*Title:* Arctic marine fungi: Biomass, functional genes, and putative ecological roles

*Running title:* Arctic marine fungi

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**Abstract.**  Recent molecular evidence suggests a global-distribution of marine fungi; however, the

ecological relevance and corresponding biological contributions of fungi to marine ecosystems

remains largely unknown. We assessed fungal biomass from the open Arctic Ocean by applying novel

biomass conversion factors from cultured-isolates to environmental sterol and CARD-FISH data.  We

found an average of 16.54 nmol m$^{-3}$ of ergosterol in sea ice and seawater, which corresponds to 1.74

mg C m$^{-3}$ (444.56 mg C m$^{-2}$ in seawater).  Using Chytridiomycota-specific probes, we observed free-

living and particulate-attached cells that averaged 34.07 µg C m$^{-3}$ in sea ice and seawater (11.66 mg C

m$^{-2}$ in seawater).  Summed CARD-FISH and ergosterol values approximate 1.77 mg C m$^{-3}$ in sea ice

and seawater (456.23 mg C m$^{-2}$ in seawater), which is similar to biomass estimates of other marine

taxa generally considered integral to marine food webs and ecosystem processes. Using the GeoChip

microarray, we detected evidence for fungal viruses within the Partitiviridae in sediment, as well as

fungal genes involved in the degradation of biomass and the assimilation of nitrate.  To bridge our

observations of fungi on particulate and the detection of degradative genes, we germinated fungal

conidia in zooplankton fecal pellets and grew fungal conidia after eight months incubation in sterile

seawater. Ultimately, these data suggest that fungi could be as important in oceanic ecosystems as they are in freshwater environments.

**Keywords:** Carbon, CARD-FISH, Chytridiomycota, Dikarya, Ergosterol, Nitrogen

**Significance Statement:** The oceans cover 71% of the world's surface; however, the contributions of marine fungi to these environments remain a gap to understanding marine ecosystem processes and associated biogeochemical cycling.  Here we provide the first biomass estimates of fungi from the Arctic Ocean, supplemented with a functional gene inventory, and experiment-based ecological data demonstrating putative niches occupied by fungi in the marine environment.  We provide evidence that marine fungi comprise a comparable quantity of biomass relative to other taxonomic groups that are generally considered to be essential ecosystem components.

**Introduction**

Fungi are ubiquitous members of global ecosystems that are responsible for biogeochemical cycling, forming symbiotic relationships, and producing secondary metabolites (1).  Fungi are among the most diverse (2) and under-studied organismal groups (<10% of the estimated species are described) on earth (3) and exhibit a wide-range of physiological plasticity enabling many taxa to survive in both freshwater and marine habitats.  Approximately 60% of recorded marine fungi are described as obligate marine species (4); however, the frequency of habitat exchange and the ecological relevance of euryhaline taxa in opposing environments remains unknown.  Regardless, the inconspicuous morphology of fungal propagules has challenged the easy identification and subsequent integration of fungal data into marine ecology.  Consequently, marine fungi receive considerably less research attention, relative to other organismal groups. In ice-covered environments, there is mounting evidence that fungi are not only active community members, but are among the most predominantly

detected eukaryotic taxa (5, 6, 7) that are seasonally dynamic (8) and abundant (9), especially in the Arctic (10).

Though little is known about the ecological contributions of marine fungi, studies of terrestrial and freshwater systems suggest that there are many potential avenues through which Arctic marine fungi could facilitate carbon flow. The Arctic marine environment is characterized by a patchwork of distinct, seasonally ice-covered seas (11) that receives 10% of the global river flux (12). As the Arctic continues to warm, thawing permafrost comprised of refractory lignins and other aromatic carbon molecules are exported into the Arctic Ocean (13). The biological fate of these substances, namely in regards to the eukaryotic microbial community at the base of the food web, remains largely speculative. However, fungi have historically been known to occupy this ecological niche in terrestrial environments by encoding lignin-degrading gene products (14). In aquatic ecosystems, animal excrement in the form of fecal pellets, can rapidly transport organic matter from the pelagic realm to benthic organisms (15). Degradation of fecal pellets by bacteria and dinoflagellates (16) are known to alter the vertical flux of this organic material. Fungi dominate biomass on bathypelagic marine snow (8) and are known to degrade and recycle animal excrement (17) in the terrestrial realm. However, the relevance of marine fungi to the degradation of fecal pellets, general degradative processes, and the effects of this metabolism on higher trophic levels remains poorly characterized. Screening of rRNA molecular barcodes and clone data has detected fungal communities comprised of an uncharacterized phylogenetic branch of Chytridiomycota (18) that appear to be important prey for Arctic zooplankton (19). These Arctic Chytridiomycota communities are primarily found with members of the Dikarya in sea ice, sediment (8, 20) and in association with driftwood (21). Analysis of rRNA barcode data can inform richness estimates for assessing community complexity; however, these data cannot detail enzymatic potential, definitively ascribe an ecological role, or be converted into carbon (C) units.

In polar environments, maintaining the fluidity and integrity of the plasma membrane is essential to survival. In the plasma membrane, sterols provide structural support and serve as precursors for hormones. Primary eukaryotic sterols generally associate with the major clades of eukaryotic life: cholesterol in vertebrates, phytosterols in plants, and ergosterol in fungi (22). Ergosterol ($C_{28}\Delta^{5,7,22}$) is the predominant membrane sterol in most of the Dikarya fungi (Ascomycota and Basidiomycota) (23) and has been used to estimate fungal biomass in a variety of ecosystems (24, 25, 26). The use of ergosterol as a fungal biomarker is not suitable for the Chytridiomycota, as they do not synthesize ergosterol (27). Understanding the ecology and biomass contributions of the fungi is important for integrating fungal contributions into food web analyses and informing ecosystem modeling efforts (28).

In this study, our objective was to take a holistic approach to examining the contributions of fungi to Arctic marine food webs. We first estimate fungal biomass in the open ocean and sea ice by generating and applying novel biomass conversion factors to integrated environmental sterol concentrations and Chytridiomycota-specific enzyme-labeled oligonucleotide counts. To supplement these biomass estimates, we explored the presence of known catalytic fungal genes in coastal surface sediment that can serve as a proxy for ecosystem functionality using the GeoChip microarray. To build on the detection of these functional genes, many with known degradative potential, we explored the ability of a marine fungus to germinate in zooplankton fecal pellets. Lastly because approximately 40% of marine fungi are known to exist in both terrestrial and marine realms, we wanted to test if one of these fungi could survive an Arctic seasonal cycle, under in situ temperature conditions without any added nutrients.

**Methods**

**Environmental sampling.** Environmental samples were collected from seawater and sea ice across the Arctic (summer) and from Tromsø air (October) in 2017 (Figure 1, STable 1). Most of the samples were collected during the expedition PS106 in June/July onboard the German icebreaker R/V

Polarstern. Additional samples were harvested close to Barrow, Alaska (station MBS) in 2014 (STable 1). Seawater was collected using a CTD/Rosette sampler in 10 l Niskin bottles from the surface, chlorophyll maximum, and bottom depths at every station.  At least one liter of seawater was collected to sample the suspended community, which was subsequently vacuum-filtered onto 47 mm, 0.2 μm nuclepore filters (Sartorius, Göttingen, Germany) for high-throughput sequencing and sterol quantification.  Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization (CARD-FISH) samples were fixed at room temperature for 30 minutes in 8% formaldehyde and subsequently filtered onto 0.2 μm nuclepore filters (Sartorius) with an underlying support filter (Sartorius). Three ice cores were extracted at each sea ice station using a 9 cm Kovacs ice corer.  The bottom 10 cm of each core was sectioned using an ethanol-sterilized handsaw.  Ice core sections were melted at room temperature into 1 l of 0.2 μm-filtered seawater.  After complete melting of the ice cores, samples were vacuum-filtered onto 0.2 μm filters. After filtration, all filters were immediately stored in sterile polypropylene tubes at -80 °C and kept frozen in the dark until analysis.  Sediment traps with 72 mm diameter and 1.8 l volume (Model 28.xxx series, KC-Denmark, Silkeborg, Denmark) were deployed at 5 and 20 m for 8 hours and 37 minutes at a single ice station (station 80).  To better interpret our ergosterol values and place our findings in a broader ecological context, we sampled a floating algal aggregate (SFigure 1) and deployed (on the ground) a Burkard cyclone spore trap (Rickmansworth, UK) to sample air (16.5 l x min$^{-1}$) in Tromsø, Norway for one hour.  Surface sediment samples were collected in Barrow, Alaska in triplicate in May and June of 2014 using a ponar grab that was deployed through a hole in the ice.  Sediment was stored in sterile polypropylene tubes and frozen at -80 °C until DNA extraction.

**DNA extraction and sequence processing.** DNA was extracted by bead beating filters in 10% wt/vol SDS lysis buffer for 10 minutes with a Precellys 24 shaker (Bertin, Frankfurt, Germany) at maximum speed, followed by a phenol-chloroform extraction. DNA was then purified using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) collection tubes and wash buffers, following the manufacturer's suggested protocol.  For 18S rRNA gene sequencing analysis, target amplicons were generated by

PCR (30 cycles) using the Earth Microbiome Project primers: Euk1391f: (5'- GTACACACCGCCCGTC-3') and EukBr: (5'- TGATCCTTCTGCAGGTTCACCTAC-3') (28) to generate ~170 base pair reads specific to the V9 hypervariable region. PCR products were generated using fusion primers with the Fluidigm CS1 or CS2 universal oligomers added to 5' ends.  Sterile MilliQ water was used throughout filtering, DNA extraction, and primary PCR to screen for the presence of unintended contamination. Secondary PCR and sequencing was performed at Michigan State University's Genomics Core Lab.  Secondary PCR was conducted with dual-indexed, Illumina-compatible primers to complete library construction. Final PCR products were batch-normalized using an Invitrogen SequalPrep DNA Normalization plate and products recovered from the plate were then pooled. The pool was quantified using a combination of Qubit dsDNA HS, Agilent Bioanalyzer DNA 1 000, and Kapa Illumina Library Quantification qPCR assays. The pool was loaded onto two (i.e. sequenced twice to increase sequencing depth) standard MiSeq v2 flow cells and sequencing was performed in a 2x150bp paired-end format using MiSeq v2 300 cycle reagent cartridges. CPCustom primers for sequence reads one and two, as well as index read one that was complementary to the Fluidigm CS1 and CS2 oligos, were added to appropriate wells of the reagent cartridge. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1.  Duplicate data output generated from the two separate runs were then concatenated (Unix cat command) before sequence analysis. Sequence analysis and processing was conducted using Mothur v1.33.3 (30, 31). Sequences with ambiguous base calls were eliminated (maxambig=0) from all datasets. Sequences were aligned using the SILVA (32) reference database (Release 123), screened for chimeras with UCHIME (33) and classified with SILVA (Release 123), using the K-nearest neighbor algorithm (bootstrap cutoff value of 75% with 1 000 iterations). Sequences classified as Bacteria, Archaea, and Metazoans were removed from datasets. Sequences were then clustered into operational taxonomic units at 97% similarity using the Average Neighbor distance.

**Isolates, carbon estimates**.  Four marine Dikarya fungi and two cosmopolitan freshwater Chytridiomycota isolates (STable2) were isolated and identified based on morphology, supplemented

by 28S rRNA and ITS1-5.8S-ITS2 DNA sequence data. Cells were harvested by inundating agar plates with sterile water. Harvested cells were sterilely sieved (35 μm, Falcon #352235, Corning, New York, USA) then counted and sorted with flow cytometry (BD FacsAria III) into sterile glass vials containing phosphate-buffered saline (Biowest, Riverside, Missouri, USA).  To estimate total carbon per cell, 100 000 sorted cells of each isolate were filtered onto precombusted glass fiber filters (Whatman, Maidstone, UK).  Precombusted filters were then fumigated for 24 hours in an enclosed container with hydrochloric acid (Sigma-Aldrich, St. Louis, Missouri, USA) and then allowed to dry for an additional 24 hours at 56 °C.  Organic carbon was quantified from fumigated filters with a CEC 440 CHN analyzer (Leeman Labs, Hundson, New Hampshire, USA). The carbon biomass per cell was estimated by dividing μg C measured with the CHN analyzer by the total number of cells sorted. These values were used to calculate the biomass per cell volume ratio, based on light-microscopically determined cell sizes assuming a shape of a sphere ($V=\frac{4}{3}\pi\,r^3$; r=range midpoint of light microscopy-measured cells) for Chytridiomycota isolates, and to create carbon:ergosterol values for the Dikarya isolates.

**Liquid-chromatography-mass-spectrometer (LCMS) methods and biomass ratios.**  10 000 cells of each Chytridiomycota and Dikarya species were harvested (as described above) and sorted in triplicate with flow cytometry (as described above), per species, to quantify sterol composition.  Each sample was run in triplicate (technical replications) and standard errors were generated from the means of these values (STable 2).  Prior to LCMS analysis, samples were treated with 1 ml of 2:1 chloroform: methanol (v/v) and allowed to extract overnight at room temperature in the dark.  Five μl of extracts were injected into an Ascentis Express C-18 Column (3 cm × 2.1 mm, 2.7 ⬚m, Sigma-Aldrich) connected to a Sciex API3200 LC-MS/MS in (+) APCI mode, run with an acetonitrile isocratic mobile phase (0.2 ml/min), and analyzed with multiple reaction monitoring. Sterols were compared to chemical standards, ergosterol (379.5 > 69.2; Sigma #45480), cholesterol (369.6 >161.3; Sigma-Aldrich #C8667), 24α-ethylcholesterol (397.0 > 161.3; Cayman Chemical #11756), 24α-methylcholesterol (393.4 > 161.1; Caymen Chemical #17344, Ann Arbor, Michigan, USA) and

quantified against isotopically labeled cholesterol (371.9 > 161.1; Sigma-Aldrich #707678).

Conversion factors were then generated for isolates by dividing µg of carbon measured on filters by

ergosterol values (µg C nmol-ergosterol$^{-1}$). These values were used to determine and subsequently

integrate (midpoint approximation) Dikarya biomass in the environment.

**CARD-FISH.** De novo probe design was conducted in ARB (34). Arctic marine Chytridiomycota 28S

rRNA clone sequences (18) were collated with all 28S rRNA Chytridiomycota sequences archived in

SILVA (29) to design two Chytridiomycota-specific probes (probe BTH that was designed to target

described taxa represented in SILVA and probe BTH2 that targets known sequences that represent

undescribed lineages) using the specification min group hits=40%, nongroup hits=30.  Using these

parameters, we generated two probes: ChyBTH: 5'-GAATGAGAGCTTCTAGTGG-3' and ChyBTH2: 5'-

TAAAAGTGTTTTCGGGGGCA-3'.  CARD-FISH was conducted using the SILVA FISH protocol v2.2 (32).

Specifically, filters were embedded in 0.2% low-melting agarose.  After embedding and deactivation

of endogenous peroxidases with $H_2O_2$, a double-hybridization was conducted to account for different

melting temperatures of our probes.  A chitinase permeabilization step was excluded, as there was a

noticeable loss of Chytridiomycota cell structure during protocol optimization.  Hybridization was

conducted with horseradish peroxidase enzyme-tagged probes (Biomers, Ulm, Germany) and

incubated with 35% and 20% formamide concentrations at 46 °C for 1.5 hours per probe. The optimal

formamide concentration for each probe was determined by identifying the concentration that

yielded the greatest specificity and signal in our target cells.  Signal amplification was conducted

using tryamide (Alexa594) at 46 °C for 45 minutes per probe.  After hybridization and signal

amplification, filters were counterstained with DAPI and then mounted in Citifluor:Vectashield

solution on glass slides and stored at -20 °C in the dark. Filters were visualized under a compound

epifluorescence microscope (Leica, Wetzlar, Germany) to enumerate labeled cells at 400x

magnification.  For illustrative purposes, laser scanning micrographs were generated on a Zeiss

LSM880.  Fluorescing cells larger than 1 µm and smaller than 20 µm were enumerated by counting 20

fields of view.  Environmental concentrations were determined by extrapolating areas observed

within our fields of view to the entire area of the exposed filter and standardizing to the volume of seawater filtered. These values were used to determine and subsequently integrate (midpoint approximation) Chytridiomycota biomass in the environment.

**Functional gene survey.** For functional gene analysis, DNA was extracted from triplicate under-ice surface sediment samples from Barrow, Alaska collected in May and June 2014. After extraction, DNA was pooled and analyzed using the GeoChip (36) functional gene microarray (GeoChip v5.0; Glomics Inc., Norman, OK, USA). Amplification, labeling, hybridization, imaging, and data processing were conducted by the Institute for Environmental Genomics at the University of Oklahoma according to published protocols (37). Signal intensity was normalized to display all positive probes detected in each sample. Probe data was removed from the output if the signal to noise ratio was below 2 or if the signal was <200 or <1.3 times the background.

**Spore longevity**. 500 µL of 1.1 x $10^6$ *Penicillium brevicompactum* spores per mL were applied to 47 mm, 0.2 µm pore filters (Sartorius). Spores were enumerated with a hemocytometer counting chamber (Hausser Scientific, Horsham, Pennsylvania, USA). Filters were folded in half and sealed with tape. Spore packets were then submersed into 0.2 µm-filtered seawater and incubated at 0 °C. Filters were then removed monthly and placed on PmTG media overnight at room temperature. Incubated filters were then stained with Rose Bengal and microscopically examined for the presence of germinating spores.

**Spore germination in fecal pellets.** *Onisimus glacialis* (amphipod) was collected onboard the R/V Polarstern in July 2017. 10 days (d) after collection, 60 fecal pellets were harvest from incubation jars and inoculated with *Penicillium brevicompactum* spores in sterile glass vials at 4 °C for 30 days. Pellets were stained with calcofluor white (Sigma) and visualized with fluorescence microscopy.

**Results**

LCMS analysis of cultured fungal isolates detected all targeted sterols, predominated by 24-ethyl cholesterol, followed by cholesterol, 24-methyl cholesterol, and finally ergosterol (STable2).

Midpoint ranges of zoosporangia (Chytridiomycota) and conidia (Dikarya) were used to establish biovolume estimates (Table 1). CHN output of carbon values exceeded the known detection threshold of the analyzer (1 µg C). Chytridiomycota isolates averaged 93.65 (22.55) pg C per cell. Dikarya isolates averaged 203.75 (79.54) pg C per propagule. Average values of ergosterol were used to create conversion factors to total carbon (Table 1). Ergosterol values were correlated with carbon ($R^2$=0.52, SFigure 2). The average ratio of carbon:ergosterol was 105.25 µg C per nmol ergosterol.

LCMS analysis of environmental samples revealed the presence of all targeted sterols at every site (Figure 2, STable3). 24-ethyl cholesterol was the most concentrated sterol detected, followed by cholesterol, 24-methyl cholesterol, and finally ergosterol. Ergosterol was detected in every sample. The average ergosterol value from all environmental sites (i.e. seawater and sea ice samples) was 16.54 (33.33) nmol m$^{-3}$; in sea ice 34.15 (38.72) nmol m$^{-3}$; and in seawater 9.69 (29.36) nmol m$^{-3}$. The greatest ergosterol value was measured in the algal aggregate (379 nmol). The lowest ergosterol value was measured from Tromsø air (0.002 nmol m$^{-3}$). Using the average carbon:ergosterol conversion factors of all cultured fungi analyzed (105.25 µg C nmol-ergosterol$^{-1}$), there was an average Dikarya biomass (i.e. seawater and sea ice samples) of 1.74 (3.50) mg C m$^{-3}$; 3.59 (4.07) mg C m$^{-3}$ in sea ice; and 1.02 (3.09) mg C m$^{-3}$ in seawater (two-tailed T-test, p=0.10). Integrated Dikarya biomass from all seawater sites averaged 444.56 (444.36) mg C m$^{-2}$. Sediment trap data revealed a Dikarya biomass sedimentation rate of 2.51 mg C m$^{-2}$ d$^{-1}$ and 5.34 mg C m$^{-2}$ d$^{-1}$ at 5 m and 20 m depth, respectively.

SILVA TestProbe of ChyBTH (using zero base pair mismatches) theoretically hybridized with 67% of all archived Chytridiomycota sequences, as well as unintended binding with three ciliate genera (*Cardiostomatella*, *Hippocomos*, *Wilbertia*), a small fraction of the Dikarya, and the Glomeromycota. SILVA Test Probe of ChyBTH2 did not theoretically hybridize with any database-archived sequences, underscoring the specificity of our probe to Chytridiomycota clone sequences. SILVA Test Probe using two and three mismatches identified ciliates, metazoans, and the dinoflagellate genus

*Procentrum* as the only non-specific taxa to which our probes would theoretically hybridize. We did not test the binding of our probes to the nearest non-specific taxa, as we believe the morphological differences between the Chytridiomycota and other non-specific probe hybridizing groups could be efficiently discerned. BLAST queries of our probes identified wheat (*Triticum aestivum)*, yak (*Bos mutus*), pole worm (*Haemonchus contortus*), and bighorn sheep (*Ovis canadensis*) as the only non-fungal eukaryotes with 100% identity and query coverage, as well as a bacterium (*Xylella fastidiosa*). CARD-FISH analysis of environmental samples detected Chytridiomycota, as well as a small (<1 μm) cluster-forming organism that was frequently associated with algae. Labeled cells larger than the 20 μm exclusion threshold were not observed. Chytridiomycota were observed at almost every station and in almost every sample; however, there were no Chytridiomycota detected in several bottom water samples. The majority (60%) of observed Chytridiomycota (sea ice and seawater) came from sea ice samples. Chytridiomycota were observed attached to particles and diatoms (Figure 3), as well as free-living cells that were not associated with any substrate. When counts were converted to carbon, Chytridiomycota biomass averaged 34.07 (50.77) μg C m$^{-3}$ across all sea ice and seawater samples; 81.57 (68.68) μg C m$^{-3}$ in sea ice, which was significantly higher than Chytridiomycota biomass in seawater, 15.60 (26.16) μg C m$^{-3}$ (two-tailed T-test, p=0.04). The Chytridiomycota derived a vertically integrated seawater biomass of 11.66 (17.96) mg C m$^{-2}$. Sediment trap data revealed a Chytridiomycota sedimentation rate of 7.09 μg C m$^{-2}$ d$^{-1}$ at both 5 m and 20 m depth. The average of summed ergosterol and CARD-FISH biomass (sea ice and seawater) approximates 1.77 (3.52) mg C m$^{-3}$; 3.67 (4.10) mg C m$^{-3}$ in sea ice; and 1.03 (3.08) mg C m$^{-3}$ in seawater (two-tailed T-test, p=0.09). Summed carbon from ergosterol and CARD-FISH values derived an average vertically integrated seawater biomass of 456.23 (447.38) mg C m$^{-2}$.

After primary PCR, in parallel with our environmental samples, visualization of DNA in agarose gel revealed no visible DNA amplification within our negative sequencing control. Using two MiSeq runs to generate sequencing data, we produced 26 677 829 assembled reads. After removal of chimeric sequences, sequences that contained ambiguous bases, unalignable sequences, and sequences allied

to the Bacteria, Archaea, and Metazoa, we retained 12 767 164 sequences.  The fungal community

comprised 8% of all retained reads and were allied to the Ascomycota, Basidiomycota,

Mucoromycota, Chytridiomycota, and unclassifiable sequences.  Fungal sequences unclassifiable to

the taxonomic phylum level represented 89% of total sequences (Figure 4).  Using the remaining

sequences classified, there was a noticeable mismatch between our amplicon sequencing data and

ergosterol/CARD-FISH counts (linear regression of ergosterol:Dikarya sequence reads, $R^2$=0.0092; no

Chytridiomycota detected in sites where CARD-FISH probes hybridized to Chytridiomycota).  The

three ciliate genera that our CARD-FISH probes theoretically hybridized with were not detected in

our high-throughput sequencing results.

GeoChip5.0 contains 167 403 unique probes to which environmental DNA can hybridize.

Environmental DNA from Barrow, Alaska sediment hybridized to 63 403 (37%) available probes.  In

May sediment, environmental DNA hybridized to 56 729 probes and 55 723 probes in June sediment.

Detected genes are known to be involved in biogeochemical cycling of carbon, nitrogen, sulfur,

phosphate, as well as secondary metabolism.  Genes from each taxonomic domain of life were

detected, including: 628 genes allied to the viruses (22% of possible viral probes), including 20 genes

allied to the Partitiviridae (16 RNA-dependent-RNA polymerases, and 4 capsid protein-encoding

genes), 3 450 genes allied to eukaryotes (30% of possible eukaryotic probes), 57 388 genes allied to

Bacteria (41% of possible bacterial probes), 1 633 genes allied to the Archaea (29% of possible

Archaea probes), and 304 genes derived from uncultured organisms.  Hybridized fungal genes (Table

2) were represented by 2 757 probes (31% of possible fungal probes) that were derived from the

Chytridiomycota, Neocallimastigomycota, Basidiomycota, and Ascomycota.  63 nitrogen-cycling

fungal genes were detected that catalyze a complete ammonification pathway from nitrite (nor,

narG, nirK) and urea (ureC) and subsequent ammonia assimilation through glutamate synthase

activity (glnA), as well as genes associated with denitrification (narG, nirK, norB).  We detected genes

involved in the degradation and conversion of phenolic compounds (e.g. lignins and naphthalene),

such as: phenylalanine N-monooxygenase, salicylate 1-monooxygenase, and peroxidase.  Out of all

functional genes detected, the fungal *pel* (pectin lyase) was the 26th strongest gene signal detected in May sediment, relative to all hybridized probes. In June sediment, this gene became the 7th strongest gene signal detected.

To explore the ability of a candidate marine fungus to degrade biological material, we inoculated the fecal pellets of *O. glacialis* (amphipod) with conidia of *P. brevicompactum*. *Penicillium* species are regularly isolated and detected in the marine realm (4) and a member of this genus was consequently selected for this study given its global distribution and presumed frequent encounters with biological material. After a month of incubation, branching hyphae were observed entwined and extending away from the fecal pellets, resulting in a loss of structural integrity to the fecal pellet (SFigure 3). *P. brevicompactum* is also regularly isolated from the terrestrial realm, consistent with approximately 40% of described marine fungal taxa (4). Consequently, we were interested to explore the longevity of terrestrial-marine fungal taxa. After eight months of incubation in sterile seawater, we were able to germinate *P. brevicompactum* spores on PmTG agar media (SFigure 4). Spores germinated overnight and produced long germination tubes that extended away from the conidium.

**Discussion**

A surge of recent molecular-based studies have demonstrated that marine fungi are abundantly present in a variety of marine ecosystems (6, 7, 38); however, the ecological relevance of marine fungi remains a knowledge gap in food web ecology and biological oceanography. In this study, we targeted a geographically distinct region of the Arctic that includes the Atlantic-influenced Norwegian Sea, the Barents Sea, and along the shelf-break region that descends into the deep Arctic Ocean (>3 000m depth). Our objective was to explore the ecological contributions of marine fungi, by focusing on the fungal fraction (Dikarya and Chytridiomycota) that comprises >95% of the entire fungal community in Arctic marine ecosystems (8, 18, 20). We quantified fungal-derived sterols, used

Chytridiomycota-specific enzyme-labeled probes, established biomass conversion factors, screened for catalytic genes, and conducted lab-based studies.

We used high-throughput sequencing to explore the relationship between ergosterol and sequence reads and to serve as a check for the presence of non-Dikarya ergosterol-synthesizing organisms, as well as unintended organisms that would hybridize to CARD-FISH probes. We detected four major fungal phyla: Ascomycota, Basidiomycota, Chytridiomycota, the Mucoromycota, as well as unclassifiable sequences, consistent with other sequencing-based studies from across the Arctic (8, 18, 20). However, we identified a strong mismatch between rRNA barcode sequences and our ergosterol and CARD-FISH quantifications. These data underscore the limited utility of amplicon-based data for quantitative measurements. Still, our rRNA results identified a small fraction of the fungal community that was unreachable with our CARD-FISH and ergosterol methods, such as the Mucoromycota. Consequently, from this perspective, our data likely underestimate the total ecological contributions of marine fungi in the sampled area.

To establish a baseline of fungal biomass, we generated carbon:ergosterol ratios for four Dikarya isolates and established cell-specific carbon values for two Chytridiomycota isolates. Our average weight ergosterol g $C^{-1}$ (6.08 mg ergosterol g $C^{-1}$) is consistent with other estimates between 2.3-11.5 mg ergosterol $g^{-1}$ biomass (39). Our Chytridiomycota biomass estimate (0.4 pg C per zoospore) is substantially lower than other (10.7 pg C per zoospore) published studies (40), assuming a zoospore diameter of 3-4 μm for *G. pollinis-pini* (41) and 5-6 μm for *C. hyalinus* (42). This low value was driven by the wide-range of sporangial diameters that were observed in culture. To circumvent this issue, we chose to use the average carbon value (93.6 pg C per cell) of all sorted particles (i.e. zoospores and zoosporangia), which we believe is more representative of actual environmental conditions, where Chytridiomycota are encountered in different stages of their lifecycle. The absence of cultured marine Chytridiomycota inhibited our use of marine isolates to generate conversion factors. Though Arctic marine Chytridiomycota form distinct phylogenetic clades, they branch among other

described taxonomic orders (18, 43) and with a mature zoosporangium diameter of ~10 µm, are within the size range reported of freshwater algal parasites (44). Consequently, we believe parallels can be drawn between freshwater and marine Chytridiomycota. Still, these biomass estimates are sensitive to the assessed carbon values generated from select fungal cultures that were applied to the environment. As a result, the average carbon value of isolates and consequently integrated biomass estimates are likely to change as the number of analyzed cultures increase.

The ergosterol synthesis pathway shares common sterol precursors with most eukaryotes, originating with squalene that is converted to lanosterol. Subsequently, lanosterol can be converted to cholesterol in vertebrates and ergosterol in fungi (45). Ergosterol biosynthesis has also arisen independently in other eukaryotic lineages, such as in the human pathogens *Trypanosoma* and *Acanthamoeba* (46) and is speculated to be present in the marine genera *Chlamydomonas* (47), *Corallochytrium,* and *Capsaspora* (48, 49). We conducted high-throughput sequencing as a check for these organisms and found minimal evidence for ergosterol contributions from non-fungal sources. Specifically, 20 sequences were classified as *Acanthamoeba* in our dataset (maximum five sequences in an ice core at station 50). We acknowledge the possibility that other uncharacterized marine organisms might produce ergosterol. In addition to ergosterol, we quantified three additional sterols known to dominate the sterol pool in fungi; however, these sterols are also synthesized by other organisms and are therefore of minimal use for exclusively quantifying fungal biomass. The concentration of these other sterols is likely elevated due to the presence of small metazoans and other cholesterol-synthesizing taxa, but are still reflective of the total quantity in our environmental samples.

To supplement our ergosterol-based fungal biomass estimates of the Dikarya, we conducted CARD-FISH counts to enumerate the Chytridiomycota. The high number of chytrids in sea ice relative to seawater is consistent with other sequencing-based studies from the Arctic (8). Furthermore, the presence of the Chytridiomycota in the water column has been confirmed in other global marine

ecosystems with CARD-FISH methods (50) and in the Arctic with sequencing data (6).

Chytridiomycota biomass estimates from other ecosystems approximate that ~15% of the carbon

biomass can be tied up seasonally by the Chytridiomycota (40). Our combined estimate of total

fungal biomass (i.e. summed ergosterol and CARD-FISH based biomass) of 1.77 mg C m$^{-3}$ from sea ice

and seawater is between <1-15% of the total carbon historically observed in this study's sampling

region (51). Comparative analysis of ergosterol and CARD-FISH counts suggest that ergosterol-

synthesizing organisms comprise the majority of marine fungal biomass. However, the absence of

the Chytridiomycota at several bottom depth sites skewed extrapolated values of biomass, especially

in deep-water sites; consequently, we believe we underestimate the biomass of the Chytridiomycota.

The carbon values of detected fungi appeared randomly distributed throughout the water column,

consistent with other sequencing-based studies across the Arctic (18). Complex hydrography (11)

and sea ice within our study region confound the elucidation of ecological drivers of fungal

abundances. Furthermore, terrestrial contributions of Dikarya fungi into the marine ecosystem likely

skews the interpretation of marine fungal biomass and sequencing data. We determined that some

spores of the terrestrial and marine-inhabiting fungus, *P. brevicompactum*, can remain viable for at

least eight months in seawater, underscoring the possible detection of viable, terrestrial-derived

fungal propagules in the marine environment. Future research should attempt to discern terrestrial

and marine-derived fungal carbon to marine ecosystems, while detailing the relative proportions of

latent and active fungal propagules.

The average fungal-derived carbon values in seawater of 1.04 mg C m$^{-3}$ is similar to other estimates

of pelagic marine fungal biomass from Chile (52). The average fungal-derived carbon value of

seawater of 444.56 mg C m$^{-2}$ in mid-summer is similar to biomass of other major morphologically

defined groups in the Arctic. For example, algal pigments in sea ice and seawater combined is

between 10 - 300 mg C m$^{-2}$ (53, 54); mesozooplankton biomass in the Arctic Ocean ranges between 1

-2 g C m$^{-2}$ (53, 55); bacterial biomass in the upper 50 m has varied between 0.2 - 0.5 g C m$^{-2}$ (56, 57).

Our fungal biomass estimates are surprisingly high relative to bacterial biomass; however, fungal and prokaryotic biomass are reported to be analogous in other oceanic regions (52). Though these late-summer estimates are comparable to other taxa, Arctic marine fungal abundances (namely the Chytridiomycota) are known to temporally modulate in association with algae and increased irradiance (8, 58). Our high values of fungi (both Dikarya and Chytridiomycota) on the algal aggregate support these findings. In other sectors of the world, members of the Dikarya also display predictable patterns of temporal modulation in association with the varying ecological niches they occupy (52, 59). We detected ergosterol at every station and observed fungi attached to non-living particulate. Based on these observations, we hypothesize that non-algal associated fungi might comprise larger fractions of total biomass under aphotic conditions (9). Unlike phototrophic organisms, fungal production can be sustained by dissolved organic material in the absence of light, such as in deep sea sediment (60) or during the polar night. Observations suggest that fungal production is at least partially constrained by consumers and routed into other trophic levels. Screening of molecular barcodes has detected fungi in the gut content of zooplankton (16); however, no additional data exists for the fate of fungal-derived carbon. We present evidence for the presence of marine mycoviruses within the Partitiviridae (61) identified in our GeoChip analysis, suggesting an additional pathway of carbon flow in the Arctic Ocean - presumably back into the dissolved organic carbon pool.

To supplement biomass estimates and conceptually explore fungal ecology outside of an exclusively trophic paradigm, a functional gene survey was conducted on surface sediment samples from Barrow, Alaska. We detected numerous genes allied to fungi that are responsible for degradative processes and nutrient cycling. For example, we detected fungal genes that assimilate nitrate/nitrite, and degrade refractory substances, such as naphthalene and lignin. The fungal pectin lyase gene was the seventh strongest probe signal detected in June sediment (the period after ice-sourced biological material settles to the seafloor), suggesting that fungi might actively be degrading biological material, while uptaking dissolved organic material and nutrients (62). The successful germination of fungal

spores in zooplankton fecal pellets augments these findings. This is particularly important in the

Arctic, where rapidly sinking zooplankton fecal pellets provide essential carbon to benthic organisms

(63). When these data are considered with our quantified fungal biomass sedimentation rates, and

microscopy observations of Chytridiomycota on particulate, these data suggest that benthic-pelagic

carbon coupling is likely influenced by the successful colonization of sinking particulate by fungi in

the upper water column, consistent with observation from the bathypelagic realm (9). The presence

and activity of nutrient-cycling gene products are likely responsible for the relationship of fungal

communities to dissolved nutrients that have been identified in other sectors of the world (64, 65).

However, the relationship between complex organic molecules and fungal communities in the

marine realm remains unknown. As thawing permafrost continues to be exported into the Arctic

Ocean, the presence of known catalytic genes suggest an ecological niche for Arctic marine fungi.

Future research should explore the potential synergy and niche portioning between the bacteria and

the fungi, especially as their biomass is similar and they both encode degradative genes.

The unique genetic signatures of marine fungi, as well as the inconspicuous morphology of fungal

propagules has challenged the easy identification and subsequent integration of fungal data into

marine ecology. Mounting data suggests that marine fungi are cosmopolitan organisms that can

comprise a sizable fraction of the eukaryotic microbial community. Here we provide several lines of

data that demonstrate the relevance and ecological potential of marine fungi in their associated

ecosystems. Ultimately, these data suggest that fungi could be as important in oceanic ecosystems

as they are in freshwater environments.

**Acknowledgements**

**Competing Interests**

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**Figure Legends**

**Figure 1.**  Map of study site area and station locations around Svalbard (grey) sampled during the RV Polarstern expedition PS106.

**Figure 2.** Heat map displaying concentrations of measured sterols at stations during the PS106 expedition and one air sample collected in Tromsø, Norway. Darker colors represent higher concentrations. The cubed root of sample values is displayed for illustrative purposes.

**Figure 3.** Fluorescence microscopy of CARD-FISH labeled Chytridiomycota cells. Scale bar represents 10 μm. Note: Our Chytridiomycota fluoresce red, but appear blue in the top four images, based on imaging limitations A) A Chytridiomycota (ch) cell attached to an unidentified algal (a) cell. B) Numerous Chytridiomycota zoosporangia attached to a particle. C) Chytridiomycota zoosporangia with various diatom cells in background. D) Two Chytridiomycota zoosporangia and one small zoospore (z) in top left of image. E) Laser scanning micrograph illustrating the red fluorescing Chytridiomycota in association with signal-saturating blue DAPI-stained diatoms. F) Laser scanning micrographs of Chytridiomycota zoosporangia in association with diatoms.

**Figure 4.** Relative abundance of fungal taxa classified from 18S rRNA V9 high throughput sequence reads generated at various stations, including surface waters, chlorophyll *a* maximum (max), bottom samples, sediment traps (ST), sea ice, and an algal aggregate (algal agg). Taxa are displayed at the taxonomic class level. Chytridiomycota sequences were unclassifiable to the class level and are consequently displayed as a phylum. Taxa that contributed less than 100 sequences are not displayed for clarity.

**Table 1:** Biovolume and biomass conversion factors of six aquatic fungi.

**Table 2:** The top-25 most intense fungal probes (proxy for abundance) detected in under-ice sediment from Barrow, Alaska using the GeoChip microarray