *Prasiola* at the genus level, unlike *Hazen* or *Planophila* (Fig. 12).

Table IV. Relative abundance within the phyla for all sampled sites and Antarctic Water treatment. 'N/A' refers to 'not analysed' due to a lack of representation in the database.

Phylum	Relative abundance (%)
N/A	32.5
Cercozoa	21.0
Rotifera	19.4
Ciliophora	14.2
Ochrophyta	3.4
Tardigrada	3.0
Nucleariidae and Fonticula group	1.68
Chlorophyta	1.33
Nematoda	0.74
Euglenozoa	0.55
Choanoflagellida	0.49
Phragmoplastophyta	0.35
Chytridiomycota	0.30
Incertae Sedis	0.24
Ascomycota	0.17
Peronosporomycetes	0.14
Dinoflagellata	0.11
Cryptomonadales	0.09
Basidiomycota	0.07
Heterolobosea	0.04
Tubulinea	0.04
Vertebrata	5.95E-03
Opalomonadea	5.95E-03

Table V. Relative abundance within the phyla for all sampled sites and Bristol Medium treatment. 'N/A' refers to 'not analysed' due to a lack of representation in the database.

Phylum	Relative abundance (%)
Chlorophyta	52.9
N/A	20.5
Rotifera	16.8
Chytridiomycota	4.10
Tardigrada	1.68
Cercozoa	1.48
Ochrophyta	1.03
Tubulinea	0.83
Ciliophora	0.17
Nematoda	0.13
Nucleariidae and Fonticula_group	0.08
Dinoflagellata	0.05
Cryptomonadales	0.04
Choanoflagellida	0.04
Basidiomycota	0.03
Phragmoplastophyta	0.03
Ascomycota	0.03
Euglenozoa	0.02
Vertebrata	4.97E-03
Incertae Sedis	0.00
Peronosporomycetes	0.00
Heterolobosea	0.00
Opalomonadea	0.00

However, *Prasiola* was discovered morphologically (Fig. 4c). It is hypothesized that *Prasiola* dominated the relative abundance in the class Trebouxiophyceae (Fig. 12), but, due to ongoing primer bias, underrepresentation in genetic databases or insufficient DNA yields under the current extraction method, it was not identified at the genus level. Rybalka *et al.* (2023) experienced similar sequencing challenges in a 2023 surface soil study. Algal samples collected from the surface soils of *Meseta* on King George Island resulted in the representation of all four targeted algal classes (Chlorophyceae, Trebouxiophyceae, Ulvophyceae and Xanthophyceae; Rybalka *et al.* 2023). Green algae (Trebouxiophyceae, *Prasiola*) dominated the sample set. Although the 830 algal operational taxonomic units (OTUs) indicated high diversity among the samples, the majority (86.1%) could not be identified at the species level due to a lack of representation in the reference databases.

Morphological observations are sufficient for identifying the presence and relative magnitude of abundance of a taxon in a sample. However, understanding how *Prasiola* presents during the various stages in its life cycle is key, and failure to do so may result in underestimation of its abundance. Its reproduction takes place through aplanospore or oogamy (John & Rindi 2003), although the sexual cycles of *Prasiola* are poorly understood (Kováčik & Pereira 2001). Understanding the life cycle of *Prasiola* may be key to

interpreting such long-term data, as changes in green mat biomass may reflect the life history of *Prasiola* as well as surrounding environmental factors such as streamflow.

This initial review of chlorophytes in the MDV green mats corroborated the sequencing results with microscopic analysis. Using these results, it may be possible to examine the preserved and archived samples of green mats collected by MCMLTER since 1995 to determine the dominant chlorophyte taxa under various environmental conditions. This analysis may reveal a transition away from *Prasiola* dominance (which would be reported as class Trebouxiophyceae). Earlier MDV surveys reported *Prasiola*, but recent periods of higher flows and generally warmer summers may have scoured away these mats (Seaburg *et al.* 1979, Izaguirre & Pizarro 1998, McKnight *et al.* 1998, Kohler 2015a, Van Horn 2016).

Future work

The species diversity and richness significantly changed when samples were cultured in a medium with a higher concentration of nitrogen and phosphorus. Green mats grown in Bristol Medium trended towards a monoculture due to the dominance of certain genera. It is unclear whether the preferential uptake or uptake

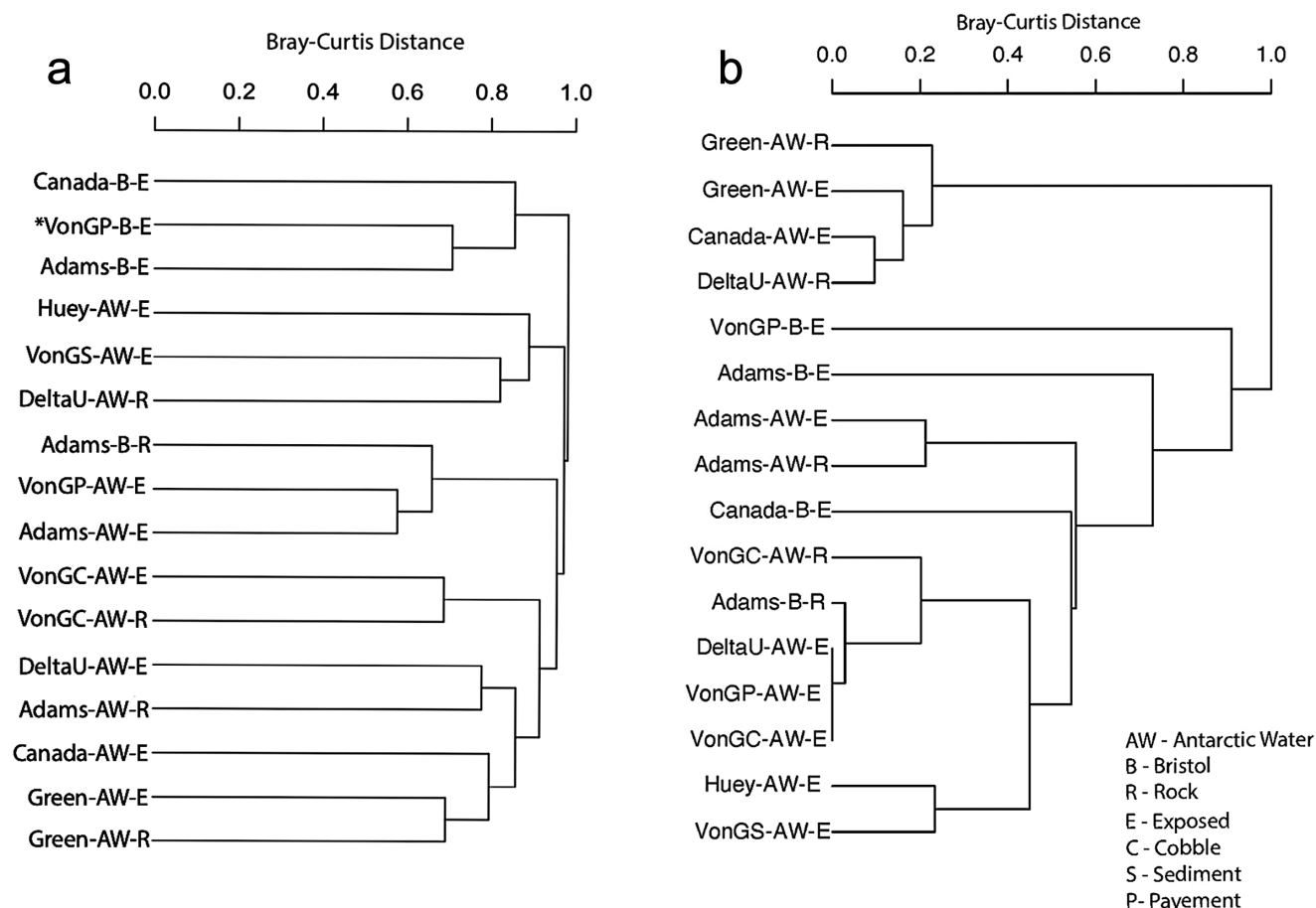


Figure 10. Bray-Curtis distance index comparing relative richness of the **a.** phyla and **b.** Chlorophyta at each sampled site, location in stream and treatment (Antarctic Water or Bristol Medium). DeltaUpper here is a site signifier from the McMurdo Dry Valleys Long Term Ecological Research (MCMLTER) project. *Von Guerard Pavement Exposed in Bristol Medium was used in Matula *et al.* (2021).

efficiency of these genera determined their dominance. Relating changes in genera in conjunction with nitrogen and phosphorus loading in streams in post-high-flow years may be an additional approach to understanding shifts in algal mat composition in response to changes in flow patterns. Furthermore, only one freshwater medium recipe was tested. By culturing these mats in other freshwater media or with various nutrient loadings, other changes in diversity could be documented that could act as biomarkers for nutrient contents or mixtures.

The presence of cyanobacteria was recorded in the morphological observations of the studied green mats, but these were not included in the sequencing analysis. Using 16S sequencing on collected samples to identify cyanobacteria would expand the list of potential photobioreactor candidates for extreme environments, mirroring current cyanobacteria uses in commercial applications (e.g. nutritional supplementation, waste remediation). Furthermore, multiple samples were collected from the featured sites, including both rock and exposed samples. However, the limited time and resources available for sequencing dictated a limited sequencing scope. Completing the sequencing for all gathered samples would strengthen the observations and statistical significance of the abundance and richness data.

Conclusion

Sequencing of the mats maintained in Antarctic Water did not result in identifying any biomarkers correlated to a specific stream. Correlations between mat diversity and flow exposure (rock or exposed) could not be made due to the limited dataset. However, culturing green mats in Bristol Medium resulted in a significant reduction in the diversity and richness of the samples, as Chlorophyta proliferated. When sequencing within the Chlorophyta database, *Pleurastrum* and *Hazenian* dominated the mat samples in Antarctic Water, whereas *Planophila* and *Pleurastrum* dominated the mat samples in Bristol Medium. This suggests that *Pleurastrum* can acclimatize to environments with varying nutrient loads, whereas *Hazenian* and *Planophila* may preferentially take up various forms of nitrogen and phosphorous or be responsive to different concentrations of nitrogen or phosphorous.

Prasiola was not observed in the molecular analysis data at the genus level in Antarctic Water or Bristol Medium, but it is hypothesized that it was represented at the class level in Bristol Medium. Morphological observations of *Prasiola* in Adams Stream Bristol Medium samples corroborated the sequenced results, thereby suggesting that there may still be a PCR bias or gaps in the database for *Prasiola*.

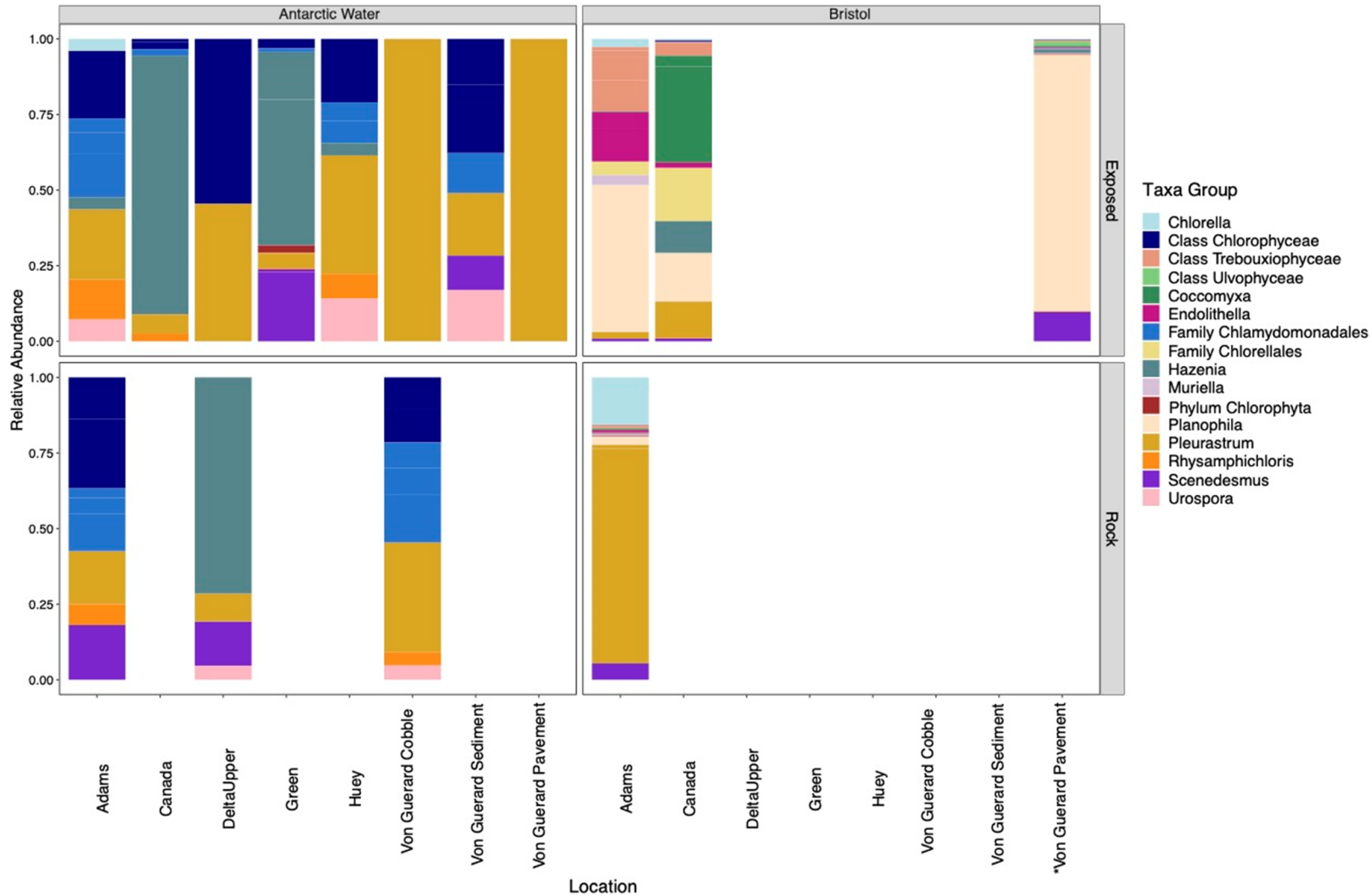


Figure 11. Relative abundance within Chlorophyta for each sampled site, location in stream and treatment (Antarctic Water or Bristol Medium). Only taxa representing 79.5% of total abundance are shown; the remaining 20.5% of taxa were unidentifiable. DeltaUpper here was a site signifier from the McMurdo Dry Valleys Long Term Ecological Research (MCMLETR) project. *Von Guerard Pavement Exposed in Bristol Medium was used in Matula *et al.* (2021).

Table VI. Relative abundance within the Chlorophyta for all sampled sites with Antarctic Water treatment. 'N/A' refers to 'not analysed' due to a lack of representation in the database, but the class or order was identified.

Genus (class or order)	Relative abundance (%)
<i>Pleurastrum</i>	34.0
<i>Hazenia</i>	24.4
N/A (Chlorophyceae)	16.9
N/A (Chlamydomonadales)	9.28
N/A (Sphaeropleales)	7.64
<i>Urospora</i>	3.98
<i>Rhysamphichloris</i>	2.98
N/A	0.41
N/A (Trebouxiophyceae)	0.32
<i>Muriella</i>	0.0
<i>Coccomyxa</i>	0.0
N/A (Ulvophyceae)	0.0
N/A (Chlorellales)	0.0

Table VII. Relative abundance within the Chlorophyta for all sampled sites with Bristol Medium treatment. 'N/A' refers to 'not analysed' due to a lack of representation in the database, but the class or order was identified.

Genus (class or order)	Relative abundance (%)
N/A (Ulvophyceae)	38.5
<i>Pleurastrum</i>	21.7
N/A (Trebouxiophyceae)	16.9
N/A (Chlorophyceae)	8.37
N/A (Chlorellales)	5.55
N/A (Sphaeropleales)	4.23
<i>Hazenia</i>	3.03
<i>Muriella</i>	1.01
<i>Coccomyxa</i>	0.95
N/A	0.11
<i>Rhysamphichloris</i>	0.08
N/A (Chlamydomonadales)	0.04
<i>Urospora</i>	0.00

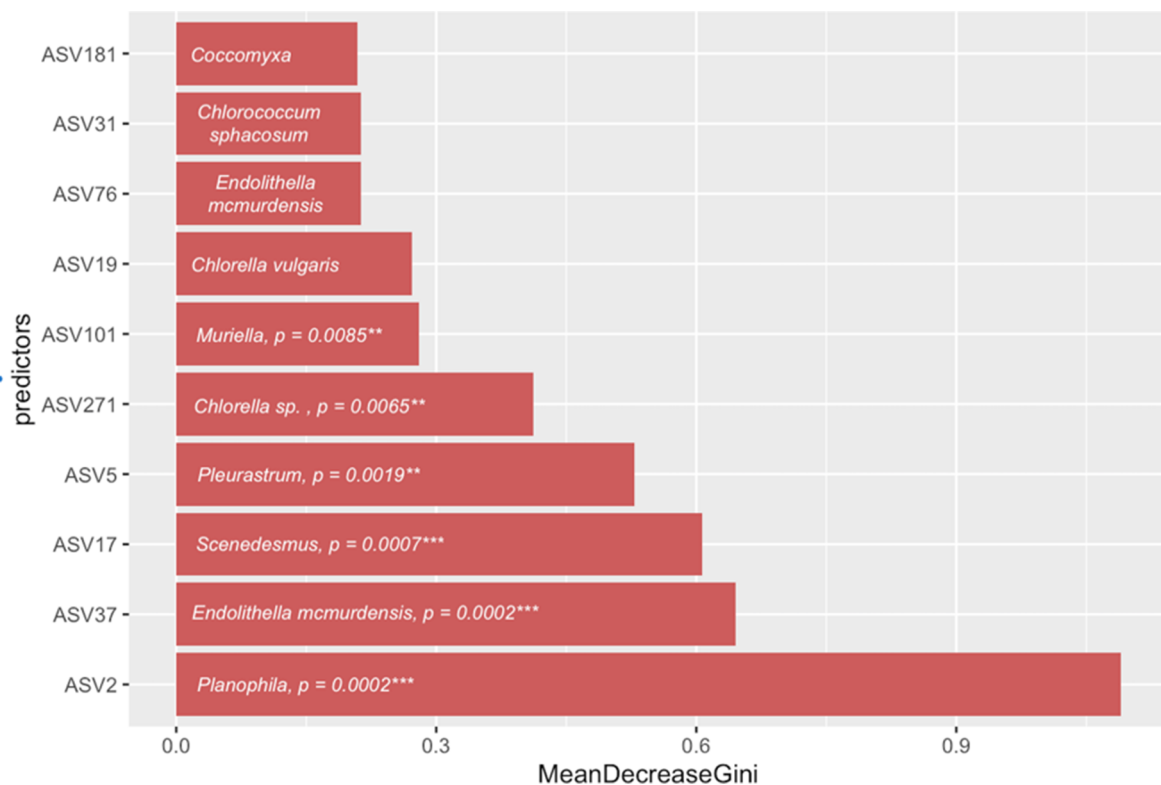


Figure 12. Random Forest classification for determining the most important amplicon sequence variants (ASVs) for classifying green mat sample species into medium type (Antarctic Water or Bristol Medium). The asterisks represent the significance of the species association with the medium treatment.

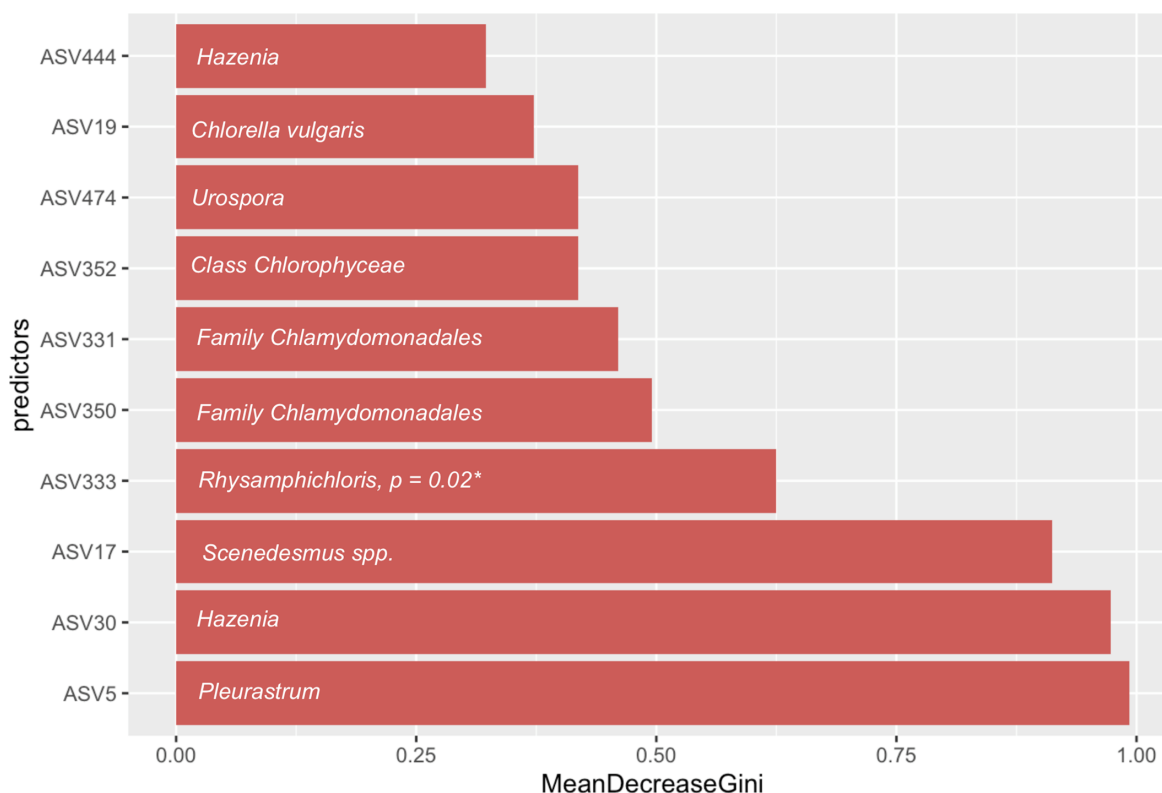


Figure 13. Random Forest classification for determining the most important amplicon sequence variants (ASVs) for classifying green mat sample species into ability to identify the source stream (Adams, Canada, Delta, Green, Huey and Von Guerard sites). The asterisks represent the significance of the species association with the source stream.

Tying the sequencing results to the Matula *et al.* (2021) experiment shows that *Planophila* was the dominant genus in the algal mat samples used in the spaceflight environment experiments.

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Competing interests. The authors declare none.

Author contributions. EMM collected the samples, executed mat isolation and sample analysis and drafted the manuscript. RK, CT-V and DMM provided sample, conducted data analysis and contributed to writing/editing the manuscript. NA contributed to figure development. DMM designed the project.

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