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Culturable fungi in Arctic cryoconite holes: A case study from Hansbreen, Spitsbergen

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Abstract: Extreme cold environments like glaciers, present substantial obstacles to the survival of organisms. Cryoconite, dark sediment covering glacier, provide unique niche for microorganisms. Therefore, we focused on understanding the diversity of fungi in Arctic ecosystems (Hansbreen, Spitsbergen), which is important in the analysis of the structure and of fungi populations. Due to a combination of two incubation temperatures (7°C or 24°C) and two media during isolation (potato dextrose agar, PDA or yeast extract peptone glucose, YPG), and classical/molecular identification approaches, we identified 20 different fungi (17 species and three unassigned species). Most belonged to filamentous fungi within the Ascomycota (19 isolates), with one identified as Basidiomycota-yeast. Regarding growth conditions, both media yielded greater number of fungal cultures at 24°C compared to 7°C. Additionally, PDA was more effective than YPG in isolating fungal cultures. On the other hand, the optimal temperature for achieving the highest CFU (colony–forming unit)/g of sediment was 7°C. The most frequently isolated species was *Cladosporium cladosporioides*, and to the best of our knowledge, we are the first to detect, the following species in an Arctic environment: *Aspergillus jensenii*, *A. tennesseensis*, *Peziza varia*, and *Trichoderma paraviridescens*. Additionally, there was a visible increase in the number of fungal propagules but a decrease in their biodiversity towards the upper parts of the glacier. Considering the Arctic amplification there is a need for further research on diversity and function of fungi in glacial ecosystems.

Keywords: Arctic, Svalbard, micromycetes, psychrophiles, glaciers.

Introduction

Environments with extreme conditions are challenging for the survival and growth of the living organisms; however, intrinsic or acquired specific features may enable the inhabitation of such places. Polar regions are considered a harsh environment for living organisms due to the frequent fluctuations of environmental conditions like freeze-thaw cycles, limited nutrient availability, UV radiation, and osmotic stress. Cryoconite holes (depressions on the glacier surface filled with water and mineral and organic material) offer occasional protection against some of these factors, thus are a specific niche for living organisms (Millar *et al*. 2021). Life within cryoconite holes is dominated by bacteria, but Archaea, microalgae, protists, fungi and metazoa taxa may also be found (Zawierucha *et al*. 2015; Millar *et al.* 2021). The vast majority of research is focused on the analysis of microbial communities residing in cryoconite holes, leading to the conclusion that the microbial composition may vary between the glaciers (Zarsky *et al.* 2013; Uetake *et al.* 2019; Segawa *et al.* 2020).

Although cryoconite holes are dominated by the bacterial taxa, the attention to the fungi residing in this peculiar environment has been recently drawn. Millar *et al*. (2021) found the presence of Ascomycota and Basidiomycota in Arctic and Antarctic glaciers, but without identification to the species level. Antarctic and Himalayan glaciers were found to be inhabited by basidiomycetous yeasts of *Mrakia*, *Leucosporidium*, *Bensingtonia*, *Curvibasidium* and *Rhodotorula* genera (Sanyal *et al*. 2018). Basidiomycota, mainly *Mrakia* sp. and *Rhodotorula* sp., were also characteristic for cryoconite holes on the glaciers in Svalbard (Edwards *et al*. 2013; Poniecka *et al*. 2020). Although basidiomycetous yeasts are the most frequently isolated from cryoconite holes, filamentous fungi, such as *Alternaria* sp., *Circinella* sp. and *Phialophora* sp. are also found on glaciers, mainly in Svalbard (Gerdel and Drouet 1960; Edwards *et al*. 2013). Filamentous ascomycetes are believed to originate from plant litter and avian feces and be windblown to the cryoconite holes and they constitute an important saprophytic group of the glacier microflora (Edwards *et al*. 2013). Feces of small animals inhabiting glaciers are known to be a reason of microbial diversity in cryoconite. The comparison of cryoconite and tardigrades microbiome from Forni Glacier showed that cryoconite material was abundant in fungi uncharacteristic to this environment (*Russula* sp. and *Xerocomus* sp.), but consistent with the food of tardigrades in cryoconite holes (Zawierucha *et al.* 2022). Due to extremality of polar climate fungal inhabitants have developed multiple strategies for the survival in such a unique environment, such as thick cell wall and dye production (*Rhodotorula* sp.), what gives them huge tolerance to freeze-thaw cycles. Dhume *et al.* (2022) have shown that the majority of fungi isolated from Himalayan glacier cryoconite were psychrotolerant with the optimum growth temperature at 15°C. Adaptation to cold environment of the isolates included utilization of simple carbon sources and secretion of enzymes (cellulase, lipase and proteinase) for the breakdown of snowpack substances (Dhume *et al*. 2022).

One of the goals of microbiological analyses of glacier materials is to identify potential threats. Although the majority of fungi found on glaciers appear to be harmless, some genera are associated with the pathogenicity towards plants, animals, or humans (Edwards 2015). Parasitic chytrids of *Rhizophidium* and *Phlyctochytrium* genera were found in infected the glacial algae in Gulkana Glacier, Alaska (Kobayashi *et al*. 2023). Yeasts of *Cryptococcus* genus, frequently isolated from glaciers, are characterized by large phenotypic plasticity and adaptation to various extreme environment including; the secretion of hydrolytic enzymes and the production of a capsule. These features are being associated also with virulence in human hosts (de Garcia *et al*. 2012).

The differences in the diversity of organisms on various glaciers have been observed and attributed, among others, to cryoconite composition and morphology (Rozwalak *et al*. 2022). However, relatively small amount of conducted research on fungal microflora encourages to continue such studies to fully understand how the location of glacier influences microbial diversity.

The work of Borzęcka *et al.* (2022) considered fungal diversity in cryoconite holes on Werenskioldbreen located in Spitsbergen, in Svalbard. High diversity of fungal species has been observed in the samples from cryoconite holes. Fungi of both, Ascomycota and Basidiomycota phyla were isolated, with the highest prevalence of *Parengyodontium album* (Limber) C.C. Tsang, J.F.W. *et al.* 2016 and *Patinella hyalophaea* Sacc. 1875 followed by the representatives of Aspergillaceae and Cladosporiaceae families. A noteworthy are the first records of some of the isolated species in the Arctic area, the examples being *A. pseudoglaucus* Blochwitz 1929, *C. allicinum* (Fr.) Bensch, U. Braun & Crous 2012, *C. ramotenellum* K. Schub., Zalar, Crous & U. Braun 2007, *P. sumatraense* Szilvinyi 1936, *P. velutinum* J.F.H. Beyma 1935, or *P. cumulodentata* (Nikol.) Parmasto 2015 (Borzęcka *et al.* 2022).

The goal of this work is the identification of fungi isolated from the samples collected from the cryoconite holes on the Hansbreen glacier in Wedel Jarlsberg Land at Spitsbergen, Svalbard and the evaluation of fungal diversity in comparison to the results obtain for Werenskioldbreen. Although both glaciers are located in South-Western Spitsbergen, they vary in multiple factors, such as solar exposure and ablation, as well as the influence of wind and Western Ocean (Kosiba 1963). These factors may affect fungal community composition, thus mycological analysis of Hansbreen will allow to detect differences in mycobiota of these two glaciers.

Study area

The samples used in this study were collected on August 13th, 2017, from cryoconite holes on Hansbreen on Spitsbergen, within the Svalbard archipelago, during the Arctic summer (Fig. 1). Located on the largest island of the Svalbard archipelago in the Arctic Ocean, Spitsbergen (latitude between 74° and 81°N) is

situated between the cold Arctic East Spitsbergen Current and the Atlantic West Spitsbergen Current, which significantly influences its climate. The Atlantic West Spitsbergen Current influences the temperature in Spitsbergen, resulting in temperatures up to 20°C higher than in other Arctic regions (Walczowski and Piechura 2011). Spitsbergen's fauna and flora are influenced by several factors, most notably the polar day and night cycles, low precipitation, as well as cold, windy winters and short summers.

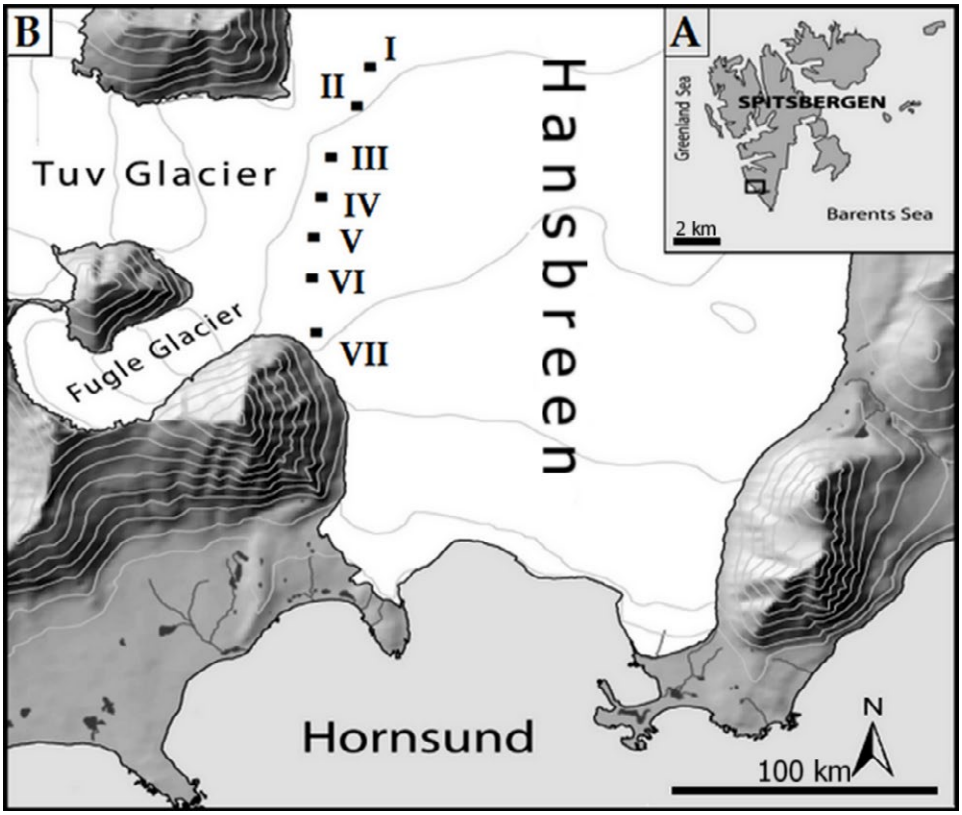


Fig. 1. Geographic location of Hansbreen on (**A**) Spitsbergen (Svalbard Archipelago, Arctic) and (**B**) study sites from I to VII.

Hansbreen is a tidewater glacier, characterized by its dynamic nature and pivotal role as a model glacier within the Polish glaciological research community, especially for research teams working in Hornsund. This glacier, exemplifying a subpolar glacier type, operates within a polythermal thermal regime, containing both cold and temperate ice. Spanning an area of approximately 55 km^2 and stretching over 16 km in length, Hansbreen's size and accessibility make it an ideal subject for diverse glaciological studies (Vieli *et al*. 2004; Oerlemans *et al*. 2011), including the integration of classical and molecular tools to investigate fungi in this unique habitat.

Samples were taken from seven locations (Table 1). Each sediment sample was aseptically placed in individually packaged, sterile conical polypropylene tubes (50 mL) with screw caps (Biologix, China). Until microbiological analyses, samples were stored at −20°C as recommended by Borzęcka *et al*. (2022).

Table 1.

The exact geographic coordinates of the study sites within Hansbreen on Spitsbergen (Svalbard Archipelago, Arctic). Coordinates according to ETRS_1989_UTM_Zone_33N.

Study sites	Latitude (N)	Longitude (E)	Altitude (m a.s.l.)
I	77°02.626′ (N8551733)	15°35.794′ (E514931)	209
II	77°02.467′ (N8551436)	15°35.794′ (E514854)	202
III	77°02.262′ (N8551054)	15°35.794′ (E514699)	194
IV	77°02.103′ (N8550758)	15°35.794′ (E514639)	193
V	77°01.942′ (N8550458)	15°35.794′ (E514594)	191
VI	77°01.777′ (N8550151)	15°35.794′ (E514584)	180
VII	77°01.559′ (N8549746)	15°35.794′ (E514610)	159

Methods

Mycological analysis of samples. — The mycological analysis of the samples followed the protocol outlined by Borzęcka *et al*. (2022). Initially, the samples underwent thawing (at 25 ± 1°C until completely defrozen, *ca.* 20 min), following which 3 g of cryoconite sediments were aliquoted into sterile conical polypropylene test tubes (25 mL) equipped with screw caps (FL Medical, Italy), each pre-filled with 12 mL of isotonic physiological salt solution (0.85% NaCl). These tubes were then shaken at room temperature (20 min; 25 ± 1°C) to achieve homogenous suspensions. Following this, the samples underwent serial dilution to concentrations of 25×, 50×, 500×, and 5000×, followed by vortexing and subsequent plating in triplicate. The inoculated plates were then subjected to incubation for a duration ranging from 5 to 56 days at temperatures of 7°C and 24 ± 0.5°C on both PDA (potato dextrose agar, BioMaxima, Poland) and YPG media (yeast extract peptone glucose media containing 10.0 g L^{-1} yeast extract, 20.0 g L^{-1} peptone, and 15.0 g L^{-1} agar). We used the temperature of 7°C to isolate psychrophilic and psychrotolerant fungi and 24°C to isolate mesophilic fungi, which is the optimal temperature for the growth of the majority of fungal species (Borzęcka *et al*. 2021). In turn, PDA is one of the most frequently used media in environmental

mycological research due to its universality and high effectiveness in culturing a wide spectrum of fungi (Kokurewicz *et al*. 2016; Ogórek *et al*. 2016; Pusz and Urbaniak 2021). Additionally, we used YPG as according to Borzęcka *et al*. (2021), this medium showed higher efficacy than PDA in isolating more fungal species. Pure cultures were acquired via the single spore technique on PDA medium and subsequently propagated on PDA slants for subsequent morphological and molecular characterization. Ultimately, the fungal colony-forming units (CFUs) per g of cryoconite sediments were enumerated.

Fungal identification. — Fungal identification relied on a combined approach employing both phenotypic and genotypic methodologies. Pure cultures were subjected to comprehensive analysis through both micro- and macroscopic evaluations. Initial phenotypic assessments encompassed a range of culture media, including PDA for all fungi and in the case of *Aspergillus* and *Penicillium* spp.: Czapek-Dox agar (1.2% agar, BioMaxima, Poland), malt extract agar (MEA, BioMaxima, Poland), and Czapek yeast autolysate agar (CYA: 30.0 g L^{-1} sucrose, 15 g L^{-1} agar, 5.0 g L^{-1} yeast extract, 3.0 g L^{-1} $NaNO_3$, 1.0 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4 \times 7H_2O$, 0.01 g L^{-1} $FeSO_4 \times 7H_2O$). Evaluation criteria encompassed colony morphology, pigmentation, and the presence of distinctive morphological features such as spores. Identification was further refined through consultation of diagnostic keys and relevant monographs (Lloyd 1921; Saccardo 1975; Sogonov *et al.* 2005; Chilvers and du Toit 2006; Schoch *et al.* 2009; Bensch *et al.* 2012; Korniłłowicz-Kowalska and Rybczyńska 2012; Visagie *et al.* 2014; Kruys *et al.* 2015; Liu *et al.* 2015; Volobuev *et al.* 2015; Soler-Hurtado *et al.* 2016; Wang *et al.* 2016; Chen *et al.* 2017; Fiuza *et al.* 2017; Dyląg *et al.* 2019; Kanegae *et al.* 2020; Kovač *et al.* 2020).

To confirm the taxonomic classification of the fungal species, the internal transcribed spacer (ITS) region of fungal rDNA was sequenced. DNA extraction from fungal colonies cultured on PDA was performed using the Bead-Beat Micro AX Gravity kit (A&A Biotechnology, Gdańsk, Poland) following the provided protocol. Amplification of the fungal rDNA ITS region (negative control was prepared without DNA) was carried out using the primer pairs ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*. 1990). PCR amplification was conducted in a T100 Thermal Cycler (Bio-Rad) following the protocol outlined by Ogórek *et al*. (2016), as follows: initial denaturation for 5 min at 94°C, 35 cycles of 30 s denaturation step at 94°C, a 30 s annealing step at 55°C, and a 45 s extension step at 72°C, followed by final extension step for 7 min at 72°C. Subsequently, the PCR products underwent electrophoretic separation on a 1.2% agarose gel to confirm their integrity. The purified PCR products were then processed using the Clean-UP kit (A&A Biotechnology) for purification before sequencing, which was carried out by Macrogen Europe (Amsterdam, Netherlands, http://dna.macrogen.com/eng/).

Data Analyses. — The primary step involved the examination of fungal sequence reads employing the BioEdit Sequence Alignment Editor (Hall 1999; Sofi

et al. 2022; Darojat *et al.* 2023), followed by their alignment with entries cataloged in the GenBank repository of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) utilizing the BLAST algorithm (http://www.ncbi.nlm.nih.gov/). Subsequently, the derived rDNA ITS fungal sequences were archived in the NCBI GenBank database. To determine the diversity of fungal communities at specific study sites, the Shannon Diversity Index (H) was used and calculated from the following equation: $H = -\sum P_i(\ln P_i)$, where P_i stands for the proportion of each community in the sample (Spellerberg and Fedor 2003).

Results

Mycological research was conducted on cryoconite collected from cryoconite holes in Hansbreen across seven locations, varying in both geographical position and altitude above sea level (Table 1). Employing a culture-dependent approach utilizing two different culture media (PDA and YPG) and two incubation temperatures (7°C and 24 ± 0.5°C), the fungi isolated from cryoconite holes were categorized into 20 distinct main fungal groups, distinguished phenotypically by factors such as macro- and micromorphology. Following this, representatives from each fungal group underwent rDNA ITS sequencing, assigned identifiers ranging from UWR_242 to UWR_261, culminating in the categorization of fungi into 20 discrete cultures (Table 2). The PCR products' lengths ranged from 357 to 516 base pairs, and the sequences were deposited in GenBank under accession numbers M045890 to MZ045909. BLAST analysis revealed E values of zero, query cover percentages of 100%, and identity percentages ranging from 96.16% to 100% The majority of cultures belonged to filamentous fungi within the Ascomycota phylum (19 isolates), with one isolate identified as Basidiomycota-yeast. Regarding growth conditions, both PDA and YPG yielded a greater number of fungal cultures at 24°C (twelve and nine, respectively) compared to 7°C (each four). Additionally, PDA (14 fungi) proved more effective than YPG (12 fungi) in isolating more fungal species in these studies (Table 2).

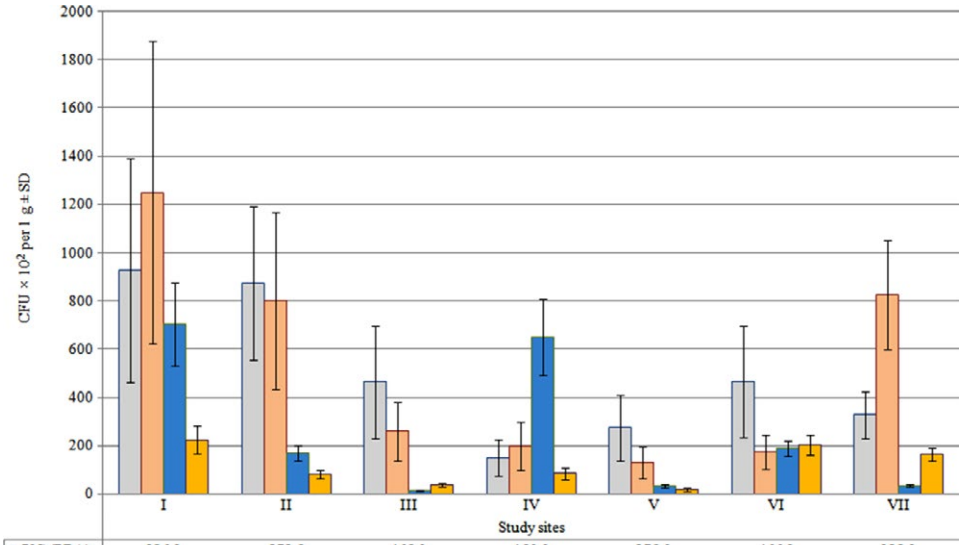
The better incubation temperature for achieving the highest fungal colony-forming units (CFUs) per g of sediment was 7°C. However, such a straightforward correlation was not consistently observed concerning the culture media, as the outcomes varied depending on the incubation temperature utilized. Fungal CFU values ranged from 150 (site no. IV) to 926.3 (site no. I) × 10^2 per g of sediment with PDA, and from 130 (site no. V) to 1250 (site no. I) × 10^2 with YPG. Conversely, CFU values of fungi incubated at 24°C ranged from 15.5 (site no. III) to 702.5 (site no. I) × 10^2 per g of sediment with PDA, and from 19 (site no. V) to 225 (site no. I) × 10^2 with YPG (Fig. 2).

The most frequently isolated fungal species from the cryoconite holes in Hansbreen, across all experimental variants, was *Cladosporium cladosporioides*, constituting 56.16% of all fungi. The presence of two other species, *Patinella hyalo-*

Table 2.

Fungi cultured from cryoconite in Hansbreen (Spitsbergen) on different media and incubation temperature. The BLAST analysis was performed on 27.04.2021 (all E values were zero and all Query Cover values were 100%). PDA – potato dextrose agar, YPG – yeast extract peptone glucose. *Species previously identified in Werenskioldbreen by Borzęcka *et al.* (2022).

Fungi isolated from glacier samples									Identity with sequence from GenBank	
Isolate number	Identified fungi	Phylum	PDA		YPG		GenBank accession No.	Sequence length (bp)	Identity, %	Accession
			7°C	24°C	7°C	24°C				
UWR_242	*Antarctomyces psychrotrophicus*	Ascomycota				+	MZ045890	401	100.00	NR_164292.1
UWR_243	*Aspergillus jensenii*	Ascomycota		+			MZ045891	459	100.00	KY351767.1
UWR_244	*Aspergillus tennesseensis*	Ascomycota		+			MZ045892	426	100.00	MH785494.1
UWR_245	*Aspergillus versicolor*	Ascomycota		+		+	MZ045893	462	100.00	MN782425.1
UWR_246	*Cladochasiella divergens*	Ascomycota		+	+		MZ045894	409	99.27	NR_153874.1
UWR_247	*Cladosporium cladosporioides**	Ascomycota	+	+	+		MZ045895	461	100.00	MT598826.1
UWR_248	*Cladosporium halotolerans*	Ascomycota				+	MZ045896	450	100.00	MN982327.1
UWR_249	*Cladosporium sphaerospermum*	Ascomycota				+	MZ045897	479	96.16	MN704699.1
UWR_250	*Goffeauzyma gilvescens*	Basidiomycota		+			MZ045898	516	100.00	LC203691.1
UWR_251	Helotiales sp.	Ascomycota		+		+	MZ045899	393	99.49	JX001632.1
UWR_252	*Lecythophora* sp.	Ascomycota				+	MZ045900	357	99.72	KJ735003.1
UWR_253	Orbiliaceae sp.*	Ascomycota		+		+	MZ045901	486	100.00	LN901113.1
UWR_254	*Patinella hyalophaea**	Ascomycota	+				MZ045902	396	100.00	MN833368.1
UWR_255	*Penicillium bialowiezense*	Ascomycota		+			MZ045903	466	98.88	NR_165994.1
UWR_256	*Penicillium chrysogenum*	Ascomycota			+		MZ045904	473	100.00	MT341466.1
UWR_257	*Penicillium concentricum*	Ascomycota	+				MZ045905	423	100.00	KC009066.1
UWR_258	*Penicillium echinulatum*	Ascomycota	+	+			MZ045906	467	100.00	MK256746.1
UWR_259	*Penicillium glandicola*	Ascomycota		+	+	+	MZ045907	484	100.00	MH860946.1
UWR_260	*Peziza varia*	Ascomycota		+			MZ045908	469	100.00	MT520565.1
UWR_261	*Trichoderma paraviridescens*	Ascomycota				+	MZ045909	427	100.00	MT187973.1



	I	II	III	IV	V	VI	VII
7°C (PDA)	926.3	872.5	465.0	150.0	275.0	466.3	328.3
7°C (YPG)	1250.0	800.0	260.0	200.0	130.0	175.0	825.0
24°C (PDA)	702.5	170.0	15.5	650.0	35.0	190.1	34.2
24°C (YPG)	225.0	82.8	37.5	85.0	19.0	203.0	165.0

Fig. 2. The number (colony–forming unit (CFU) × 10^2 per 1 g ± SD, standard deviation represented by 'whiskers') of fungi cultured from the cryoconite holes in Hansbreen (Spitsbergen) and incubated at 7°C or 24°C on PDA (potato dextrose agar) and YPG (yeast extract peptone glucose) media: I–VII — study sites.

phaea and *Penicillium echinulatum* Raper and Thom ex Fassat. 1977, was noticeable, accounting for 11.57% and 12.07% of all fungi, respectively. In turn, the proportion of other species ranged from 0.02% to 2.63% of all fungi (Fig. 3).

Overall, seven different fungal species were cultured from the cryoconite holes in Hansbreen under cultivation conditions typical for psychrophilic and psychrotolerant fungi (7°C) using PDA and YPG media: *C. cladosporioides* obtained on both PDA and YPG, *Cladochasiella divergens* Marvanová 1997 obtained on YPG, *Penicillium concentricum* Samson, Stolk and Hadlok 1976 obtained on PDA, *P. hyalophaea* obtained on PDA, *Penicillium chrysogenum* Thom 1910 obtained on YPG, *P. echinulatum* obtained on PDA, and *Penicillium glandicola* (Oudem.) Seifert & Samson 1986 obtained on YPG. Among all the species mentioned in the study, *C. cladosporioides* was also the most frequently cultured at 7°C on both PDA and YPG, with the exception of study site no. I on PDA where *P. hyalophaea* was isolated as the sole species. In turn, *C. cladosporioides* was the only species obtained in six research cases, such as study site no. I on YPG, study site no. III on PDA, study site no. IV on PDA and YPG, study site no. V on PDA and YPG (Table 3). The highest number of species (three species) and the highest Shannon Diversity Index value (0.385) were obtained in study site no. VII on YPG. In the remaining study sites, one to two species were obtained, and the Shannon Diversity Index values ranged from 0 to 0.299 (Table 3).

Table 3.

Fungi cultured from cryoconite holes (colony-forming unit (CFU) × 10^2 per 1 g) in Hansbreen (Spitsbergen) at 7 and 24°C on different media: [1]I–VII—study sites; [2]"—" means not detected. PDA – potato dextrose agar, YPG – yeast extract peptone glucose.

Fungi		Study sites													
	°C	I		II		III		IV		V		VI		VII	
		PDA	YPG	PDA	YPG	PDA	YPG	PDA	YPG	PDA	YPG	PDA	YPG	PDA	YPG
A. psychrotrophicus	24	—[2]	—	—	—	—	5.0	—	—	—	1.0	—	—	—	50.0
A. jensenii	24	—	—	100.0	—	—	—	—	—	—	—	—	—	—	—
A. tennesseensis	24	—	—	—	—	1.5	—	—	—	—	—	—	—	0.6	—
A. versicolor	24	—	180.0	—	—	—	25.0	—	—	—	0.5	—	50.0	0.7	—
C. divergens	7	—	—	—	—	—	—	—	—	—	—	—	—	—	100.0
	24	102.5	—	5.0	—	1.0	—	—	—	—	—	10.0	—	—	—
C. cladosporioides	7	—	1250.0	672.5	750.0	465.0	250.0	150.0	200.0	275.0	130.0	460.8	150.0	178.3	525.0
	24	—	—	—	—	4.5	—	—	—	—	—	7.5	—	0.5	—
C. halotolerans	24	—	—	—	—	—	—	—	—	—	—	—	—	—	10.0
C. sphaerospermum	24	—	—	—	—	—	—	—	5.0	—	—	—	—	—	—
G. gilvescens	24	—	—	—	—	—	—	—	—	—	—	15.0	—	—	—
Helotiales sp.	24	—	—	—	50.0	—	—	100.0	—	15.0	—	—	25.0	12.5	25.0
Lecythophora sp.	24	—	45.0	—	20.3	—	—	—	70.0	—	17.5	—	3.0	—	75.0
Orbiliaceae sp.	24	—	—	10.0	—	—	7.5	—	—	—	—	—	125.0	—	—
P. hyalophaea	7	926.3	—	200.0	—	—	—	—	—	—	—	—	—	—	—
P. bialowiezense	24	—	—	55.0	—	—	—	—	—	17.5	—	59.8	—	1.0	—
P. chrysogenum	7	—	—	—	—	—	10.0	—	—	—	—		25.0	—	200.0
P. concentricum	7	—	—	—	—	—	—	—	—	—	—	—	—	150.0	—

Fungi		Study sites													
	°C	I		II		III		IV		V		VI		VII	
		PDA	YPG	PDA	YPG	PDA	YPG	PDA	YPG	PDA	YPG	PDA	YPG	PDA	YPG
P. echinulatum	7	—	—	—	—	—	—	—	—	—	—	5.5	—	—	—
	24	600.0	—	—	—	8.5	—	550.0	—	—	—	2.8	—	9.0	—
P. glandicola	7	—	—	—	50.0	—	—	—	—	—	—	—	—	—	—
	24	—	—	—	12.5	—	—		10.0	—	—	95.0	—	—	—
P. varia	24	—	—	—	—	—	—	—	—	2.5	—	—	—	10.0	—
T. paraviridescens	24	—	—	—	—	—	—	—	—		—	—	—	—	5.0
Total species	7	1	1	2	2	1	2	1	1	1	1	2	2	2	3
	24	2	2	4	3	4	3	2	3	3	3	6	4	7	5
Shannon Diversity Index	7	0.000	0.000	0.234	0.102	0.000	0.071	0.000	0.000	0.000	0.000	0.028	0.178	0.299	0.385
	24	0.180	0.217	0.412	0.406	0.474	0.375	0.186	0.251	0.390	0.142	0.545	0.419	0.602	0.557

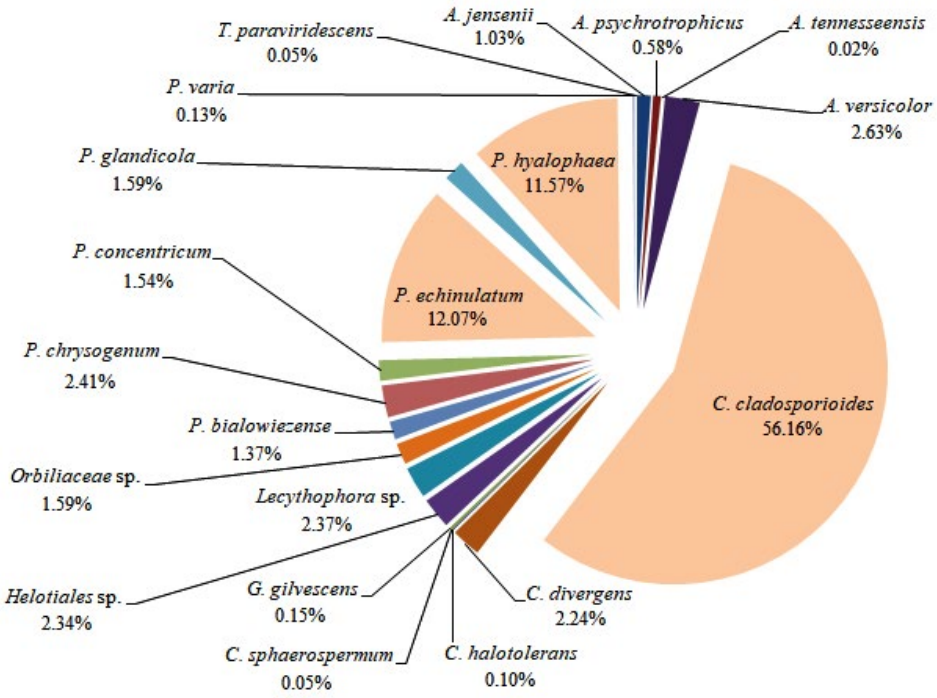


Fig. 3. Percentage of each instance of the fungus occurring (individual colony–forming units (CFUs)) in the study contributing to the total fungal number (total colony–forming unit (CFU)) cultured from the cryoconite holes in Hansbreen (Spitsbergen) for all incubation temperatures and for all culture media.

In the case of incubation at 24°C, 17 distinct fungal cultures were isolated, comprising 8 on PDA, 5 on YPG, and 4 on both media. For this incubation temperature, clear dominance tendencies towards species in individual study locations were not observed as for 7°C. Specifically, *P. echinulatum* dominated on PDA in study sites no. I, III and IV, *A. versicolor* on YPG in study sites no. I and III, *A. jensenii* on PDA in study site no. I, Helotiales sp. on YPG in study site no. II and on PDA in study site no. VII, *Lecythophora* sp. on YPG in study sites no. IV, V and VII, Orbiliaceae sp. on YPG in study site no. VI, *Penicillium bialowiezense* K. Zaleski 1927 on PDA in study site no. V and *P. glandicola* on PDA in study site no. VI. Moreover, the study sites differed from each other in terms of the diversity of the fungal species isolated at 24°C, as illustrated by the Shannon Diversity Index values. Overall, more species of fungi were isolated at this temperature than at the incubation temperature of 7°C, which also resulted in higher values of the biodiversity index. The greatest number of species (7 species) and the highest Shannon Diversity Index value were obtained in study site no. VII on PDA. In the remaining study sites, one to six species were obtained, and the Shannon Diversity Index values ranged from 0.142 to 0.557 (Table 3).

Discussion

Despite that the Arctic is a challenging environment for survival, there is an ongoing discovery of a diverse array of microorganisms, encompassing both prokaryotic and eukaryotic forms, flourishing in freshwater and terrestrial environments (Maccario *et al*. 2015). Advancements in our understanding of microbial diversity in these distinct ecosystems are crucial. They enhance our knowledge regarding the structure and dynamics of microbial populations, as well as their functions within the Arctic ecological framework (Garcia-Lopez *et al*. 2021; Winkel *et al*. 2022). By the end of the century significant shifts in Arctic vegetation patterns and soil carbon reservoirs are anticipated (Ludley and Robinson 2008). These shifts, predominantly towards shrubbier vegetation and altered soil organic matter composition, underscore the necessity of comprehending how decomposer fungi adapt and influence these evolving ecosystems. Their role in carbon transformation and nutrient cycling becomes increasingly critical as these environmental changes progress.

In this study, we detected 20 different fungi, while on a nearby glacier, characterized by similar conditions 23 different fungi were detected using the same test conditions as ours (media and incubation temperatures; Borzęcka *et al.* 2022). However, CFU values of fungi inhabiting cryoconite holes in Werenskioldbreen were from one to two orders of magnitude lower than the values in the present work (Borzęcka *et al*. 2022). This consideration highlights the complexity of factors influencing fungal diversity in cryoconite ecosystems and the necessity of using a combination of culturing and molecular approaches to accurately assess biodiversity in these unique polar habitats. Here, amplicon sequencing of the ITS region was pivotal in elucidating the fungal diversity within cryoconite ecosystems, enabling precise identification. This molecular approach complemented our culture-based findings, revealing the presence of certain species not detected through traditional methods and allowing for a more comprehensive assessment of fungal communities (Raja *et al.* 2017; Lücking *et al.* 2020).

Interestingly, the heterogeneity observed in the fungal species distribution across cryoconite samples underscores the complex ecological interactions within these microhabitats. This variability suggests that each cryoconite hole may act as a distinct ecological niche, potentially influenced by factors such as its physical structure, and the input from surrounding flora and fauna. Recognizing the diversity and heterogeneity of these micro-ecosystems is crucial for understanding the broader ecological dynamics of glacier surfaces and the potential impact of environmental changes on these unique communities.

We also confirm the reports of Borzęcka *et al*. (2022) that the better medium in terms of obtaining the highest number of fungal CFUs per 1 g of sediment from cryoconite holes is PDA at both incubation temperatures. Moreover, when it comes to the conditions of temperature increase, we also confirm the reports of Borzęcka *et al*. (2022) that the incubation temperature at 24°C is more effective

to obtain a larger number of species than 7°C, but the optimal incubation temperature for achieving the highest fungal CFUs per g of sediment was 7°C rather than 24°C. This observation could be linked to findings by Pittino *et al*. (2023), which suggest that while organisms may survive in cryoconite at low temperatures, the actively functioning bacterial community diverges from what DNA sequencing might indicate as potential inhabitants (Pittino *et al*. 2023). Consequently, our research also shows an increase in the number of fungal propagulas (CFU) but a decrease in their biodiversity (number of species and Shannon diversity index) towards the upper reaches of the glacier. A similar relationship regarding the number of fungal propagulas was also noted by Borzęcka *et al*. (2022) who studied cryoconite holes on Werenskioldbreen.

Borzęcka *et al*. (2022) noted that the most frequently isolated species inhabiting cryoconite holes in Werenskioldbreen on Spitsbergen was *P. album*. In our study the most cultured fungal species in the study was *C. cladosporioides.* This species is similar to others belonging to *Cladosporium* genus exhibiting a wide distribution across various environmental habitats, spanning from soil and plants to indoor and outdoor air and water sources. They thrive in damp or humid conditions and demonstrate adaptability to both natural and human-altered environments (De Hoog *et al*. 2000; Domsch *et al*. 2007; Ogórek *et al*. 2012; Pashley *et al*. 2012; Summerbell 2019). *Cladosporium cladosporioides* is xerophilic and psychrophilic species, capable of growth at very low temperatures, previously identified in fruit-filled pastries, chicken pies, and other frozen food products (Frisvad 2008). Interestingly, this species was also isolated in the previous studies in the cryoconite holes of Werenskioldbreen (Borzęcka *et al*. 2022). Literature data also indicate isolation *C. cladosporioides* from environments such as bottom sediments of the Barents and Kara seas (Bubnova and Nikitin 2017) or open surfaces in residential and working areas of the Arctic and Antarctic Research Institute station on the Bolshevik Island of the Northern Land archipelago (Vlasov *et al*. 2019). Moreover, *Cladosporium* sp. was also present in air samples from the 'Heroic Era' historic huts on Ross Island, Antarctica (Duncan *et al*. 2010). Our present study revealed the presence of *C. cladosporioides* within the cryoconite holes of Hansbreen on Spitsbergen (Svalbard Archipelago, Arctic) during the Arctic summer.

The additional strains which were isolated during the investigation that belong to the *Cladosporium* sp. include *C. divergens*, *C. halotolerans* Zalar, de Hoog & Gunde-Cim. 2007, and *C. sphaerospermum* Penz. 1882. There are evidence indicating their prevalence (especially *C. sphaerospermum*) in extremely frigid environments, such as highland soils, as well as Arctic and Antarctic territories (Robinson 2001; Hassan *et al*. 2016), but the species is globally distributed, without any discernible preference for a specific habitat (Zalar *et al*. 2007).

Understanding the role of fungi as decomposers in Arctic ecosystems is crucial for a comprehensive anticipating of biogeochemical cycles and ecosystem dynamics in the face of climate change. Decomposer fungi, through their ability

to degrade organic matter, play a fundamental role in the recycling of nutrients, thereby affecting the structure and functioning of ecosystems. In Arctic ecosystems, where temperature and water availability limit the rate of organic matter decomposition, decomposer fungi are key agents in accelerating these processes, contributing to the maintenance of ecological balance and ecosystem health (Condron *et al*. 2010; Lindahl and Clemmensen 2016). Here, the relative abundance of *Penicillium* spp., particularly *P. echinulatum*, suggests a significant role in organic matter decomposition and nutrient cycling within these unique habitats.

A multitude of *Penicillium* species, characterized by their psychrotolerance and robustness, are distributed extensively across various environments, including food, soil, and the atmosphere. In polar regions, members of the Aspergillaceae family, particularly *Penicillium* spp. and *Aspergillus* spp., are frequently isolated (Sonjak *et al*. 2006; Cong *et al*. 2017). It is therefore not surprising that in our studies of cryoconite we isolated 5 strains of *Penicillium* spp., *i.e.*, *P. bialowiezense*, *P. chrysogenum*, *P. concentricum*, *P. echinulatum*, *P. glandicola* among which the most numerous was *P. echinulatum*. *Penicillium echinulatum*, similar to other isolated species, is regarded as safe and is not known to be a common cause of infections or health problems in humans or animals. These species are commonly found in soil, decaying organic matter, and various indoor environments, as well as diverse outdoor habitats such as agricultural soils and plant debris. This suggests that the presence of *Penicillium* spp., adapted to thrive in cold environments, may play a pivotal role in the ecological processes of cryoconite ecosystems, potentially influencing both the microbial diversity and the biogeochemical cycles. Additionally, in studies of samples from the Antarctic Peninsula, it has been described as an endophytic fungus associated with algae (seaweeds) (Sonjak *et al*. 2006; Teixeira *et al*. 2019). Interestingly *P. echinulatum* is of interest in biotechnology due to its ability to produce various secondary metabolites, including enzymes and bioactive compounds. It has been studied for its potential applications in industries such as food and pharmaceuticals. One notable characteristic of *P. echinulatum* is its ability to produce cellulase enzymes, which enable it to degrade cellulose, a complex carbohydrate found in plant cell walls. This makes it potentially valuable for biomass conversion and biofuel production (dos Santos Costa *et al*. 2016; Schneider *et al*. 2016).

In our previous work, we have reported the presence of *Aspergillus fumigatus* Fresen. 1863 in the droppings of *R. tarandus platyrhynchus* (Ogórek *et al*. 2022) and identified *A. pseudoglaucus* and *Aspergillus sydowii* (Bainier & Sartory) Thom and Church 1926 in the cryoconite holes of the Werenskioldbreen (Borzęcka *et al*. 2022). In our current research, we have discovered *A. jensenii*, *A. tennesseensis*, and *A. versicolor* (Vuill.) Tirab. 1908 in the cryoconite holes of Hansbreen. To the best of our knowledge, *A. jensenii* and *A. tennesseensis* have not been previously reported in polar regions. On the other hand, *A. versicolor* is known for its widespread distribution in terrestrial ecosystems, ranging from polar to southern latitudes (Fomicheva *et al*. 2006).

In the earlier study of the Werenskioldbreen, *P. hyalophaea* ranked as the second most prevalent species, particularly thriving in fungi isolated on YPG incubated at 7°C (Borzęcka *et al*. 2022). In our current research, this species has been identified as the third most common, showing a notable shift in its growth medium preference to PDA. Consistent with previous findings, *P. hyalophaea* was again exclusively isolated at 7°C. Since the last report, there have been no additional reports of this species' occurrence. Recent isolations of *P. hyalophaea* include wood samples from Deception Island (Antarctica) (Held and Blanchette 2017) and Western Greenland (Pedersen *et al*. 2020). However, this fungus has been identified in lacustrine sediment cores from a lake on King George Island (Antarctica) (Ogaki *et al*. 2020), suggesting a broader ecological presence.

In the earlier exploration of the mycobiota associated with the droppings of the Svalbard reindeer (*Rangifer tarandus platyrhynchus*) on Spitsbergen, we had documented the occurrence of *Goffeauzyma gilvescens* (Chernov and Babeva) Xin Zhan Liu, F.Y. Bai, M. Groenew and Boekhout 2015 (previously known as *Cryptococcus gilvescens*) in polar regions, as reported by Ogórek *et al*. (2022). Notably, at that time, the species was isolated exclusively at 5°C. In contrast, our current findings show its presence solely in cultures incubated at 24°C. This suggests potential physiological intra-species variations in *G. gilvescens*. Despite this, like most *Cryptococcus* species, *G. gilvescens* is known for its polysaccharide capsule production and is commonly noted in Arctic environments (Carrasco *et al*. 2012; Białkowska *et al*. 2017).

Among the fungi we isolated, three could not be precisely identified at the species level and were classified as *Lecythophora* sp., Helotiales sp., and Orbiliaceae sp. Notably, the latter two represent the only macromycetes identified in this study. This aligns with the previous findings in cryoconite holes, where four macromycete species were reported: *Bjerkandera adusta* (Willd.) P. Karst. 1879, *Holwaya mucida* (Schulzer) Korf and Abawi 1971, *Trametes versicolor* (L.) Lloyd 1920, and the aforementioned Orbiliaceae sp. (Borzęcka *et al*. 2022). The Orbiliaceae family encompasses a variety of fungi, including genera such as *Arthrobotrys*, *Dactylella*, *Dactylellina*, *Monacrosporium*, and *Orbilia* (Chmiel 2006; Mułenko *et al*. 2008). Based on our current and the previous research on cryoconite holes, we propose the hypothesis that this environment may harbor unique, yet-to-be-discovered Orbiliaceae species.

Regarding the Helotiales order, research has shown that the roots of Arctic tundra plants like *Cassiope tetragona* (L.) D. Don 1834, *Empetrum nigrum* L. 1753, and *Vaccinium vitis-idaea* L. 1753 host diverse fungal communities predominantly composed of Helotiales (Walker *et al*. 2011). Interestingly, Edwards *et al.* (2013) reported that most of the fungi cultured from cryoconite sediments from three valley glaciers at Kongsfjorden, (Svalbard) belong to the order Helotiales or Pleosporales. Meanwhile, members of the *Lecythophora* (*Coniochaeta*) genus are occasionally isolated from polar regions (Blanchette *et al*. 2016). In some instances, *Lecythophora* spp. have been known to exhibit pathogenic characteristics

towards humans and plants (Damm *et al*. 2010; Irfani *et al*. 2022).

Also, we have isolated fungi such as *C. divergens*, *A. psychrotrophicus* Stchigel and Guarro 2001, *P. varia*, and *T. paraviridescens*. Notably, none of those were cultivated at 4°C; their growth was observed only at 25°C. Both *C. divergens* and *A. psychrotrophicus* have been previously reported in polar environments. Specifically, *C. divergens* was found in ice algae and has been identified as a contamination during the isolation of aquatic hyphomycetes related to Leotiomycetes (Baschien *et al*. 2013; Perini *et al*. 2019). Meanwhile, *A. psychrotrophicus*, discovered in Antarctica, is known for producing antifreeze or ice-binding proteins (Stchigel *et al*. 2001; Xiao *et al*. 2010; Arai *et al*. 2019). These proteins, found in various cold-adapted organisms, are structurally diverse polypeptides with thermal hysteresis activity.

To the best of our knowledge, *P. varia* and *T. paraviridescens* have not been previously identified in polar regions. The classification within the core group of *Peziza*, particularly *P. varia*, is a subject of significant debate. This group, exemplified by *P. vesiculosa* Bull. 1790, is morphologically distinct, but species delineation, especially in the '*P. varia* complex'—encompassing 27 specimens known as *P. cerea* Fr. 1822, *P. micropus* Pers. 1800, and *P. repanda* Pers. 1808 from various substrates and locations—is contentious (Hansen *et al*. 2002). In contrast, *T. paraviridescens* is a widespread species found in cellulose-rich environments, such as freshwater ecosystems in Korea or in decaying wood in Central European mountain forests (Błaszczyk *et al*. 2016; Goh *et al*. 2018).

Conclusions

Our research enhances the understanding of the diversity of culturable fungi within the cryoconite holes of Hansbreen, located on Spitsbergen in the Svalbard Archipelago of the Arctic. Our study led to the isolation of 20 distinct fungi, comprising 17 identified species and three not yet classified at the species level. Among the most frequently isolated species was *Cladosporium cladosporioides*. Moreover, to the best of our knowledge, we are the first to report the occurrence of *Aspergillus jensenii*, *A. tennesseensis*, *Peziza varia*, and *Trichoderma paraviridescens* in cryoconite, highlighting the need for continued study of fungal diversity in Arctic environments. *Cladosporium* species, including xerophilic and psychrophilic *C. cladosporioides*, exhibit wide distribution highlighting the need for monitoring and understanding these fungi in shifting environmental dynamics. Variations in species prevalence and growth conditions, such as the shift in growth medium preference for *Patinella hyalophaea*, suggest different ecophysiological requirements of the same species represented by different strains from different sites. Identification of possibly new fungal species by assignment of *Lecythophora* sp., Helotiales sp., and Orbiliaceae species to the species level highlights the potential for discovering unique fungi in cryoconite holes. Addi-

tionally, our research suggests that polar regions may harbor novel fungal strains beneficial for biotechnological uses, notably *Penicillium* species, known for their psychrotolerance and widespread distribution, hold potential for biotechnological applications. Our observations also indicate an increase of the number of fungal propagulas (CFU) but a decrease in their biodiversity (number of species and Shannon Diversity Index) towards the upper parts of the glacier. Considering Svalbard and its cryoconite holes, employing the suggested culture-dependent and independent methods, future research could ascertain whether the species previously identified remain unchanged or if new fungal species have been introduced to the region. Moreover, there is a prevailing consensus that ongoing research is essential to enable comprehensive comparisons across contemporary, past, and future findings.

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