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Extremophile hypolithic communities in the Vestfold Hills, East Antarctica

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Abstract: The Vestfold Hills are a 400 km^2, isolated ice-free oasis in eastern Antarctica featuring large areas with translucent quartz rocks that provide habitat for hypolithic microbial communities underneath. We used high-throughput DNA sequencing of 16S and 18S ribosomal RNA amplicons to characterize bacterial and eukaryotic hypolithic communities across the Vestfold Hills. We found high-level, local heterogeneity in community structure consistent with limited dispersal between hypoliths. Hypolithic communities were dominated by heterotrophic Bacteroidetes (mean bacterial relative read abundance: 56%) as well as Cyanobacteria (35%), with the eukaryote component often dominated by Chlorophyta (43%). Small but significant proportions of the variation in microbial community composition and function were explained by soil salinity (5–7%) and water availability (8–11%), with distinct taxa associated with different salinities and water availabilities. Furthermore, many inferred bacterial metabolic pathways were enriched in hypolithic communities from either dry or high-salinity sites. Vestfold Hills hypolithic habitats are likely to be local refuges for bacterial and eukaryotic diversity. Gradients in soil salinity and water availability across the Vestfold Hills, in addition to the number and diversity of lake types and fjords as potential source populations, may contribute to the observed variation in the extremophile, hypolithic microbial community composition.

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Introduction

Terrestrial Antarctic ecosystems are characterized by limited available water, organic carbon and nitrogen, coupled with freezing temperatures. Terrestrial macroscopic plants and animals are rare, with microorganisms dominating Antarctic diversity, biomass and biogeochemistry in nutrient-poor mineral soils (Cowan *et al.* 2002, Chown *et al.* 2015). Hypolithic (sublithic) environments can be hotspots for diversity and productivity below translucent rocks and gravels (e.g. quartz, marble, limestone) in polar (as well as hot) deserts, with life adhering either to the rock or soil beneath. These micro-habitats provide a milder microclimate in terms of temperature and water availability (Broady 1981, Smith *et al.* 2000), as well as protection from ultraviolet radiation and abrasion, although growth is limited to areas that receive sufficient light (Cockell & Stokes 2004, Cary *et al.* 2010). Such habitats can support photosynthetic taxa such as cyanobacteria, algae and mosses, along with associated heterotrophic communities, often on substrates otherwise devoid of macroscopic vegetation.

Hypolithic and surrounding soil microbial communities are typically distinct, both in terms of community composition (Smith *et al.* 2000, Pointing *et al.* 2009, Khan *et al.* 2011, Makhalanyane *et al.* 2013a) and metabolic profile (Chan *et al.* 2013, Le *et al.* 2016, Wei *et al.* 2016). Polar hypolithic microbial communities form unique tiny 'islands' of diversity and primary productivity, containing orders of magnitude more bacteria than the surrounding soil (Smith *et al.* 2000). Hypolithic communities can contribute significant amounts of organic carbon to largely inorganic polar desert mineral soils (Cockell & Stokes 2004, Mergelov *et al.* 2020).

Hypolithic communities are common in a number of ice-free Antarctic oases, including the Dry Valleys (reviewed by Cary *et al.* 2010), Schirmacher Oasis

(Pankow *et al.* 1991), the Larsemann Hills (Mergelov *et al.* 2020) and the Vestfold Hills. Glacial till containing translucent quartz provides habitat for hypolithic communities across the Vestfold Hills, East Antarctica (Broady 1981, 1986).

The distribution of marine salts across the Vestfold Hills and its influence on soil salinity has led to the recognition of two broad habitat types divided by the 'salt line', with salt-enriched ground to the west and north nearest the coast and salt-poor ground to the east and south nearest the ice sheet (Adamson & Pickard 1986, Gore *et al.* 2004). The salt line is known to influence vegetation distribution across the Vestfold Hills, with mosses and lichens being less tolerant of saline conditions compared to hypoliths, which are found more commonly to the west of this line (Pickard 1986, T. Travers *et al.*, unpublished data 2024). In contrast to hypolithic communities, at a local level, surface mosses, lichens and algae have relatively restricted distributions in the Vestfold Hills (Pickard 1986, T. Travers *et al.*, unpublished data 2024), suggesting that hypolithic communities may have greater biomass than surface vegetation in the area (Broady 1981).

Broady (1981, 1986) noted the presence of several cyanobacterial and eukaryotic algal species in Vestfold Hills hypolithic communities based on traditional morphology and culturing, with algal species composition varying with gradients in soil salinity, nutrients and moisture. Smith *et al.* (2000) examined a small number of bacterial hypolithic communities from the narrow coastal region of the Vestfold Hills (an area of salt-enriched ground or high soil salinity) using Sanger DNA sequencing and culturing techniques, finding many heterotrophic psychrophilic bacteria with marine bacteria as their closest relatives. However, a more in-depth understanding of Vestfold Hills hypolithic communities and how they vary over environmental gradients would be gained by applying modern high-throughput DNA sequencing methods to hypolithic communities from across the area.

In this study, we characterize both the photosynthetic and heterotrophic components of bacterial and eukaryotic hypolithic communities throughout the Vestfold Hills using high-throughput DNA sequencing of parts of the 16S and 18S ribosomal RNA genes. Although polar hypolithic communities are known to support eukaryotes such as chlorophyte algae (e.g. Broady 1981), moss and fungi (e.g. Cowan *et al.* 2010), the eukaryotic component of Antarctic hypoliths has rarely been characterized with high-throughput DNA sequencing (although see Khan *et al.* 2011). We examine the community composition and the inferred metabolic profiles of bacterial communities over water availability and salinity gradients. Specifically, we explore:

- Hypolithic bacterial and eukaryotic diversity in terms of zero-radius operational taxonomic unit (zOTU) richness (alpha-diversity) and the effects of soil salinity and water availability on zOTU richness.
- Hypolithic bacterial and eukaryotic community composition (beta-diversity), including the effects of soil salinity and water availability on community composition and inferred bacterial metabolic pathways, and we examine in more detail zOTUs and inferred metabolic pathways that showed differential relative abundance between sites with high and low salinity or water availability.
- The contribution of dispersal and natural selection processes to spatial community turnover by testing for correlations between geographical distance and hypolithic community dissimilarity (Mantel test) and null modelling approaches (Stegen *et al.* 2013, 2015).

Materials and methods

Sample collection

Samples of hypolithic microbial communities were collected from 77 sites as part of a systematic vegetation survey of the Vestfold Hills, East Antarctica (Fig. 1a,b) between December 2019 and February 2020 (detailed sample methodology in T. Travers *et al.*, unpublished data 2024). Hypolithic communities of the Vestfold Hills are most common in areas with high wind exposure and flat or shallow north-east-facing slopes of salt-enriched ground, and they are found in areas far from snowmelt (T. Travers *et al.*, unpublished data 2024). Topographical factors including wind exposure, aspect and slope create the conditions that expose and orientate quartz cobbles of an appropriate size at the surface of glacial valley beds, allowing colonization by hypolithic communities. The ice-free area was divided into 10 strata based on elevation, slope, solar radiation (as a proxy for aspect) and terrain roughness derived from the Reference Elevation Model of Antarctica (Howat *et al.* 2019), with 200 random points generated within each stratum. These points were used as prospective site locations for this survey, with sampling occurring widely to maximize regional cover and locally to maximize habitat variability.

At each study site, quartz rocks were carefully lifted and turned over. If hypolithics were present (Fig. 1c,d), a small portion was scraped with the edge of a sterile tube, such that scrapings fell into the tube. Variation in colour of microbial communities across the hypolithic surface was observed. Scrapings attempted to include all colour variations. Additional material on the soil surface directly under the rock was also collected, if abundant. Each sample was taken from a single rock, such that more than one sample could be collected per site if multiple hypolithic communities were found. No equipment was reused between samples. At a small

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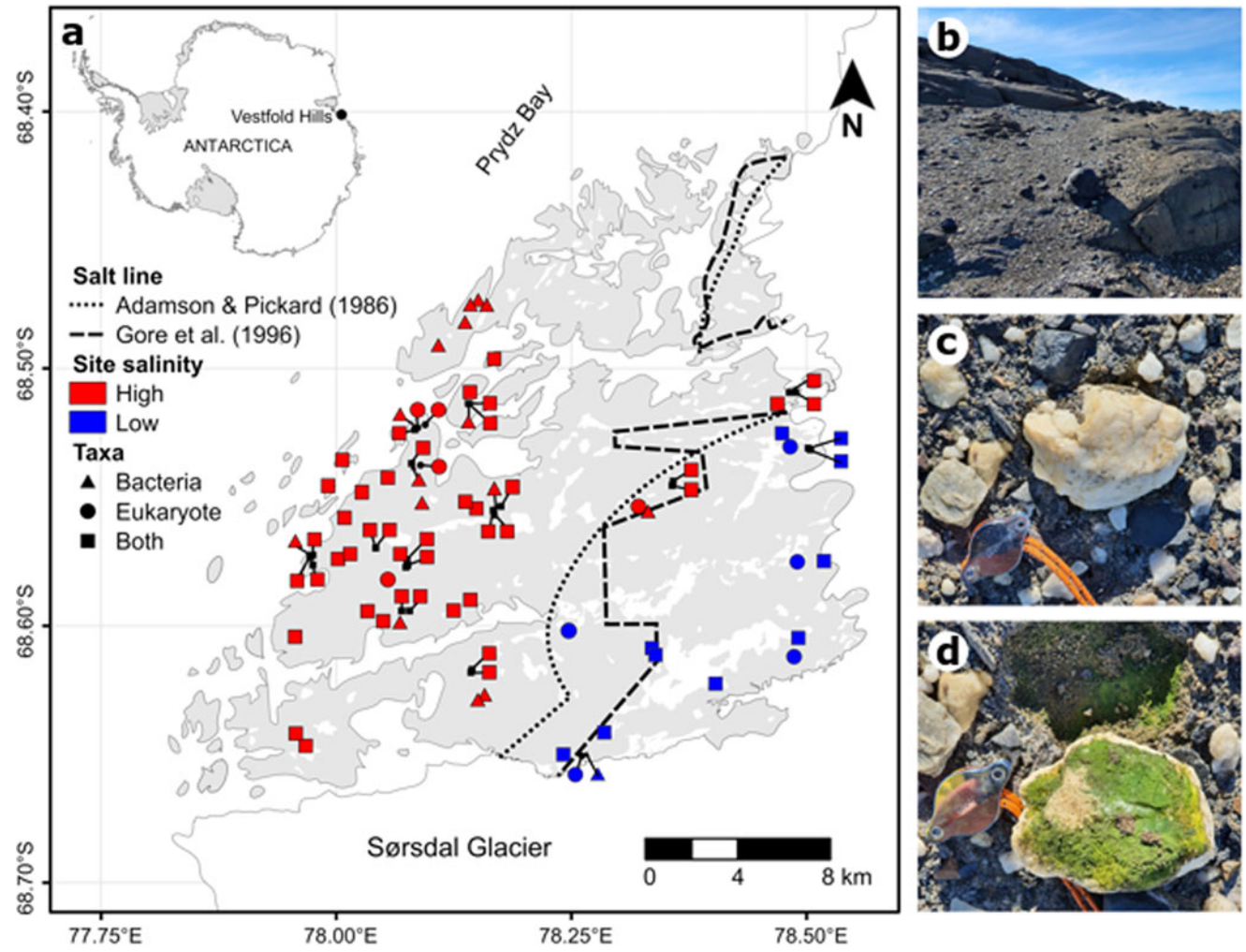

Figure 1. a. Map of hypolithic communities examined across the Vestfold Hills. The filled grey area is ice free. There are two types of water bodies: 1) marine areas including fjords and 2) lakes. Continental ice lies to the east and south. Sites are colour-coded by salinity (red = high, blue = low). There were 77 sites in total (51 with both bacteria and eukaryotic analyses, 15 with bacteria only, 9 with eukaryote only; 16 were low salinity, 61 were high salinity). The positions of two interpretations of the regional soil salinity transitional boundary termed the 'salt line' are shown. **b.** Example field site and **c.** hypolithic community *in situ* and **d.** showing community.

number of sites, entire rocks supporting hypolithic communities were transferred to sterile Ziplock bags; otherwise, rocks were carefully returned to their original position. Visible disturbance was kept to a minimum (e.g. footprints) and, if needed, restored before leaving the site. Samples were stored at -20°C in Antarctica and in transit and at -80°C at the Australian Antarctic Division, Tasmania, for ~3 months prior to analysis.

Edaphic factors

We refer to salt-enriched sites to the west and north of the 'salt line' as high salinity and sites with salt-poor ground to the east and south nearest the ice sheet as low salinity. Water availability at each site was categorized as dry, moist, wet-ephemeral or wet-permanent (sample metadata in Table S1). This schema attempted to identify site water availability over the summer despite each site being visited only once. Features such as the size and position of snowbanks and seeps relative to hypoliths informed site classification, with a smooth surface and lack of cobbles indicating the presence of local snowbanks earlier in the season. Relative rock size was estimated from site photographs. Attempts to measure soil conductivity and water content were complicated by dry soils and rocky sites, respectively, leading to sparse, incomplete datasets that were not used.

DNA extraction and sequencing

DNA was extracted from ~100 mg of hypolithic material and an extraction control (no material) using the

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QIAGEN DNeasy PowerSoil kit. We followed the manufacturer's instructions with the exception that samples were homogenized in the PowerBead tube using a FastPrep-24 (MP Biomedicals) at 4.5 m/s for 45 s. The extracted DNA was quantified using the Qubit 2.0 (dsDNA High Sensitivity kit), and sample DNA concentrations were normalized to 2 ng/µl.

The V4 and V5 regions of bacterial 16S ribosomal RNA (rRNA) genes were amplified with the primers 515F-Y and 926R (Quince *et al.* 2011, Parada *et al.* 2016). The V4 region of the eukaryote 18S rRNA gene was amplified using the primers V4 18S Next.Rev and V4 18S Next.For (Piredda *et al.* 2017). High-throughput sequencing libraries were prepared as per Clarke *et al.* (2017). Specifically, polymerase chain reaction (PCR) amplifications were performed in two rounds: the first to amplify the target gene and add sample-specific 6 bp multiplex-identifier (MID) tags (forward and reverse primer) and Illumina sequencing primers and the second to add sequencing adapters and additional 8 bp MID tags. Using two rounds of tagging reduces the chances of contamination or sequencing artefacts such as tag-jumping leading to false positives, as reads must include two pairs of unique MID tags to be assigned to a given sample.

The first round of PCR for the 16S primers was 98°C for 30 s, followed by 25 cycles of 98°C for 5 s, 65°C for 20 s and 72°C for 20 s, and then a final extension at 72°C for 5 min. The first round for the 18S primers was 98°C for 30 s, followed by 10 cycles of 98°C for 10 s, 44°C for 30 s and 72°C for 15 s, and then 20 cycles with an annealing temperature of 62°C and a final extension at 72°C for 7 min (Piredda *et al.* 2017). Each reaction mix contained either 0.2 µM (16S) or 0.5 µM (18S) each of forward and reverse primer, 2 µg bovine serum albumin, 1 × Phusion Master Mix (New England BioLabs, Ipswich, MA, USA) and 2 ng DNA extract in a total reaction volume of 10 µl. The PCR products were diluted 1:10, and Illumina sequencing adapters were added in a second round of PCR (10 cycles with an annealing temperature of 55°C) using the same conditions as the first round, except primer concentrations were reduced to 0.1 µM each.

Products from each round of PCR were separated by electrophoresis and visualized on 2% agarose gels. Equal volumes of second-round 16S and 18S PCR products were pooled separately to create two amplicon libraries and then purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA), and the size distribution and concentration of the two libraries were assessed on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The pools were diluted to 2 nM, and paired-end reads were generated on separate MiSeq runs (Illumina, San Diego, CA, USA) with the MiSeq Reagent Kit v3 (2 × 300 bp).

Data analysis

High-throughput DNA sequencing data for the 16S and 18S ribosomal RNA genes were processed following Suter *et al.* (2020). Sequences were assigned to sample-specific FASTQ files (available in the National Center for Biotechnology Information (NCBI) database with metadata under BioProject: PRJNA912313) using the 8 bp MID tags on the MiSeq. Paired reads were merged using *USEARCH* v11.0.667 (Edgar 2010). Only reads with exact matches to first-round 6 bp MID tags and the forward and reverse primer sequences (identified with the *R* package 'ShortRead'; Morgan *et al.* 2009, R Core Team, 2017) were kept, as non-exact matches are more likely to contain sequencing errors in the remainder of the sequence. Processed sequences were then pooled and dereplicated using the *USEARCH* command 'fastx_uniques'. The *USEARCH* command 'unoise3' was used to remove sequencing errors and chimaeras and to create a list of unique zOTU sequences with a minimum abundance of eight reads. Reads for each sample were then mapped to unique zOTUs to create separate zOTU tables for the 16S and 18S datasets using the *USEARCH* command 'otutab'. Taxonomy was assigned to bacterial and eukaryote zOTUs using the Ribosomal Database Project (RDP) Bayesian classifier (Wang *et al.* 2007) based on the *SILVA* v132 database (Yilmaz *et al.* 2014) and a 60% probability cut-off.

Hypolithic microbial diversity (*alpha-diversity*)

To compare alpha-diversity (zOTU richness) in hypolithic communities, we explored the number of bacterial and eukaryote zOTUs detected at each site using rarefaction curves generated with the 'iNEXT' *R* package (Hsieh *et al.* 2016). We then tested the influence of categorical edaphic attributes (soil salinity and water availability) on alpha-diversity by modelling counts of zOTUs (based on zOTU tables rarefied to 8000 or 1200 reads per sample for bacteria and eukaryotes, respectively) for each site using a negative binomial generalized linear model in the 'MASS' *R* package (Venables & Ripley 2002).

Spatial variation in community composition (*beta-diversity*)

We computed the Aitchison distance (Euclidean distance for centred log-ratio (clr)-transformed data; Aitchison 1986) using the 'phyloseq' (McMurdie & Holmes 2013) and 'microbiome' (Lahti & Shetty 2017) *R* packages to explore the ecological distance (beta-diversity) amongst communities as recommended for compositional high-throughput sequencing data (Gloor *et al.* 2017). We used the *adonis* function from the 'vegan' *R* package (Oksanen *et al.* 2020) to perform permutational analysis of variance

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(PERMANOVA; a non-parametric test that is robust to unbalanced sample sizes and is suitable for multivariate data), testing for effects of soil salinity and water availability on bacterial and eukaryotic community composition as well as inferred metabolic pathways (see below). We identified zOTUs and inferred metabolic pathways (see below) that showed differential relative abundance between sites with high compared with low salinity and between dry compared with permanently wet sites using a Welch *t*-test in *ALDEx2* (Fernandes *et al.* 2013). We report the zOTUs and pathways with effect sizes > |1.0|, being the difference in clr-transformed relative abundance between groups divided by the overall standard deviation.

Correlations between geographical distance and hypolithic community dissimilarity

We used a Mantel test to investigate correlations between geographical distance separating individual hypoliths and community dissimilarity (Aitchison distance) amongst bacterial and eukaryotic hypolithic communities, and we report the Pearson correlation coefficient to detect a positive or negative relationship.

Inferring bacterial metabolic pathways

In the absence of metagenomic data, we used *PICRUSt2* (version 2.4.1; Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; Langille *et al.* 2013, Douglas *et al.* 2020), which wraps several tools (*EPA-ng*, Barbera *et al.* 2019; *Gappa*, Czech *et al.* 2020; *castor*, Louca & Doebeli 2018; *SEPP*, Mirarab *et al.* 2011; *MinPath*, Ye & Doak 2009) to predict functional gene content and metabolic pathway abundances for hypolithic bacterial communities based on our 16S data. *PICRUSt2* infers the genomic content for a given 16S rRNA sequence based on averaging over bacterial genomes with the most similar 16S rRNA sequences available in the Integrated Microbial Genomes database (Markowitz *et al.* 2012). The Nearest Sequenced Taxon Index (NSTI) was calculated for each zOTU to estimate the similarity of zOTUs to available genome data; only zOTUs with NSTI < 2 were used for the analysis (98.7% of zOTUs, 99.8% of reads). The accuracy of predicted metagenomes for each sample was estimated based on the NSTI weighted by the abundance of each zOTU. Pathways were categorized into superclasses based on their classification in the curated MetaCyc database of experimentally deduced metabolic pathways from all domains of life (Caspi *et al.* 2018).

Contribution of selection and dispersal processes to community turnover

We explored the contribution of selection and dispersal processes to ecological turnover between hypolithic communities using a series of null models as per Stegen *et al.* (2013, 2015). The first null model estimates the contribution of selection (both homogeneous and variable) using phylogenetic turnover between communities. Where phylogenetic turnover was consistent with the first null model (see below), a second null model using the Raup-Crick metric incorporating Bray-Curtis dissimilarities (RC_{Bray}) compares observed and expected levels of community turnover without using phylogenetic information to infer the influence of homogenizing dispersal and dispersal limitation. The same approach was applied to both bacterial communities and microbial eukaryotic communities (excluding metazoans) so that the results could be compared with those for lake microbial communities in the Vestfold Hills (Logares *et al.* 2018).

To explore phylogenetic turnover between communities, bacterial and eukaryote phylogenetic trees were generated in *QIIME* 1.8 (Caporaso *et al.* 2010) by aligning 16S and 18S sequences using *MUSCLE* (Edgar 2004) or *PyNAST* (Caporaso *et al.* 2009), filtering the alignment (removing 0.0005% most variable positions and those that were > 80% gaps) and building the tree using *FastTree* 2.1.3 (Price *et al.* 2010). The mean phylogenetic distance between each zOTU in one community and its closest relative in a second community was calculated as the between-community mean nearest taxon distance (βMNTD). A null-model distribution of βMNTD was generated by randomly shuffling zOTUs across the tips of the phylogeny (999 permutations). β-nearest taxon indices (βNTIs) were calculated as the difference between the observed βMNTD and the mean of the null distribution, expressed in units of standard deviations; significant deviations (βNTI values < -2 or > +2) represent variable selection (βNTI > 2) or homogeneous selection (βNTI < -2).

Where phylogenetic turnover was consistent with the null model (|βNTI| < 2), we used the RC_{Bray} to compare observed and expected levels of community turnover without using phylogenetic information. Significant deviations from the null distribution represented homogenizing dispersal (RC_{Bray} < -0.95) or dispersal limitation (RC_{Bray} > +0.95), whereas comparisons consistent with the null distributions for both βNTI and RC_{Bray} suggested no single ecological process dominated compositional turnover (referred to as 'drift' in Stegen *et al.* 2013).

Results

We analysed bacterial community composition from 68 sites with hypoliths across the Vestfold Hills. Most hypolithic communities were collected from sites classified as dry with high salinity (35/68 bacterial communities, 28/60 eukaryotic communities). Of the

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77 total hypoliths (including those analysed for either bacterial or eukaryotic communities), only 16 were from sites classified as low salinity, and only 26 were from sites classified as wet (permanently wet: 8, ephemerally wet: 7) or of intermediate water availability (11). After filtering and quality control, the 16S dataset included 3.9 million paired-end sequencing reads (8038–112 837 reads per sample), representing 2890 zOTUs with eight or more reads (Table S2). Archaea were represented by only three zOTUs (all Thaumarchaeota, family Nitrososphaeraceae and known ammonia oxidizers) and 124 of 3.9 million reads (0.003% of reads), and they were excluded from further analysis. High-throughput sequencing of the 60 eukaryotic hypolithic communities yielded 970 000 paired-end reads (1236–39 941 reads per sample), representing 1016 zOTUs with eight or more reads (Table S3). Extraction controls yielded zero and one read for the bacterial or eukaryote primer sets, respectively, following filtering and quality control, and they were removed.

The number of bacterial zOTUs was marginally higher in hypolithic communities from low-salinity sites (low-salinity mean ± SD: 194 ± 122, high-salinity mean ± SD: 128 ± 71, negative binomial GLM: $P = 0.019$; Figs 2 & S1). Hypoliths from both ephemeral and permanently wet communities contained significantly more bacterial zOTUs than dry and intermediate communities, and the latter two contained similar numbers of bacterial zOTUs (Fig. 2). Furthermore, bacterial hypolithic communities from both permanently and ephemerally wet sites had more inferred metabolic pathways than communities from dry sites ($P < 0.05$), although the numbers of pathways were similar between ephemerally wet and intermediate sites ($P > 0.05$). The number of inferred bacterial metabolic pathways did not differ significantly between low- and high-salinity sites ($P = 0.60$).

Hypoliths from permanently wet sites supported more eukaryote zOTUs than those from dry sites (negative binomial GLM: $P < 0.05$; Fig. 2) but did not differ between other levels of water availability. The number of eukaryote zOTUs was not significantly different between hypoliths from low- and high-salinity sites (low-salinity mean ± SD: 30 ± 16, high-salinity mean ± SD: 34 ± 28).

Community composition

Community composition for both bacteria and eukaryotes was highly variable between hypoliths, even for sites with similar soil salinity and water availability profiles.

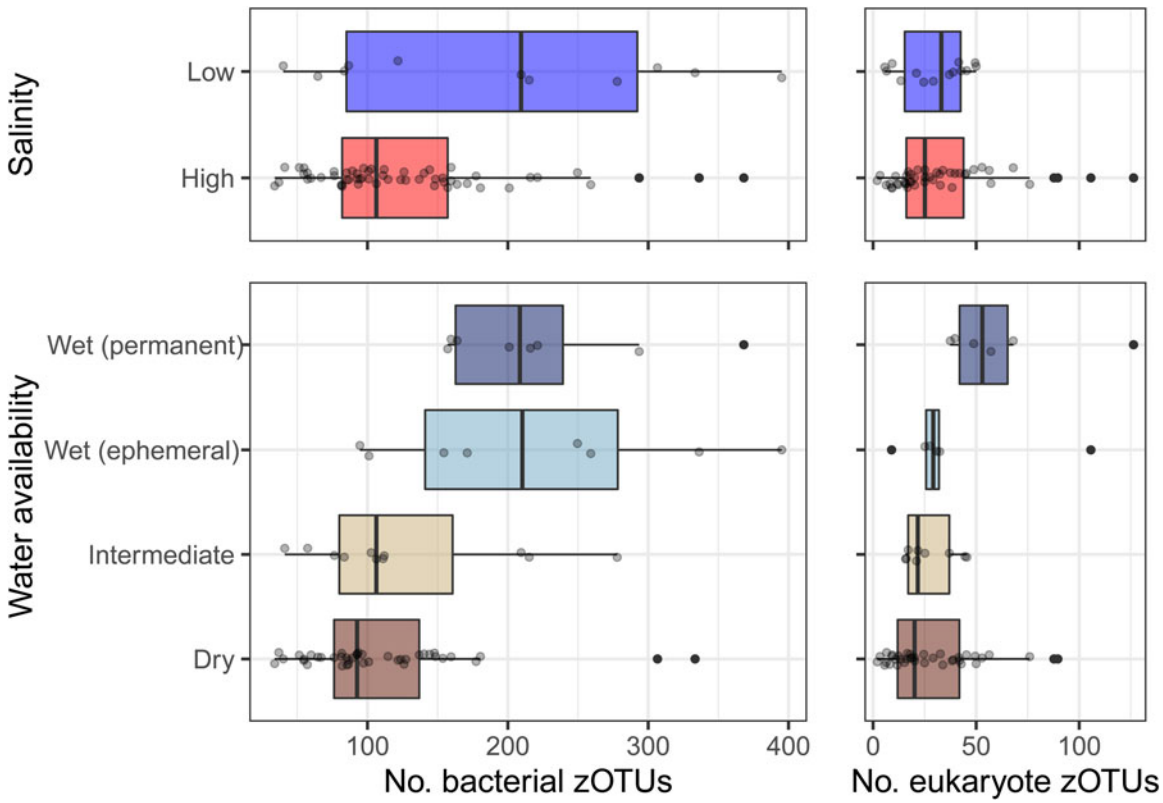

Figure 2. Number of bacterial and eukaryote zero-radius operational taxonomic units (zOTUs) in hypolithic communities from the Vestfold Hills from 68 (left plots) or 60 sites (right plots) based on soil salinity and water availability, respectively. Boxes enclose the 25th and 75th percentile values and outliers are black. The number of bacterial zOTUs was based on a rarefaction depth of 8000 reads; the number of eukaryote zOTUs was based on a rarefaction depth of 1200 reads.

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The phyla Cyanobacteria and Bacteroidetes comprised the majority of each bacterial community (mean ± SD: 91% ± 11%; Fig. 3a). However, the dominant taxa at the order level were distinct between high- and low-salinity sites; within the Cyanobacteria, many hypolithic communities within the high-salinity sites had high proportions of reads assigned to Phormidesmiales (25% ± 21%), whereas communities from low-salinity sites had high proportions of Nostocales (11% ± 15%) and Oxyphotobacteria (*incertae sedis*, 11% ± 17%). Within the Bacteroidetes, communities from both high- and low-salinity sites had relatively high proportions of Cytophagales (high: 25% ± 18%, low: 11% ± 13%), but the other dominant Bacteroidetes orders differed (high salinity: Flavobacteriales, 21% ± 21%, Chitinophagales, 7% ± 10%; low salinity: Flavobacteriales, 2.5% ± 6.0%, Chitinophagales, 32% ± 29%).

Eukaryotic communities showed similar variability within and between hypoliths from high- and low-salinity sites (Fig. 3b). At the class level, all communities had on average high proportions of Trebouxiophyceae (Chlorophyta, high salinity: 28% ± 37%, low salinity: 47% ± 43%). Many high-salinity sites also had high relative abundances of Ulvophyceae (20% ± 30%) that were near-absent from hypolithic communities at low-salinity sites (0.4% ± 1.0%). Diatoms comprised 30–94% of reads in three high-salinity samples but were not detected in samples from low-salinity sites. Unclassified eukaryotes represented > 60% of reads in six high-salinity communities. A phylogenetic tree of eukaryote zOTUs showed many of the abundant unclassified zOTUs were most closely related to ciliates (Fig. S2). Ciliates show substantive variation in the number of ribosomal DNA copies per cell (> 500 000 copies in some species; Gong *et al.* 2013, Wang *et al.* 2017), which could explain the high relative abundance of these unclassified zOTUs in some samples. Other eukaryote taxa with high relative abundance in at least some communities were cercozoans, chrysophytes (golden algae), ascomycota (fungi) as well as mosses (Embryophyta) and metazoan, including rotifers, tardigrades and unclassified arthropods that most probably represent mites (Acari).

Differential abundance of zOTUs and metabolic pathways between high- and low-salinity sites

Three bacterial zOTUs showed significant differential abundance (effect size - the difference in clr-transformed relative abundance between groups divided by the overall standard deviation > |1.0|) between hypoliths from high- and low-salinity sites; a *Phormidesmis* ANT.LACV5.1 zOTU (Cyanobacteria) and a zOTU assigned to the family MWH-CFBk5 (order: Cytophagales) were enriched in hypoliths from high-salinity sites (Fig. 4). A *Blastocatella* zOTU (Acidobacteria) was enriched in the communities from low-salinity sites. No eukaryote zOTUs showed differential abundance between high- and low-salinity sites with an effect size > |1.0|.

Seven inferred metabolic pathways were significantly enriched in hypoliths from high-salinity sites (effect size > |1.0|; Fig. 4), including synthesis of ppGpp (guanosine tetraphosphate and pentaphosphate), a secondary metabolite that regulates gene expression during nutrient or energy starvation or other environmental stress, phosphorus compound (methylphosphonate) degradation, NAD (nicotinamide adenine dinucleotide) biosynthesis from tryptophan and a sub-pathway of tryptophan degradation.

Differential abundance between dry and permanently wet habitats

Just three bacterial zOTUs showed differential abundance between hypoliths from dry and permanently wet sites (effect size > |1.0|), and all of them were enriched in wet sites: these zOTUs were from the genera *Cryomorpha*, *Crocinitomix* (both Flavobacteriales) and the cyanobacterial *Phormidesmis* ANT.LACV5.1 (Fig. 5).

Although only three bacterial zOTUs showed differential abundance between hypolithic communities from dry and permanently wet sites, predicted community metabolic profiles suggested 41 pathways were enriched in communities from dry sites (effect size > |1.0|). Many of these pathways were involved in biosynthesis, including biosynthesis of nucleotides (11), amino acids (4), cofactors (4), cell structure or cell walls (4), aromatic compounds (2), secondary metabolites (2) and vitamins (2). Six energy metabolism pathways were also enriched in dry habitats, including four related to glycolysis. Two aromatic compound degradation pathways (aerobic toluene degradation) were enriched in permanently wet hypolithic communities.

Just two eukaryotic zOTUs, both assigned to Chlorophyta, were differentially abundant between hypoliths from dry and permanently wet sites (effect size > |1.0|), all of them enriched in wet sites; one *Chlorococcum* sp. (order Chlamydomonadales) and one unclassified Ulvophyceae zOTU.

Due to the high variability in hypolithic community composition, PERMANOVA showed that water availability and salinity (high *vs* low) explained a small but significant proportion of variation for bacterial (water availability: 8.7%, salinity: 6.7%, both $P < 0.001$, interaction: 4.6%, $P = 0.022$; Fig. 6b) and eukaryotic communities (water availability: 8.0%, $P = 0.003$, salinity: 5.0%, $P < 0.001$, interaction: 1.8%, $P = 0.3$; Fig. 6a & Table S4). Inferred bacterial metabolic profiles also showed a small but significant difference between the habitat types (water availability: 10.6%, salinity: 5.3%, both $P < 0.001$; Fig. 6c).

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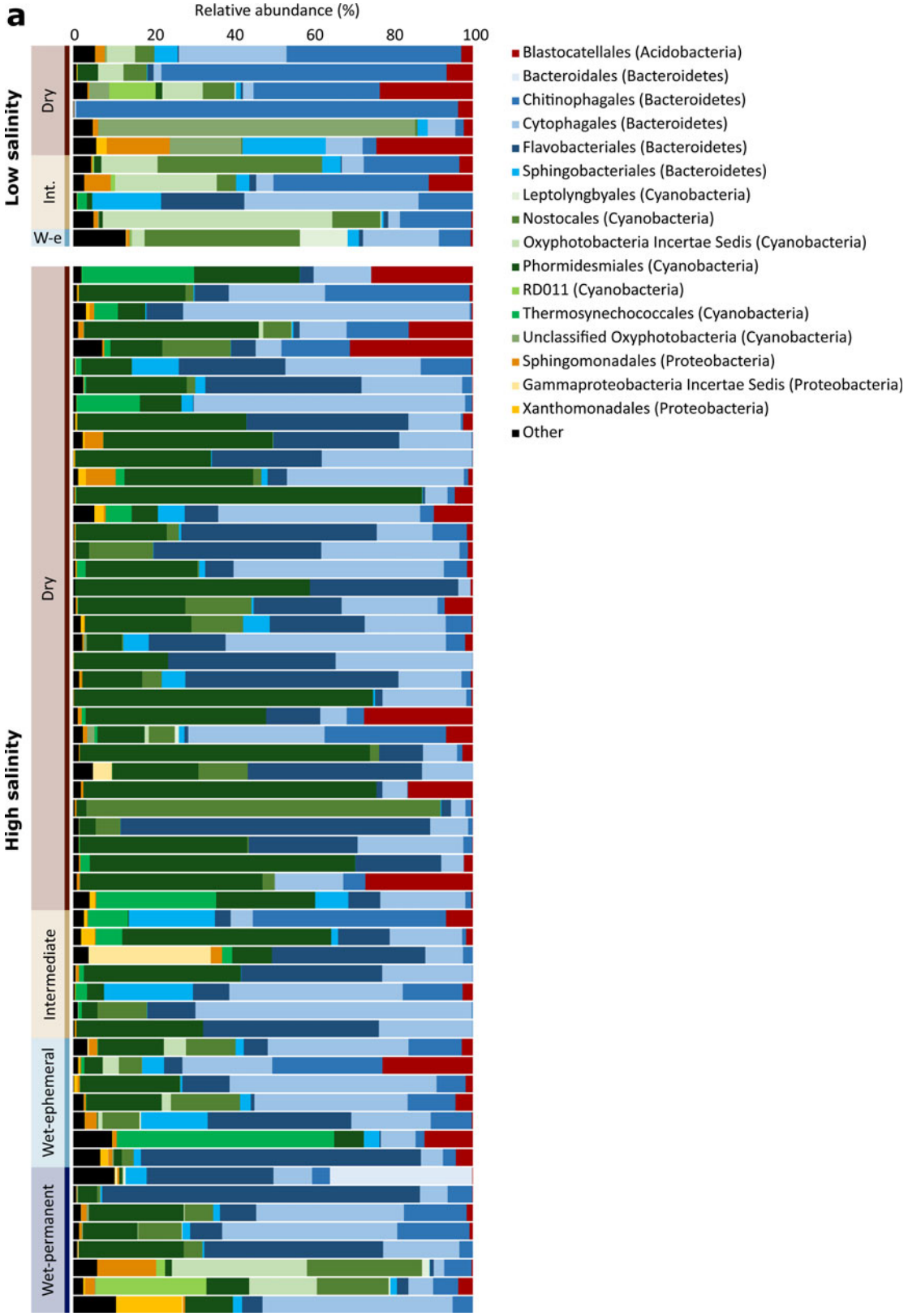

Figure 3. a. Relative abundance of reads from dominant bacterial orders in hypolithic samples from the Vestfold Hills. Phyla are in brackets and colour-coded (e.g. Cyanobacteria are all green). **b.** Relative abundance of reads from dominant eukaryote classes in hypolithic samples from the Vestfold Hills. Phyla are in brackets and colour-coded (e.g. Chlorophyta are all green). Int. = intermediate; NA = not applicable; W-e = wet-ephemeral.

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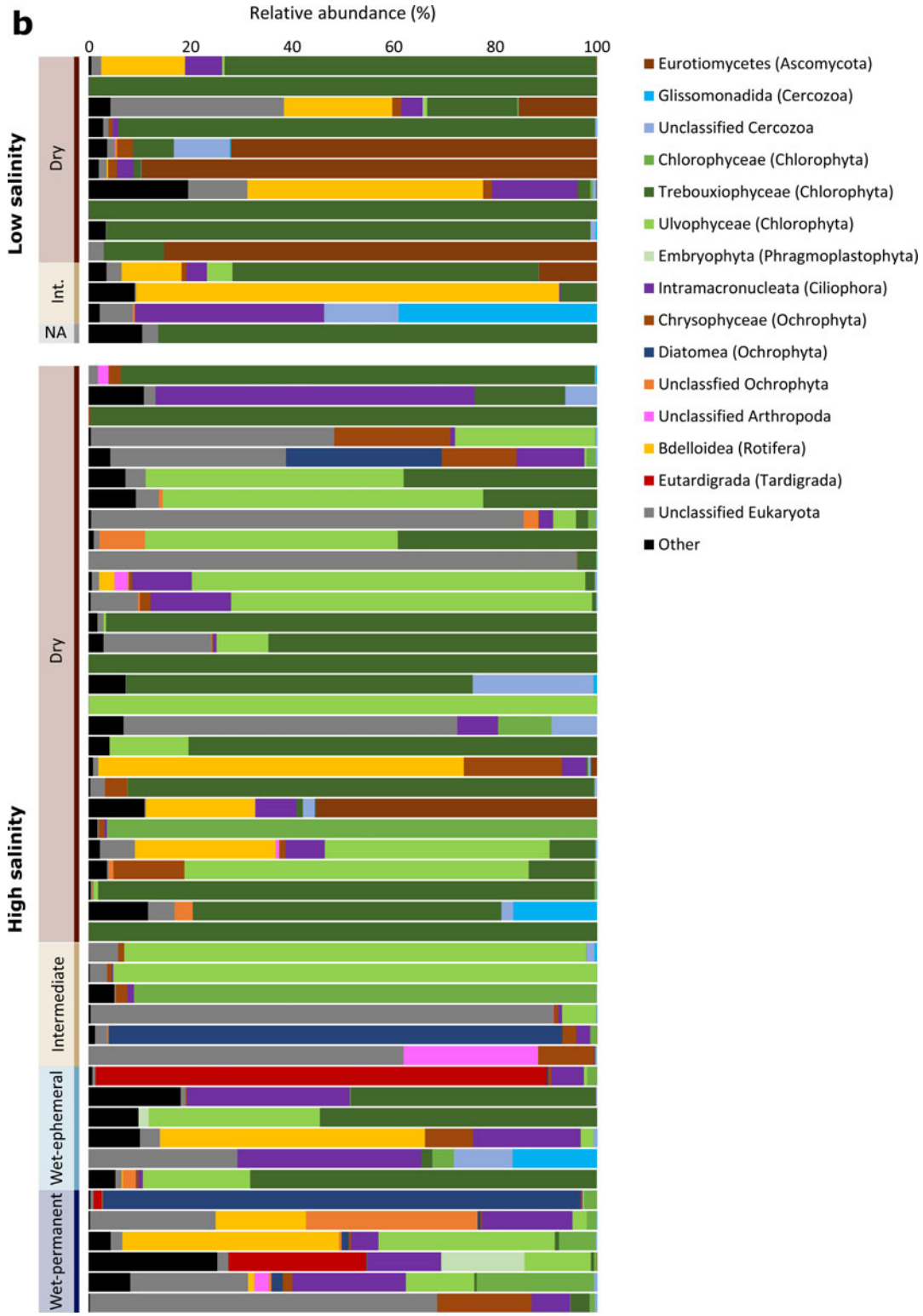

Figure 3. (Continued)

Spatial turnover

Mantel tests revealed a significant correlation between geographical distance and bacterial community composition (Aitchison distance, Pearson's $r = 0.33$, $P = 0.0001$), suggesting community similarity decreased with increasing geographical distance (Fig. S3). No

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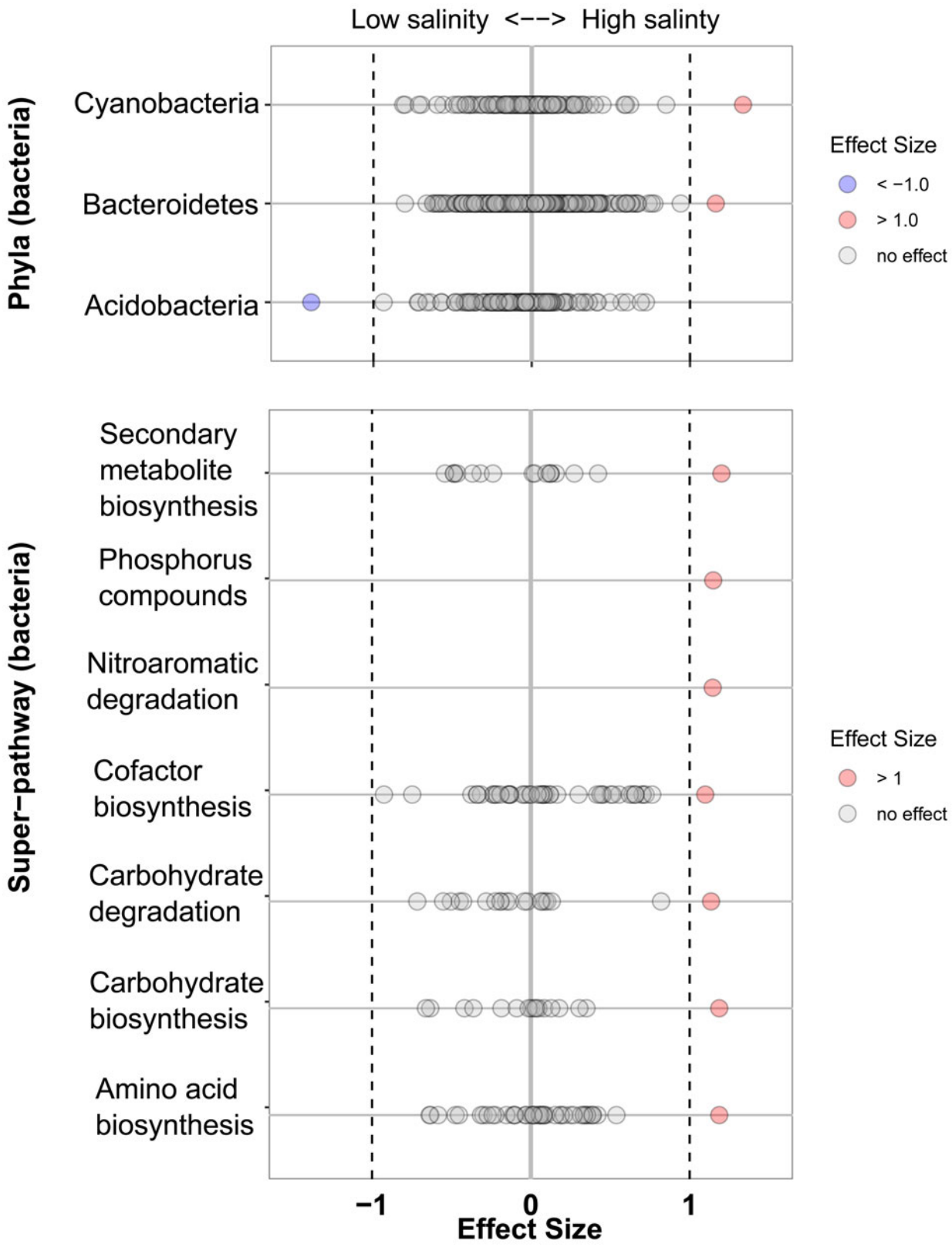

Figure 4. Differential abundance of bacterial zero-radius operational taxonomic units (zOTUs) and inferred bacterial metabolic pathways between low- and high-salinity hypolithic sites. No eukaryote zOTUs showed differential abundance between high- and low-salinity sites with a significant effect size > |1.0|.

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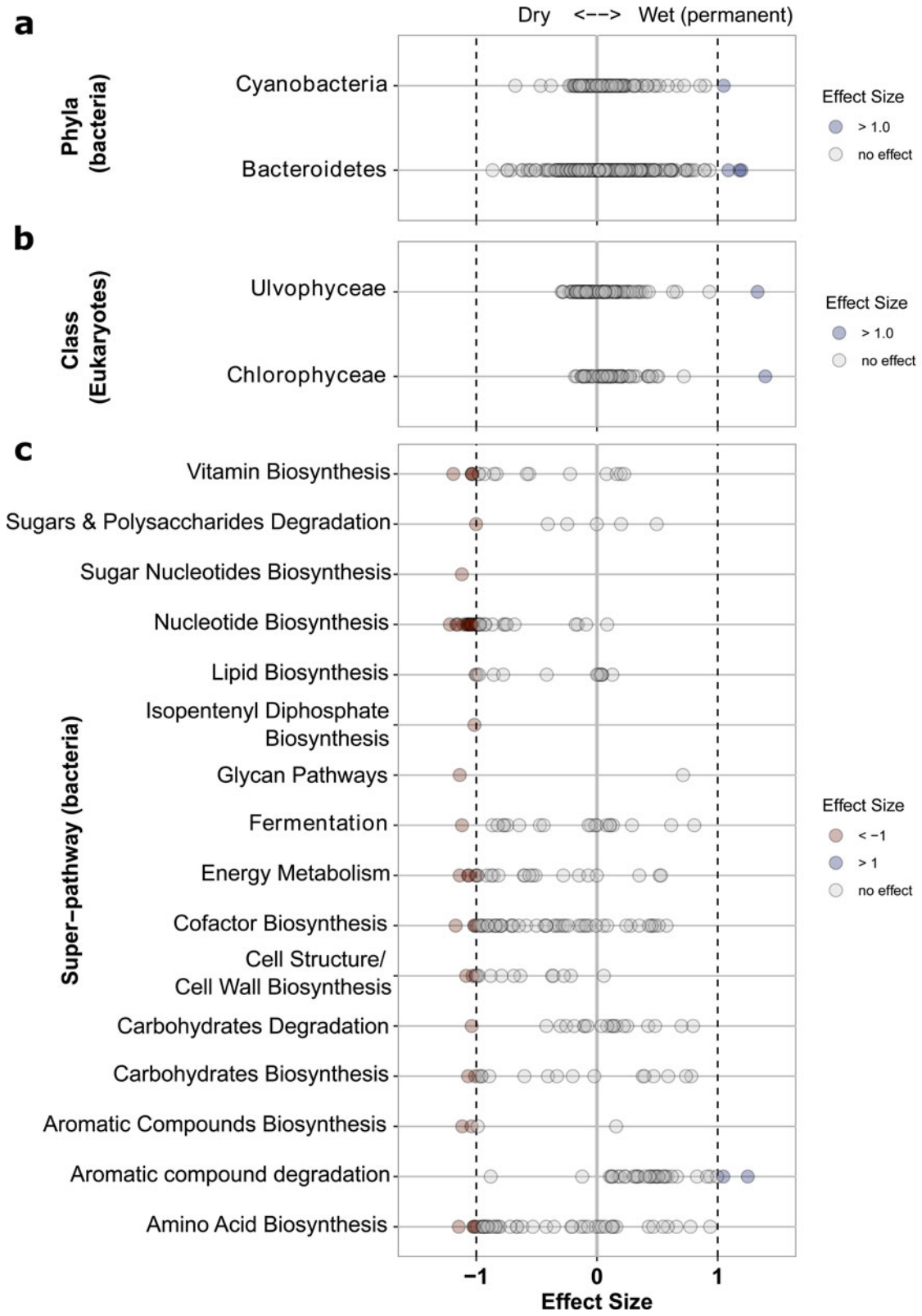

Figure 5. Differential abundance of **a.** bacterial and **b.** eukaryotic zero-radius operational taxonomic units (zOTUs) and **c.** inferred bacterial metabolic pathways between dry and permanently wet hypolithic habitats.

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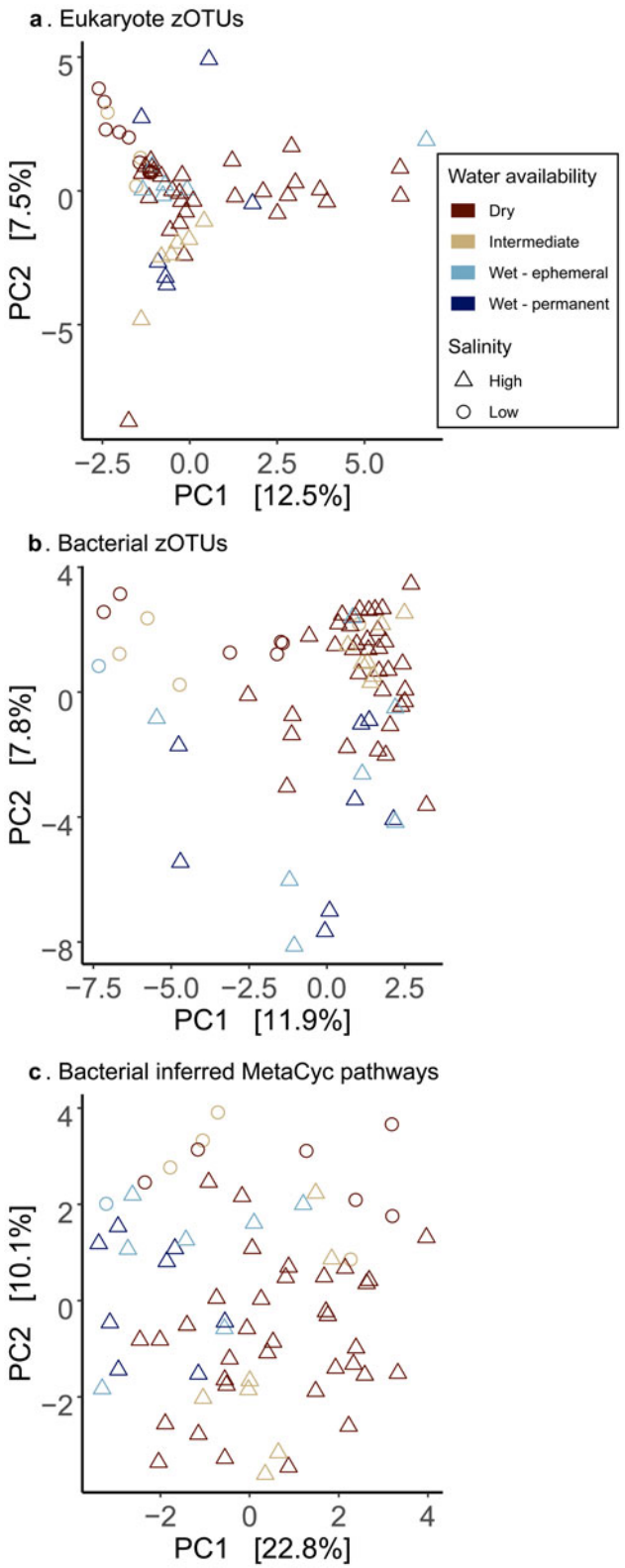

Figure 6. Unconstrained principal component (PC) ordination of the Vestfold Hills hypolithic **a.** eukaryotic communities, **b.** bacterial communities and **c.** bacterial metabolic profiles based on Aitchison distances with centre-log-transformed data. zOTU = zero-radius operational taxonomic unit.

significant distance-decay relationship was observed for eukaryotic communities (Fig. S3), either overall or amongst high- or low-salinity sites ($P > 0.05$).

Contribution of selection and dispersal processes to hypolithic community turnover

We used null modelling approaches to estimate the contribution of selection *vs* stochastic processes to hypolithic community turnover. We found dispersal limitation drives bacterial community differentiation, but selection processes differed for pairwise comparisons between sites within the same salinity type (high or low) compared to between high- and low-salinity sites. For bacterial communities, neither dispersal nor selection was the primary cause of community turnover for 45% of pairwise comparisons (termed 'undominated'; Fig. 7), with dispersal limitation being the next most important process (41%). Eukaryotic hypolithic communities showed similar patterns to bacteria but with more turnover attributed to 'undominated' (85%; Fig. 7), as observed in Vestfold Hills lake microbial communities (Logares *et al.* 2018), followed by dispersal limitation (9.9%). The small number of pairwise comparisons identified as homogenizing dispersal (bacteria: 8, eukaryotes: 50) did not necessarily involve sites that were close to each other or sites with higher water availability. Most comparisons identified as homogenizing dispersal were between sites classified as 'dry' from different locations (bacteria: 5/8, eukaryotes: 41/50).

The proportion of comparisons attributed to selection was sensitive to the alignment method used to generate the bacterial phylogenetic tree (decrease from 19.8%

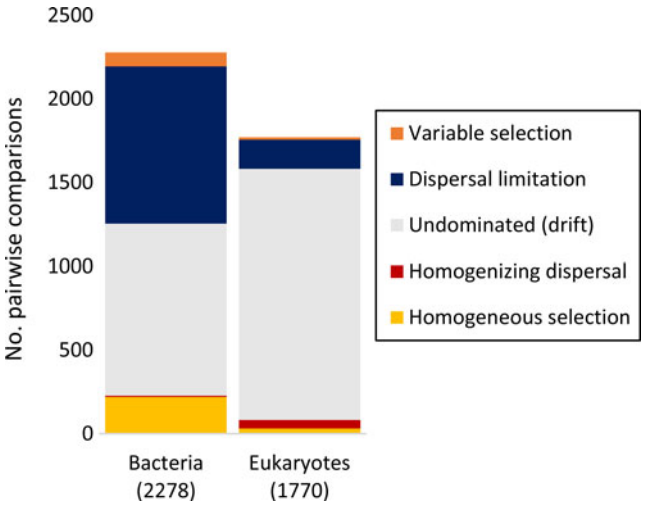

Figure 7. Contribution of ecological processes to hypolithic bacterial or microbial eukaryotic community turnover overall. The number of pairwise comparisons for each category is shown in parentheses.

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using *PyNAST* to 14.5% using *MUSCLE*), probably due to the introduction of many more gaps in the *MUSCLE* alignment. However, general trends were consistent between methods (majority of turnover attributed to 'undominated', followed by dispersal limitation).

Discussion

Our study is the first characterization of Vestfold Hills hypolithic bacterial and eukaryotic communities using high-throughput DNA sequencing. Although polar hypolithic communities have long been known to support eukaryotes such as chlorophyte algae (e.g. Broady 1981), moss and fungi (e.g. Cowan *et al.* 2010), the eukaryotic component of Antarctic hypoliths has rarely been characterized with modern genetic methods (although see Khan *et al.* 2011). High-throughput DNA sequencing revealed that Vestfold Hills hypoliths support a diverse suite of micro-eukaryotes, including ciliates, cercozoans and chrysophytes, as well as Chlorophyta and fungal taxa. Hypolithic communities from high-salinity sites supported several diatom genera, including *Navicula* (noted by Smith *et al.* 2000), *Pinnularia* and *Nitzschia*, which were not detected in hypoliths from low-salinity sites (Table S3). *Pinnularia* diatoms occur globally in freshwater (low-salinity) habitats but are also known from marine coastal environments (Souffreau *et al.* 2011). We also detected metazoans, including rotifers, tardigrades and unclassified arthropods (most probably mites), similar to the nematodes and mites observed by Smith *et al.* (2000). We did not quantify the proportion of eukaryote *vs* bacterial DNA (e.g. Pointing *et al.* 2009), thus it is not clear yet whether the eukaryotes represent a minor or major component of Vestfold Hills hypolithic communities. Regardless, Vestfold Hills hypolithic communities appear to be refuges of both bacterial and eukaryotic diversity compared to the limited microbial biomass in soils from the region (Smith *et al.* 2000). Broady (1986) reported distinct zonation between green alga and cyanobacteria under rocks, with light green banding being dominated by alga. We also observed this distinct banding under many rocks. Although detected archaeal diversity was at least an order of magnitude lower than bacterial diversity in Vestfold Hills soils (Zhang *et al.* 2020), and Archaea represented only 0.43% of metagenomic sequences from Dry Valley hypoliths (Le *et al.* 2016), further investigation is required to verify whether the extremely low hypolithic archaeal diversity in this study is an artefact of primer bias or poor representation in sequence databases (Bowman 2018, Lambrechts *et al.* 2019).

Hypolithic bacterial communities from the Vestfold Hills have a similar composition at the phylum level to that found in previous genetic studies of Antarctic hypolithic communities, with Cyanobacteria and Bacteroidetes being the common dominant phyla detected in hypoliths from both the Vestfold Hills and Dry Valleys (Smith *et al.* 2000, Pointing *et al.* 2009, Makhalanyane *et al.* 2013a, Le *et al.* 2016). However, we found that Acidobacteria (in particular the order Blastocatellales) was present at high relative abundance (mean ± SD: 5% ± 8%, maximum 31%), whereas previous studies found that Actinobacteria were more common than Acidobacteria (Smith *et al.* 2000, Khan *et al.* 2011, Makhalanyane *et al.* 2013a, Le *et al.* 2016). Differences in relative abundance could reflect biases in PCR primers or methods (e.g. 16S rRNA sequencing *vs* metagenomics) or different colonization sources (see below).

Studies have concluded that polar hypolithic microbial community composition is driven by homogeneous selection and stochastic processes such as dispersal limitation, with hypolithic communities creating productive but isolated enclaves in polar desert landscapes (Pointing *et al.* 2009, Lebre *et al.* 2020). We demonstrate that variable selection also influences hypolithic bacterial communities in the Vestfold Hills, as communities are shaped by historical and ongoing inputs of marine salt. Selection has also led to distinct community metabolic profiles in areas with distinct soil salinity or water availability profiles. The greater influence of selection for hypolithic bacterial community assembly compared to microbial eukaryotes (Fig. 7) was also observed in Vestfold Hills lakes (Logares *et al.* 2018). A higher relative contribution of selection *vs* dispersal for bacteria compared to microbial eukaryotes has been observed at local (Vass *et al.* 2020), continental (Bock *et al.* 2020) and global scales (Logares *et al.* 2020) and could indicate tighter biogeochemical coupling for bacterial communities. The sensitivity of the estimated contribution of selection to community turnover to the alignment method used to generate the bacterial phylogenetic tree (*PyNAST vs MUSCLE*) suggests researchers should be careful when comparing the contribution of selection and dispersal processes between studies.

Hypolithic communities are heterogeneous

Hypolithic microbial communities from the Vestfold Hills were heterogeneous both within and between habitat types (low *vs* high salinity, differences in water availability). Similar compositional heterogeneity between hypolithic bacterial communities was observed at very small spatial scales in the McMurdo Dry Valleys (Wood *et al.* 2008, Lebre *et al.* 2020). The absence of liquid water to transport microbes between hypoliths limits dispersal of both bacterial and eukaryotic communities (Fig. 7; Lebre *et al.* 2020). Although ~20% of sites were classified as either ephemerally or permanently wet

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(16/68 bacteria, 12/60 eukaryotes), these were typically spatially separated and would not be connected by surface or ground water. However, two samples in close proximity from a permanently wet site on Partizan Island were one of the small number of pairs in which homogenizing dispersal drove bacterial community turnover. We found no significant relationship between ecological and geographical distance for hypolithic eukaryotic communities, which is consistent with limited connectivity between communities. Our results suggest that the distance-decay relationship for hypolithic bacterial communities in the Vestfold Hills may be partially driven by spatial clustering of samples from high- and low-salinity habitats (Fig. 1). Alternatively, the high wind exposure typical of Vestfold Hills sites with hypolithic communities (T. Travers *et al.*, unpublished data 2024) may facilitate stochastic dispersal of bacteria between nearby hypoliths and from potential source environments (see below). We also cannot discount past connectedness or isolation, especially considering the complex history of glaciation, sea-level rise and isostatic uplift in the Vestfold Hills. Hypolith communities can be stable in Antarctica over the 1000 year timescale (Mergelov *et al.* 2020).

Implications for community metabolic function

Although there is evidence that metabolic profiles can be inferred from 16S rDNA profiles for well-characterized communities (e.g. Ward *et al.* 2017, Raes *et al.* 2021), inferred bacterial pathways should be interpreted with caution. We found little difference in the inferred metabolic profiles of hypolithic bacterial communities from low- and high-salinity sites (Fig. 4c). However, several pathways enriched in the high-salinity communities can be explained based on the contrasting environmental features of the low- and high-salinity habitats. The enrichment of a phosphorus degradation pathway may be due to higher concentrations of phosphorus towards the coast (west of the salt line) in the Vestfold Hills, as saline and hypersaline lakes in the Vestfold Hills tend to have higher phosphorus concentrations than fresh lakes (Logares *et al.* 2013). Biosynthesis of the metabolic regulator ppGpp may be enriched in high-salinity communities due to osmotic stress (Srivatsan & Wang 2008). The enrichment of two pathways involving tryptophan present in *Cytophaga hutchinsonii* (NAD *de novo* biosynthesis II from tryptophan and l-tryptophan degradation to 2-amino-3-carboxymuconate semialdehyde; Caspi *et al.* 2018) may reflect higher relative abundances of MWH-CFBk5 (order Cytophagales) in hypolithic communities from high-salinity sites (Fig. 4a). We found inferred pathways tended to be enriched under what we would consider the harsher conditions, namely dry and/or high-salinity sites. Most inferred pathways (38/41) with differential abundance based on water availability were enriched in hypolithic communities from dry sites. Similarly, the seven inferred pathways with differential abundance based on salinity were all enriched in hypolithic communities from high-salinity sites. Future studies of Vestfold Hills hypolithic communities could apply metagenomic approaches to determine whether stress-response genes are differentially expressed amongst hypolithic communities in the Vestfold Hills (Chan *et al.* 2013, Le *et al.* 2016, Albanese *et al.* 2021).

Freshwater and marine environments as potential sources of hypolithic microbes

There is some debate over whether hypolithic communities are colonized from microbes in the surrounding soil (e.g. Makhalanyane *et al.* 2013b) or nearby aquatic environments, such as sea ice, lakes and associated microbial mats (e.g. Wood *et al.* 2008, Pointing *et al.* 2009). Although our study did not attempt to identify the source of hypolithic microbial communities, we can make comparisons based on previous studies. The distinction between soil and hypolithic communities (and between high- and low-salinity sites) suggests a role for freshwater and marine environments as source populations for hypolithic communities in the Vestfold Hills, with habitats under rocks creating refugia that can support aquatic taxa, whereas open soil cannot. For example, Actinobacteria and Proteobacteria were present in high relative abundance in Vestfold Hills soils (~30% and 15%, respectively; Zhang *et al.* 2020) but represented much lower proportions of the hypolithic bacterial community ($0.2\% \pm 0.4\%$ and $3\% \pm 6\%$, respectively; this study). Similarly, Smith *et al.* (2000) were able to culture Cytophagales (Bacteroidetes), a common component of hypolithic communities in both high- and low-salinity habitats (Fig. 2a), from Vestfold Hills hypoliths but not the underlying soil. The most frequently detected cyanobacterial zOTU in high-salinity samples was *Phormidesmis* ANT.LACV5.1. This taxon was originally described based on material from the saline Ace Lake in the Vestfold Hills (Taton *et al.* 2006), supporting a link between hypolithic and aquatic environments. Some of the Cytophagales taxa, such as *Gelidibacter* sp., identified by both Smith *et al.* (2000) and in this study, were most similar to bacteria from Antarctic sea-ice algal assemblages (Bowman *et al.* 1997). We also found diatoms that often dominate the surface sediments of Vestfold Hills lakes (predominantly *Pinnularia*, *Nitzschia* and *Navicula* species; Roberts & McMinn 1996) in eukaryotic hypolithic communities from high-salinity sites.

Wood *et al.* (2008) suggested that the absence of lakes and ponds in one of the Dry Valleys may be why there is

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a low abundance of cyanobacteria in the soils there. By contrast, the vast number and diversity of lake types in the Vestfold Hills may contribute to the observed variation in hypolithic microbial community composition, with wind-driven dispersal of material from benthic 'lift-off' microbial mats (e.g. Wharton *et al.* 1983) being a plausible mechanism for hypolith colonization. Direct comparison of microbial communities from soils, sea ice, lakes, aquatic microbial mats and hypolithic environments could confirm the importance of aquatic environments as sources of Antarctic hypolithic microbial communities (Wood *et al.* 2008, Pointing *et al.* 2009).

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S0954102023000408.

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Author contributions

PV and DMB conceived the study and completed the fieldwork. LJC performed the laboratory work and led the data analyses and manuscript preparation. EJR and TT contributed to the data analyses. All authors contributed to interpreting the findings and editing the manuscript prior to submission.

Competing interests

The authors declare none.

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