Evaluating Best Practices for Isolating Pyrophilous Bacteria and Fungi from Burned Soil

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Abstract

A live microbial culture is invaluable to assess traits and functions, yet culturing immediately from fresh soil is logistically challenging and media selection is not trivial. Isolating pyrophilous bacteria and fungi after wildfires from burned soil, we tested the best: 1) soil storage method to retain culturable viability; 2) media for highest microbial diversity. We tested four soil storage methods (dried, stored at 4ºC, stored at -80ºC alone or in glycerol) and compared to fresh soil obtained 6 months after a severe California shrubland wildfire. We also tested 3 media types: rich (Lysogeny Broth (LB) for bacteria; Malt Yeast Agar (MYA) for fungi), oligotrophic (Reasoner's 2 Agar (R2A)) and media made from pyrogenic organic matter (PyOM). For bacteria, storing soil frozen at -80ºC alone yielded the highest diversity of genera and with glycerol preserved the most species. For fungi, storing soil at -80ºC preserved the most species but preserved equivalent diversity of genera as fresh and refrigerated soil. R2A yielded the highest bacterial and fungal diversity but some species of interest were only captured with

PyOM. Using a combination of methods from 2018-2022, we cultured >500 isolates (286 bacteria; 258 fungi) after 7 California wildfires for testing pyrophilous microbial traits.

Introduction

Although advances in the fields of molecular biology and DNA sequencing have freed microbiologists from the need to work with culturable species exclusively (Franzosa et al. 2015), few laboratory resources are as valuable as live cultures. Pure cultures are essential components for high quality whole-genome assembly (Hugenholtz 2002; Haridas et al. 2011), genetic engineering experiments (Malik and Claus 1987), and the phenotypic profiling of microorganisms (Kämpfer 2014; Alster et al. 2021; Lustenhouwer et al. 2020; Day et al. 2019; Maynard et al. 2019; Camenzind et al. 2024). Additionally, microbial isolates greatly improve the interpretation of metabolomics data (Swenson et al. 2018), and possession of a diverse culture collection can unlock a plethora of applied microbiological research directions (Malik and Claus 1987; Reyes-César et al. 2014; Carini 2019). Once isolated and properly stocked, culture collections become a permanent resource for future microbiological research and create a veritable breeding ground for collaborative research (Caktu and Turkoglu 2011; Stackebrandt et al. 2014; Arnold and Lutzoni 2007). However, there are many hurdles to creating microbial culture collections and each collection has unique challenges (Caktu and Turkoglu 2011).

The various factors that can be modified when attempting to culture microorganisms are as varied as there are microbes to isolate. For example, media selection may require grappling with an endless number of variables including consideration of the proper carbon sources, nitrogen and phosphorus content, salt concentrations, pH, and a host of other micronutrient

considerations (Carini 2019; Davis, Joseph, and Janssen 2005; Stevenson et al. 2004). One must also consider incubation temperature and duration, water and oxygen availability, and whether to alternate light and dark cycles or to make the media nutrient rich or poor (oligotrophic) (Davis, Joseph, and Janssen 2005; Stevenson et al. 2004; Leadbetter 2003). This complexity contributes to many microorganisms remaining uncultured (Prakash et al. 2013). Though recent advances in our understanding of microbiology have begun to make the adage of "99% of microbes are unculturable" outdated (Carini 2019; Stevenson et al. 2004; Leadbetter 2003; Martiny 2019), culturing the majority of microorganisms still remains beyond our reach.

One group of understudied microorganisms that is of growing interest are the pyrophilous (or "fire-loving") microbes. Pyrophilous microbes are the first to increase in abundance following wildfire, and they can persist in the environment for many years post-fire (Seaver and Clark 1910; Pérez-Valera et al. 2018; Pressler, Moore, and Cotrufo 2019). While there are many studies identifying which microbes become more abundant post-fire, there are very few studies characterizing how pyrophilous microbes interact with their environment (Fox et al. 2022). Wildfires dramatically change landscapes and create legacy effects that can impact soil microorganisms and their overall ecosystems for many years following fire (Wan, Hui, and Luo 2001; Pierson et al. 2019; Michelotti and Miesel 2015). With rising global frequency and severity of wildfires across many ecosystems (de Groot, Flannigan, and Cantin 2013; Hantson, Pueyo, and Chuvieco 2015), understanding how pyrophilous microbes modify and interact with their environments is of critical importance. An important step to characterizing the ecology of pyrophilous microbes is laboratory testing of cultured isolates to provide data on how individual pyrophilous taxa compete for resources, cycle post-fire nutrients, and interact with the post-fire

environment. However, there are numerous technological and logistical challenges to creating a culture collection of pyrophilous microbes.

Beyond the well-known hurdles of creating a microbial culture collection, attempting to culture microbes associated with wildfires presents its own set of challenges. First, there are logistical challenges associated with working with post-fire environmental samples. Wildfires are unpredictable natural phenomena. As such, it is often not possible to have the infrastructure for large-scale culturing efforts (personnel available, media selected and created, broth tubes or petri dishes ordered, culturing conditions decided and established) in place when a wildfire occurs. As part of this logistical navigation, post-fire soil samples may have to be stored for varying lengths of time before they can be used. This is a logistical problem shared among many environmental microbial culturing efforts ranging from marine systems (Joint, Mühling, and Querellou 2010) to Antarctic soils (Malosso et al. 2006). While many insights have been gained regarding these logistical hurdles such as culturing from samples as quickly as possible (Caktu and Turkoglu 2011; Černohlávková et al. 2009), and methods for freezing samples that improve cultural viability, their efficacy is not always consistent and the viability of culturable microorganisms may be reduced depending on the soil storage method (Černohlávková et al. 2009; Rubin et al. 2013).

In addition to the logistics, wildfires radically alter environmental nutrient and resource landscapes (Caon et al. 2014; Pierson et al. 2019). Wildfire pyrolysis turns labile, easily accessible carbon sources into difficult to degrade pyrogenic organic matter (PyOM) (Michelotti and Miesel 2015; Preston and Schmidt 2006). This PyOM is often enriched in polycyclic aromatic hydrocarbons (PAHs) that may require specialized metabolic pathways to process (Reyes-César et al. 2014; Woolet and Whitman 2020; Aranda et al. 2017; Fischer et al. 2021;

Pulido-Barriga et al. 2025). This can lead to accumulated char persisting in the environment for up to and exceeding 100 years (Knicker 2011). Additionally, wildfires can deposit an enormous amount of inorganic nitrogen into the environment when organic nitrogen previously locked in plant biomass becomes released during burning (Caon et al. 2014; González-Pérez et al. 2004; Wan, Hui, and Luo 2001). This high-nitrogen environment can radically alter the nutrient strategies that microorganisms may employ post-fire and limit the microbes capable of surviving. For example, many species of fungi are known to be nitrophobic (Lilleskov et al. 2019). These radical nutrient shifts may result in commonly used laboratory media not providing appropriate resources to cultivate post-fire microbes.

Here, our goal was to create a large culture collection of pyrophilous microbes to use to test the traits that govern pyrophilous microbial adaptation to fire and the post-fire environment via biophysical assays and genomics. Keeping in mind the above described logistic and resource and nutrient-related challenges, we used soil from a local high severity California chaparral wildfire to test the best 1) methods to store post-fire soil to retain pyrophilous microbial culturable viability and 2) laboratory media to capture the highest diversity of pyrophilous microbes. For soil storage, we tested culturing immediately from fresh soils versus soils stored for 3 months in four ways (air-dried, refrigerated at 4°C, and frozen at -80°C with and without glycerol). For media type, we tested rich (lysogeny broth (LB) agar for bacteria, malt-yeast agar (MYA) for fungi), oligotrophic (Reasoner's 2 Agar (R2A)), or a media created in-house from PyOM. We tested the impact of our treatments on abundance (CFUs: Colony Forming Units) and microbial richness and composition (assessed via Sanger sequencing of 16S for bacteria or ITS for fungi; Figure 1). We hypothesized (H1) that air-dried soil would retain the greatest diversity of pyrophilous bacteria and fungi compared to fresh soil since fires reduce soil moisture, so we

expected air drying soils would be the least dramatic disturbance for soil borne microorganisms. We further hypothesized (H2) that a combination of the rich and PyOM media would yield the greatest diversity of pyrophilous microbes as the rich media could grow a wide range of bacteria and fungi and the PyOM media would capture those pyrophilous species with unique metabolic requirements specific to post-fire burn scars. Although we specifically sought to create a pyrophilous culture collection, the insights gleaned from this study not only showcase potential strategies for culturing fastidious pyrophilous microorganisms but can also aid environmental microbiologists in decision making about storage and media selection for many types of environmental culturing. The culmination of this research was the creation of a large and diverse collection of pyrophilous microorganisms of >540 isolates (286 bacteria; 258 fungi) from burned soils, mushrooms, and smoke derived from 7 California wildfires.

Methods

Site Description: To test the best soil storage method and media to cultivate pyrophilous microbes, we sampled from The El Dorado wildfire, which burned ~92km^2 Manzanita (*Arctostaphylos)* dominated chaparral shrublands, in Yucaipa, CA from September through November of 2020. We examined high-resolution successional data of bacterial and fungal communities after a similar nearby high-severity Manzanita dominated chaparral wildfire (Pulido-Chavez et al. 2023), and compared to other post-fire studies that had successional data (Bárcenas-Moreno et al. 2011; Caiafa et al. 2023), to identify 6 months post-fire as the ideal time to attempt to isolate a wide range of pyrophilous bacteria and fungi. We thus collected samples from high severity burned soils in June 2021, 6 months post-fire. Burned sites were characterized

as having 50 to 75 percent slopes with dry, sandy soils in the Springdale family-Lithic Xerorthents association (https://websoilsurvey.nrcs.usda.gov/app/).

Soil Collection and Storage: Approximately 250mL of soil from 0-10 cm below the ash layer of burned soil was collected from 9 plots with ethanol cleaned releasable bulb planters and placed into whirl-pak bags 6 months post-fire following methods used in a similar ecosystem (Pulido-Chavez et al. 2023). After collection, soil was 2mm sieved and either used as fresh inoculum for cultivation or stored in four different ways: air dried, refrigerated (4ºC), frozen (-80ºC), or suspended 50:50 %w/v soil with glycerol to create a soil slurry and then frozen (-80ºC) (Figure 1).

Culturing Medias Used: Fresh soil samples were cultured immediately onto rich (LB for bacteria, MYA for fungi), oligotrophic (R2A), or PyOM media. After 3 months of storage, stored soil samples were cultured onto rich media. This means that the cultures isolated from fresh soils on rich media served as both the “rich media” condition for the media experiment and the “fresh” comparison for the soil storage experiment (Figure 1).

We produced LB broth by adding 15g of powdered LB Broth (Fisher Scientific, CAS# 73049-73-7) and MYA broth by adding 5g each of powdered Malt Extract (Research Products International, Genessee, CAS# 8002-48-0) and Yeast Extract (Sigma-Aldrich, Cat # 70161) per liter of DI water. For solid agar media, 15g per liter of bacteriological grade agar (Apex Chemicals, Cat # 20-273) was added to either the LB or MYA broth. R2A agar was produced using powdered pre-mixed R2A agar (Criterion, Cat # 16721) following manufacturer directions.

We developed an in-house recipe of PyOM media using biochar harvested from the El Dorado Fire burn sites (Figure S1). First, we ground biochar into a fine powder with a coffee

grinder (Chefman, Model: RJ44-OPP-RED) for 3-5 minutes at maximum speed. Then, we added 50g of ground biochar to a 2L Erlenmeyer flask, added 1200mL of DI water, covered the mouth of the flask, stirred on a hotplate for 24 hours at 40°C, then removed from heat to settle for 8 hours. We then siphoned liquid contents into a clean Erlenmeyer flask, being careful to not disturb the bottom sediment. We passed the liquid through cheesecloth, and then twice through a coffee filter to remove any remaining sediment before transferring to clean containers for autoclaving. We added 13g of bacteriological grade agar (Apex Chemicals, Cat # 20-273) to each liter of PyOM media, since adding 15g resulted in under-dissolved agar in the final product. We used DI water for all media types (LB, MYA, R2A, PyOM) and all were autoclaved at 121ºC for 45 minutes.

We added antimicrobials to the liquid agar after autoclaving, once it had cooled to 60ºC. For media used to isolate fungi and intended to resist bacterial growth, we added filter sterilized gentamycin and ampicillin (Hardy Diagnostics, Santa Maria, CA) to a final concentration of 10mg/mL each. For media used to isolate bacteria and intended to resist fungal growth, we added filter sterilized cycloheximide (Research Products International, CAS # 66-81-9) to a final concentration of 20mg/L.

<u>Experimental Design</u>: We selected 12 soil samples collected from high-severity burn locations (3 plots with four 1m^2 subplots each). We took 3g of soil from each of the 12 samples and mixed them to create a single homogenous soil sample that was then subsampled for culturing treatments (fresh soil for LB or MYA culturing, R2A, or PyOM, or stored for 3 months air-dried, refrigerated, frozen, or frozen in glycerol). For each treatment, we made 4 separate 50 mL falcon tubes by combining 1g soil with 5mL of sterile DI water. We then created dilution series ranging from 1:10 to 1:10,000 for each treatment and plated 100µL of each dilution onto

10 plates per target organism. We applied ampicillin and gentamycin to all plates targeting fungi and cycloheximide to all plates targeting bacteria. We had a total of 560 experimental plates (2 Kingdoms (bacteria, fungi) x 4 dilutions (1:10, 1:100, 1:1,000, 1:10,000) x 10 replicates x 7 treatments [4 storage treatments (dried, 4ºC, -80ºC, -80ºC + glycerol) + 3 media types for fresh soils (Rich, R2A, PyOM)]. After spreading solution onto plates, we wrapped them in parafilm and kept them in a drawer at room temperature for ~1 week, after which we counted Colony Formining Units (CFUs) and unique morphologies. Plates containing between 30-500 colonies of well-defined growths of bacteria or fungi were considered "countable" plates and the initial CFU concentrations were calculated by multiplying the number of colonies by the dilution factor resulting in 6-9 countable replicate plates per treatment. We then isolated, extracted DNA, Sanger sequenced, and stocked each bacterial and fungal unique morphology (Figure 1). Diversity was assessed based on the percentage (total or pyrophilous) of genera or species captured by each treatment.

Microbial Isolation Method: Fungi and bacteria were morphotyped using size, color, and growth rate. For bacteria or yeasts, we used a sterile 10µL pipet tip to pick a well isolated colony and then used the streak plate method onto either fresh LB plate without antifungals for bacteria or a fresh MYA plate without antibacterials for fungi. For filamentous fungi, we used a flame-sterilized scalpel to cut a 1 cm^2 square of the growing edge of a fungus from the agar plate, then transferred the square growing-side down into the center of a fresh MYA plate without antibacterials. In cases where microbes would not establish onto LB or MYA, they were maintained on the media they originated from (either R2A or PyOM). We incubated plates at room temperature for 1 week before re-isolating following the same procedure. This was repeated until three clean isolations were obtained prior to DNA extraction and stocking.

Microbial Stocking Method*:* Each isolate was stocked for future use after a thrice purified isolate was obtained and DNA was extracted. For bacteria, a colony was picked aseptically from the last isolation streak plate using a sterile 10µL pipet tip and placed into 3mL of LB broth in a sterile 15mL Falcon tube, then grown at shaking incubation at 180rpm for 24-72hrs until broth became turbid. We added 1mL of the turbid culture to a 2mL cryovial and combined with 1mL of sterile 40% glycerol, resulting in a final 20% glycerol suspension. This was vortexed briefly and then frozen at -80ºC. For fungi, a 1cm^2 cube of fungus was allowed to grow until it came within approximately 1cm of the edge of the 100mm Petri dish. Then, using a sterile transfer tube (Spectrum Laboratories Inc., San Clemente CA), 5mm disks were punched from the growing edge of the fungus. We transferred 8-10 disks to a 2mL screw-cap tube containing 1mL of autoclaved tap water (McGinnis, Padhye, and Ajello 1974) then stored at room temperature. We made duplicate stock tubes for each isolate.

DNA Extraction: DNA was extracted from all unique isolates using home-made Extract-N-Amp Extraction Solution made by combining 10 ml of 1M pH 8.0 Tris-HCl, 1.86g KCl, 0.37g EDTA, and 80 mL DI water, then adding 1M NaOH until pH 9.5 and then diluting to a total volume of 100mL and filter sterilizing. For bacteria, one well-isolated colony was picked using a sterile 10µL pipet tip, and for fungi, a 1mm^2 area of the growing edge of the fungus was scraped with a flame-sterilized probe. The tip or probe was then submerged and swirled in 10µL of Extraction solution, which was heated at 65ºC for 10 minutes followed by 95ºC for 10 minutes in a Thermocycler (BioRad). Once finished, 10µL of 3% BSA was added and the samples were left to chill overnight at 4ºC before being PCR amplified or frozen at -20ºC.

PCR and Sanger Sequencing: The bacterial 16S rRNA region was amplified using a PA forward and PH reverse primer (Edwards et al., 1989) and the fungal ITS region was amplified

with the ITS1F forward (Gardes & Bruns, 1993) and ITS4 reverse primer (White et al., 1990). Each 20 µl PCR reaction contained 13.64 µl of molecular grade water, 0.16µL of Taq polymerase (New England Biolabs, Ipswitch MA) and 2µL of 10x Taq Polymerase Buffer containing $MgCl_2$, 2µL of 10x solution of equally mixed dNTPs, 0.6µL of each primer at 10µM, and 1µL of DNA. Thermocycler conditions were as follows: 94°C for 2 minutes, then 35 cycles of 57°C (for fungi) or 62°C (for bacteria) for 1 minute, 72°C for 1 minute, then 94°C for 1 minute, ending with a final extension of 72°C for 8 minutes (Bruns and and Taylor 1990). The DNA was cleaned using 3µL of diluted Exosap mastermix (Applied Biosystems, Waltham MA) per 7µL PCR product before diluting and preparing for Sanger sequencing. Samples were Sanger sequenced in the forward direction using either Genewiz (San Diego, CA), Eurofins Genomics (Louisville, KY), or the UCR Institute for Integrative Genome Biology Genomics Core services.

BLAST Identification*:* Raw sequences as .ab1 chromatogram files were trimmed in either SnapGene or Geneious Prime to remove low quality regions then saved as FASTA files. Taxa were identified using NCBI BLASTn to create hit tables of top 10 hits. IDs were assigned using a combination of query cover, error value, percent identity, and a consensus of hits with the same quality scores. In general, an isolate was identified at species level if the query cover was 100%, max e score was < 0.01, and percent identity was >98%. If percent identity fell to ~95% then isolate identifications were left to genus, at 90% to family, at 85-90% to class, and at < 85% to phylum level. All isolate names were preserved from the best-matching BLAST identification, including any additional modifiers indicating potentially problematic taxonomy.

Statistical Analysis: All statistical analyses and figures were produced in R 4.0.2 (R Core Team 2020) and all scripts are available on GitHub (https://github.com/DylanEnright93/Culturing_Pyrophilous_Microbes). Normality was tested

with a Shapiro test, then a two-way ANOVA was used to test the effect of soil storage or media treatment on bacterial and fungal CFU counts, followed by a post-hoc Tukey HSD test. Heatmap-like visualization of genera and species level diversity were created using the "geom_tile" function in ggplot2 (Wickham et al. 2019). Diversity among treatments was evaluated by calculating the number of species, genera, families, and orders per treatment and comparing the percent of total or total pyrophilous isolates (based on literature support) obtained per treatment. All bacterial 16S and fungal ITS FASTA sequences have been deposited to NCBI GenBank under accession numbers PV662515 - PV662757 and PV620023 – PV620259, respectively.

Results

Microbes Obtained: Throughout our experiments we isolated a remarkable array of bacterial and fungal morphotypes (Figure 2). We cultured a total of 170 bacterial isolates representing 4 phyla, 10 orders, 31 genera and 83 species from burned soils collected 6 months after the 2020 El Dorado Fire across all our storage and media type experimental treatments. We also cultured 160 fungal isolates representing 3 phyla, 9 orders, 15 genera and 35 species (Table S1). Of these isolates, 138/170 bacterial isolates composed of 17 genera and 71 species and 152/160 fungal isolates composed of 8 genera and 30 species had literature support for inclusion as putative or confirmed pyrophilous taxa (Enright et al. 2022; Pulido-Chavez et al. 2023; Woolet and Whitman 2020; Aranda et al. 2017; Caiafa et al. 2023; Seaver and Clark 1910; Petersen 1970; Whitman et al. 2019; Pierson et al. 2019; Bárcenas-Moreno and Bååth 2009; Ferrenberg et al. 2013; Fox et al. 2022) (Table S2). We used the total (Table S1) and pyrophilous isolates (Table S2) to test the effect of media type and soil storage method on CFUs and

microbial diversity. In addition to testing storage methods and media selection, here we present our culture collection of >540 pyrophilous microbes (286 bacteria; 258 fungi) that we isolated from 2018-2022 from burned soils, mushrooms, and smoke of 7 California wildfires for future testing of pyrophilous microbial traits (Table S3). For bacteria, we had 286 isolates from 8 phyla, 16 orders, 35 genera, 76 species. For fungi, we had 258 isolates from 3 phyla, 11 orders, 13 genera, and 34 species. The full table includes the fires of origin, collection times, substrate cultures derived from, isolate names, FASTA sequences, and BLAST summary statistics (Table S3).

Effect of Storage Method Selection on CFUs: Soil storage method significantly impacted the number of CFUs obtained per gram soil for both bacteria (ANOVA: $F_{1,4} = 19.97$, $p < 0.001$; Figure 3A) and fungi (ANOVA: $F_{1,4} = 19.78$, $p < 0.001$; Figure 3B). For both bacteria and fungi, soil stored air-dried (Bacteria: 2.31×10^5 CFUs/gram, Fungi: 1.33×10^4 CFUs/gram) and stored at -80ºC with glycerol (Bacteria: 1.31×10^5 CFUs/gram, Fungi: 6.69×10^3 CFUs/gram) retained the fewest mean CFUs/gram soil. For bacteria, soils stored at 4ºC (6.98×10^5 CFUs/gram) or at -80ºC without glycerol (6.39×10^5 CFUs/gram) were not significantly different from fresh soils (5.73×10^5 CFUs). For fungi, fresh soils had the highest mean CFUs/gram soil (3.07×10^4 CFUs/gram), followed by soil frozen at -80ºC without glycerol (2.66×10^4 CFUs/gram), and then stored at 4ºC (2.16×10^4 CFUs/gram).

Effect of Media Selection on CFUs: Media type significantly impacted the number of CFUs obtained per gram of fresh soil for both bacteria (ANOVA: $F_{1,2} = 39.43$, $p < 0.001$; Figure 3C) and fungi ($F_{1,2} = 7.77$, $p < 0.01$; Figure 3D). For both bacteria and fungi, PyOM media yielded the fewest mean CFUs per gram (Bacteria: 1.66×10^5; Fungi: 1.03×10^4), and rich media (LB for bacteria and MYA for fungi) had the highest average CFUs per gram (Bacteria:

$5.73x10^5$; Fungi: $3.07x10^4$). R2A media had the second highest per gram mean CFU counts for bacteria ($3.20x10^5$) and for fungi, CFU counts ($2.20x10^4$) were statistically equivalent to MYA.

Effect of Storage Method Selection on Bacterial Diversity*:* Soil storage method strongly affected diversity of bacterial genera with fresh soil retaining the highest total (Figure 4A) and frozen soil retaining the highest pyrophilous diversity (Figure 4B). Dried soil retained the lowest diversity of bacterial genera among both total (Figure 4A) and pyrophilous isolates (Figure 4B). Figures are ordered by number of genera first, which covers greater phylogenetic diversity, then by number of species. Across all storage treatments, we cultured a total of 21 bacterial genera (Figure S2) and 64 species (Figure S3) and we found literature support as pyrophilous for 14 genera (Figure 6A) and 51 species (Figure S4). Considering total bacterial taxa, fresh soil was the most diverse (62% of genera, 41% of species), followed by frozen soil (62% of genera, 30% of species), soil frozen with glycerol (57% of genera, 39% of species), refrigerated soil (48% of genera, 22% of species) with dried soil as least diverse (38% of genera, 23% of species). Only 3 genera (*Arthrobacter, Curtobacterium, Streptomyces*) were isolated across all 5 storage treatments whereas 8 genera (*Brevibacterium, Fontibacillus, Glutamicibacter, Massilia, Ornithinimicrobium, Paeniglutamicibacter,* an unidentified Firmicute, and *Pseudoarthobacter*) were isolated from a single treatment (Figure S2). Finally, 64% of species were isolated one time with only one species *Curtobacterium oceanosedimentum* observed in all storage treatments (Figure S3). Among the pyrophilous subset (Figure 4B), frozen soil was the most diverse (85% of genera, 33% of species), followed by fresh soil (62% of genera, 35% of species), soil frozen with glycerol (54% of genera, 39% of species), refrigerated soil (54% of genera, 22% of species) with dried soil as least diverse (38% of genera, 24% of the species).

Effect of Storage Method Selection on Fungal Diversity*:* Soil storage method strongly affected fungal diversity with fresh soil retaining the highest total genera (Figure 4C) and fresh, frozen and refrigerated retaining equal numbers of pyrophilous genera (Figure 4D). Soil frozen with glycerol retained the lowest diversity of fungal genera among total (Figure 4C) and pyrophilous isolates (Figure 4D). Across all storage treatments, we cultured a total of 6 fungal genera (Figure S5) and 23 fungal species (Figure S6), of which we found literature support as pyrophilous for 3 genera (Figure 6B) and 20 species (Figure S7). For total fungi, fresh soil was the most diverse, capturing 100% of all 6 genera (*Aspergillus, Holtermaniella, Ochrocladiosporium*, *Penicillium*, and 2 uncultured Helotiales), followed by frozen and refrigerated soil capturing 50% of the genera, and dried soil capturing 33% of total fungal genera (Figure S5). Soil frozen with glycerol performed the worst, capturing only *Penicillium* (17% of the total; Figure S5). For total fungal species, fresh soil captured 52%, followed by 43% in frozen, then 30% in refrigerated and dried soil, and finally 26% in frozen with glycerol (Figure S6). For pyrophilous fungi, fresh, frozen, and refrigerated soil all captured 100% of the 3 genera (*Aspergillus, Holtermanniella, Penicillium*), whereas dried captured 2 (*Aspergillus, Penicillium*) and frozen with glycerol only captured 1 (*Pencillium*; Figure 6B). For pyrophilous fungal species, frozen soil captured 50%, followed closely by 45% in fresh, 35% in refrigerated and 30% in air-dried (Figure S7).

Effect of Media Selection on Bacterial Diversity*:* Media type strongly affected bacterial diversity with R2A and LB both capturing the highest numbers of total bacterial genera and species (Figure 5A), R2A yielding the highest diversity of pyrophilous genera and species (Figure 5B), and PyOM capturing the fewest total and pyrophilous bacterial genera and species. Across all media types from fresh soil, we cultured 24 total bacterial genera (Figure S8), 61 total

bacterial species (Figure S9), and 16 pyrophilous bacterial genera (Figure 7A) and 49 pyrophilous bacterial species (Figure S10). Among total bacteria, LB and R2A obtained equally diverse communities (58% of genera, 44% of species), while PyOM captured the least diverse (33% of genera, 18% of species). Among pyrophilous bacteria, R2A captured the greatest diversity (69% of genera, 49% of species), followed by LB (56% of genera, 39% of species) with PyOM yielding the lowest diversity (44% of genera, 20% of species). Although PyOM media yielded the lowest diversity, it was the only media to isolate some important pyrophilous bacterial genera (Whitman et al. 2019; Caiafa et al. 2023; Pulido-Chavez et al. 2023) like *Noviherbaspirillum* and *Peribacillus* and interestingly, R2A was the only media to capture *Massilia* (Figure 7A).

Effect of Media Selection on Fungal Diversity: Media type affected fungal diversity with R2A capturing the most total (Figure 5C) and pyrophilous (Figure 5D) fungal genera and species whereas MYA captured the fewest. Across all media types from fresh soil, we cultured 14 total fungal genera (Figure S11), 29 total fungal species (Figure S12), and 8 pyrophilous fungal genera (Figure 7B) and 23 pyrophilous fungal species (Figure S13). Among total fungi, R2A media captured the most diversity (57% of genera, 48% of species) while both MYA and PyOM were equally diverse (43% of genera, 41% of species). Among pyrophilous fungi, R2A also captured the most diversity (89% of genera, 57% of species), followed by PyOM (50% of genera, 43% of species), and MYA was the least diverse (38% of genera, 39% of species). While PyOM did not capture the most diversity, it was the only media to capture *Neurospora* (Figure 7B), a well-documented post-fire fungus with relatively little knowledge about its ecology (Kuo et al. 2014).

Discussion

Here, we present results of an experiment to test soil storage and media selection to obtain the best diversity and abundance of bacterial and fungal isolates, which have informed our continual culturing efforts resulting in a collection of >540 pyrophilous microbes (286 bacteria; 258 fungi) (Table S3). To our knowledge, this is the first study examining the impact of soil storage on fungal diversity, and this is also the first large-scale culturing effort of pyrophilous bacteria, and among the largest soil and mushroom based culturing efforts of pyrophilous fungi since 1910 (Seaver and Clark 1910). For both bacteria and fungi, freezing soil without glycerol yielded the highest number of pyrophilous genera and R2A yielded the most genera and species. Although it did not capture the highest number of CFUs or highest number of taxa, novel solid agar media we created from field-harvested PyOM was necessary to capture some key pyrophilous genera of interest like *Neurospora* for fungi and *Noviherbaspirillium* for bacteria.

For bacteria, we conclude that freezing soil at -80°C is the best storage method relative to fresh soil, yielding the highest number of CFUs and total and pyrophilous bacterial genera. Interestingly, adding glycerol increased the number of species but reduced the number of total and pyrophilous genera isolated. While CFU based analyses are useful in giving an approximation of the total number of culturable cells present in the original sample and a broad overview of the effectiveness of certain culturing practices at cultivating cells, it does not capture the important distinction of isolate diversity. However, in this case, freezing soils without glycerol captured both the highest number of CFUs and yielded the vast majority of the pyrophilous bacterial genera of interest based on the post-fire bacterial succession literature (Enright et al. 2022; Pulido-Chavez et al. 2023; Bárcenas-Moreno et al. 2011; Caiafa et al. 2023; Brown et al. 2019; Whitman et al. 2019). While culturing from fresh soil did capture the greatest

number of total genera and species, freezing soil captured the greatest diversity of the pyrophilous genera (Figure 4). Interestingly, freezing soil resulted in more total and pyrophilous bacterial genera being isolated, but adding glycerol increased the number of species and was necessary to isolate *Massilia,* which is a bacterial genus repeatedly observed post-fire (Pulido-Chavez et al. 2023; Caiafa et al. 2023; Whitman et al. 2019; 2022), with very little understanding about its ecology, making it a prime candidate for further study. Therefore, it may be of value for researchers to freeze some soil with glycerol as well as without, to capture some of these more potentially vulnerable taxa. Finally, dried soils performed the worst in capturing total and pyrophilous bacterial diversity, which surprised us given how dry post-fire soils usually are. One previous study found that refrigeration at 4ºC was better than freezing at -20ºC at retaining microbial biomass and respiration (Černohlávková et al. 2009). This study examined unburned grassland and forest soils, which could have different soil chemistry and texture from our burned chapparal soils. A study examining how storage time (0-14 days) and temperature (room temp, 4°C, and -20°C) affected 16S sequencing results rather than culturing results similarly found that freezing preserved the most bacterial diversity, though they did not test freezing to -80°C as we did (Rubin et al. 2013). Both of these studies only froze at -20ºC rather than -80ºC, which may have resulted in fewer taxa retained (Pulido Barriga, Randolph, and Glassman 2024).

Soil storage method seemed to matter less for pyrophilous fungi than bacterial genera. While there was one method that stood out as the worst (freezing at -80°C with glycerol), the rest of the methods captured most of the diversity observed (Figures 4D and 6B). This could be due to the resilience of fungal spores to a variety of disturbance effects (Falconer, Otten, and White 2015), but it is also very possible that MYA was not the ideal culture medium for pyrophilous fungi, given the success of R2A and PyOM media at cultivating pyrophilous fungi (Figures 5D

and 7B). Future work repeating the storage experiments with R2A and PyOM might improve pyrophilous fungal isolate retention. Even with the media selection caveat to our storage experiments, we were able to draw some very surprising conclusions from our experiments. For example, our hypothesis that air-drying soil would be the most effective storage method was fully unsupported by our results. Indeed, air-drying yielded the lowest CFU counts and total and pyrophilous diversity. This is an important finding since drying soils prior to laboratory use is not uncommon (Rubin et al. 2013; Castaño et al. 2016; Cui et al. 2014). It is worth noting that we were unable to find other studies exploring the impact of soil storage on fungal communities for comparison, indicating a strong need for these methods to be tested across kingdoms.

In assessing the most effective culturing media for capturing pyrophilous bacteria and fungi, we were surprised at how well the oligotrophic media R2A performed. R2A produced the second highest number of CFUs after the rich medias for bacteria (LB) and fungi (MYA) (Figure 3) and the most diverse pyrophilous bacterial (Figure 5B) and fungal (Figure 5D) isolates. Considering that R2A was developed to culture bacteria from potable drinking water (Reasoner and Geldreich 1985) which is extremely oligotrophic, this would suggest that a large number of pyrophilous microbes prefer a more oligotrophic environment. While shifting life-strategies of microbial communities under stress can be extremely varied and nuanced (Ho, Di Lonardo, and Bodelier 2017), under an increasing stress gradient (such as carbon stress, soil moisture stress, or heat stress) oligotrophic bacteria and fungi can be favored (Hernandez et al. 2021), which might be ideal for capturing a wide diversity of microbes thriving in the stressful post-fire environment. However, this is also a surprising result as post-fire environments tend to be high in nitrogen and phosphorus, even if the carbon landscape has radically changed (Wan, Hui, and Luo 2001; Michelotti and Miesel 2015). While previous studies exist examining the role of media

conditions on microbial culturability (Vieira and Nahas 2005; Sánchez-Clemente et al. 2018; Malosso et al. 2006; Stevenson et al. 2004), these studies often take either a fine-scale approach to tuning a specific culture medium or focus on very broad-scale patterns of culturability such as relying on CFU-based metrics alone or use only rich media. Continued exploration of media conditions is essential to increasing the number of cultured microbial species, and insights gained from our study may help open new paths in culture media development.

Finally, although our home-made PyOM media captured the lowest CFUs of bacteria and fungi, and the lowest bacterial diversity, it captured the second highest fungal diversity and was necessary to isolate key bacterial (i.e. *Noviherbaspirillum soli*) and fungal (i.e. *Neurospora crassa*) pyrophilous taxa of interest. In this case, isolating finicky pyrophilous taxa of interest was improved by the low-density growth we observed in PyOM, which likely resulted from the lower nutrient content similar to R2A media. Rich media like LB, MYA, and potato dextrose agar are often plagued by fast growing ruderal species that can overwhelm the growth of taxa that grow at lower density (Cray et al. 2013). These taxa are particularly interesting because *Noviherbaspirillum* has been repeated observed increasing in abundance post-fire (Enright et al. 2022; Pulido-Chavez et al. 2023; Caiafa et al. 2023) despite very little understanding of its ecology, and *Neurospora crassa* is famously found post-fire and is widely used as a genetics model organism despite very little understanding about its ecological role in the environment (Kuo et al. 2014). Therefore, we conclude that the best media to use to maximize pyrophilous bacterial and fungal diversity if you can only select one is R2A, but best to also include a PyOM media if specifically interested in isolating the most finicky and perhaps interesting pyrophilous microbes.

While we made significant progress regarding testing effective culturing practices for environmental microbes and specifically pyrophilous bacteria and fungi, much additional work remains and caveats exist. Except for the home-made PyOM media, we limited ourselves to commonly available laboratory culturing media. We acknowledge there is a near endless range of possible medias to use (Vieira and Nahas 2005) and there may be more effective media available for the culturing of pyrophilous microorganisms. However, we assert that the broad trends (rich vs. oligotrophic vs. PyOM media) yield valuable insights into the culturing of these microorganisms. As for the PyOM media itself, it is a complex undefined media and uses a water-extraction based method of incorporating nutrients. This method might have resulted in our media missing some key nutrients required for post-fire metabolism that are less water-soluble in PyOM than others (Zeba et al. 2024), which could have led to us missing some pyrophilous microbial taxa. Additionally, the culturing effort in this study all took place aerobically at room temperature. Further, future work could endeavor to test a wider variety of culturing and incubation factors including testing a range of growth temperatures (Bárcenas-Moreno and Bååth 2009; Vieira and Nahas 2005) and media pH (Davis, Joseph, and Janssen 2005; Sánchez-Clemente et al. 2018). Finally, while we acknowledge that separating microbial cultures based on colony morphology is difficult at best (Ben-Jacob, Cohen, and Gutnick 1998; Sousa et al. 2013; Watrud et al. 2006), we decided that choosing colonies based on morphotype and then identifying with Sanger sequencing was an acceptable concession to the technical and logistic demands of isolating, stocking, and identifying every single colony we grew. This does mean that we could have missed some species-level diversity since some species can share extremely similar colony morphologies (Sousa et al. 2013; Watrud et al. 2006) and techniques like dilution to extinction culturing might capture more isolates identified to species level

(Collado et al. 2007; Yang, Kang, and Cho 2016). Given these caveats, we still obtained a remarkable diversity of total (Table S1) and pyrophilous (Table S2) bacteria and fungi and found meaningful differences in media and storage types.

Conclusions

Here, we present the first study to systematically test how soil storage methods impact fungal cultivability, and the largest scale test of how soil storage and media type affect bacterial and fungal cultivation. We found that storing soils at -80°C without glycerol and isolating with oligotrophic R2A media preserved the greatest diversity of culturable pyrophilous bacteria and fungi compared to fresh soils from burned chaparral. However, certain pyrophilous taxa of interest were only captured from soil frozen with glycerol, like *Massilia*, and on our novel PyOM media, like *Neurospora* and *Noviherbaspirillium*, indicating a potential value in using two media and storage types. This experiment contributed to a larger culturing effort resulting in a collection of >540 pyrophilous microbes (286 bacteria; 258 fungi) isolated from soil, smoke, and mushrooms isolated from 7 California wildfires (Table S3), which can be used for future multi-omics, phenotypic, and applied microbiological research. For example, a culture collection is necessary to perform biophysical assays and genomics to assess traits that can yield insights into ecological processes and trade-offs (Maynard et al. 2019). Sequencing genomes from isolates with concurrent biophysical assays is a powerful yet under-used tool to gain insight into microbial traits and connect genomic information to trait expression (Chase et al. 2017). Obtaining the cultures is the first step to characterizing their traits via ‘omics and phenotypic methods.

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Competing Interests

The authors declare no conflicts of interest or competing financial interests in relation to the work described.

Data Accessibility and Benefit-Sharing:

Sequences are accessioned and will be available at the National Center for Biotechnology at Genbank database at time of publication. All scripts used to analyze the data are likewise available on GitHub at https://github.com/DylanEnright93/Culturing_Pyrophilous_Microbes.

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Figures:

Figure 1:

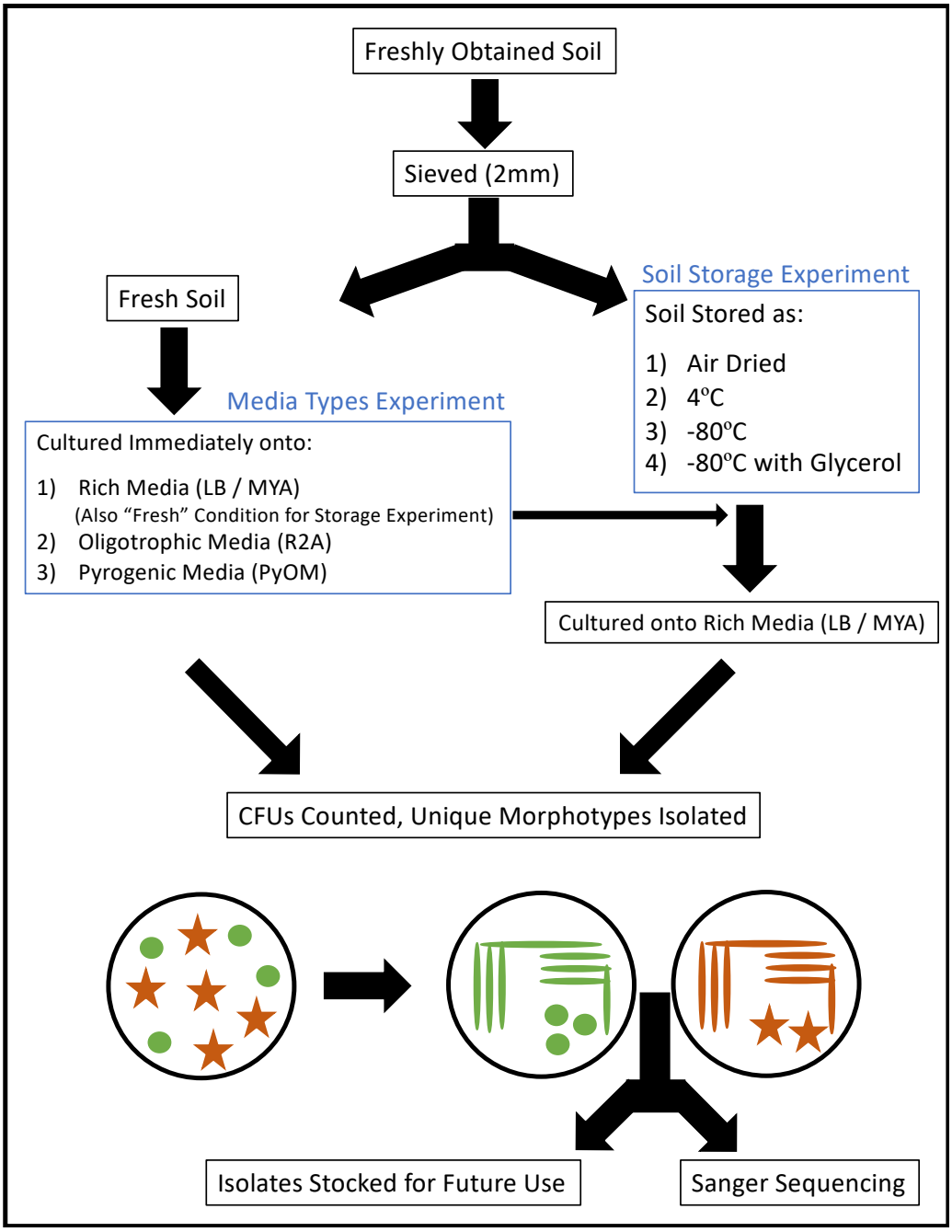

Figure 1: A conceptual diagram of the experimental design used in this experiment. "LB" is abbreviated for Lysogeny Broth, "MYA" is Malt Yeast Agar, "R2A" is Reasoner's 2 Agar, and "PyOM" is Pyrogenic Organic Matter. Note that the cultures obtained from fresh soil plated onto rich media also serve as the "Fresh" storage condition for the soil storage experiment. Green circles and orange stars symbolize two different morphotypes collected from the same initial plate.

Figure 2:

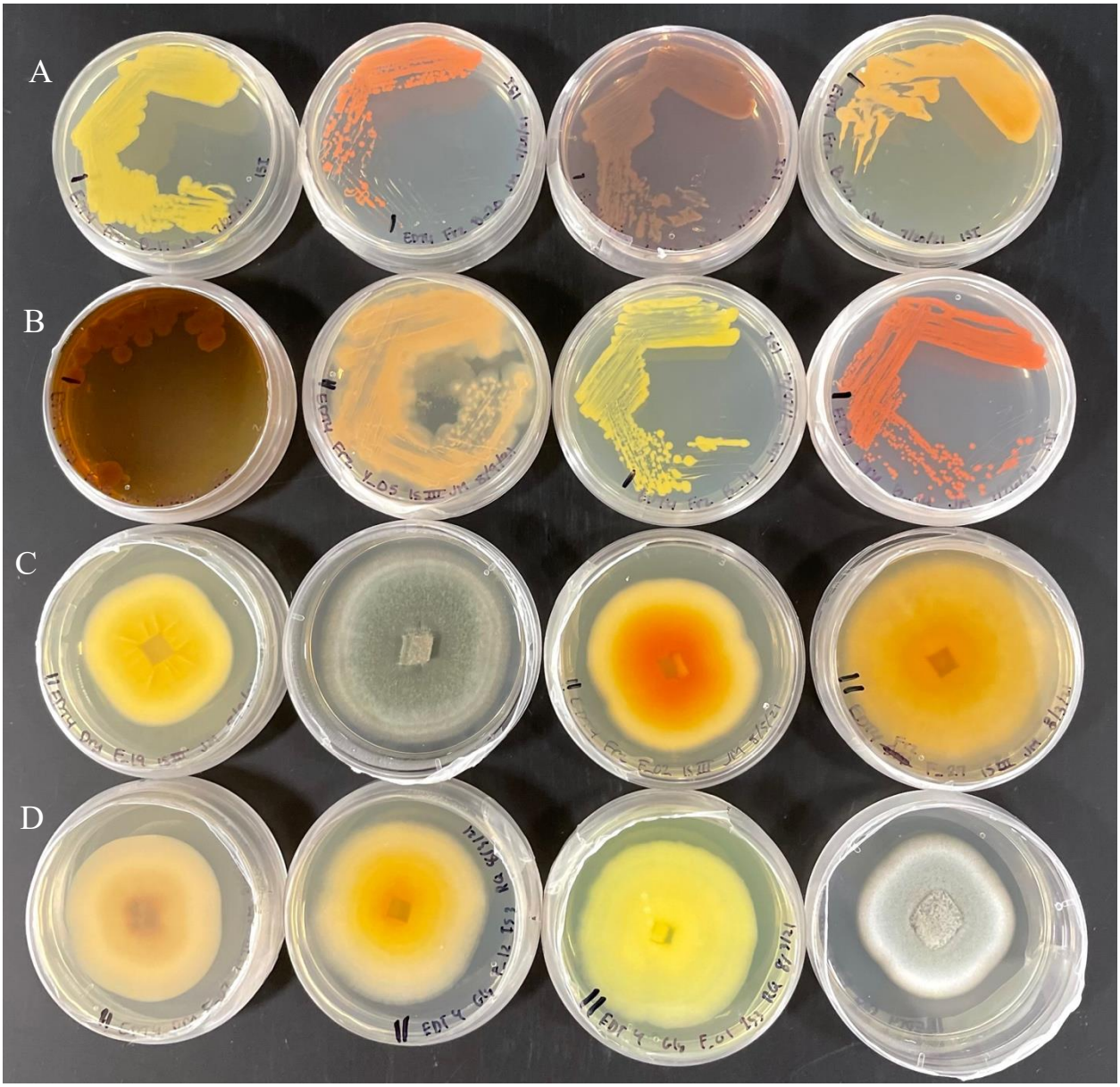

Figure 2: A snapshot of the morphological diversity of the cultures obtained from the El Dorado Fire across all soil storage and media type experiments. Identities were obtained via Sanger sequencing of the 16S or ITS regions for bacteria and fungi, respectively. Moving left to right across each row, the isolates shown are A)*Glutamicibacter bergerei, Arthrobacter agilis, Streptomyces mauvecolor, Kocuria rosea,* B) *Arthrobacter bussei, Holtermaniella festucosa, Pedobacter panaciterrae, Curtobacterium oceanosedimentum,* C) *Penicillium radiatolobatum, Penicillium fagi, Penicillium glabrum, Penicillium thomii,* D) *Penicillium chalabudae, Penicillium radiatolobatum, Penicillium murcianum, Penicillium adametzii.*

Figure 3:

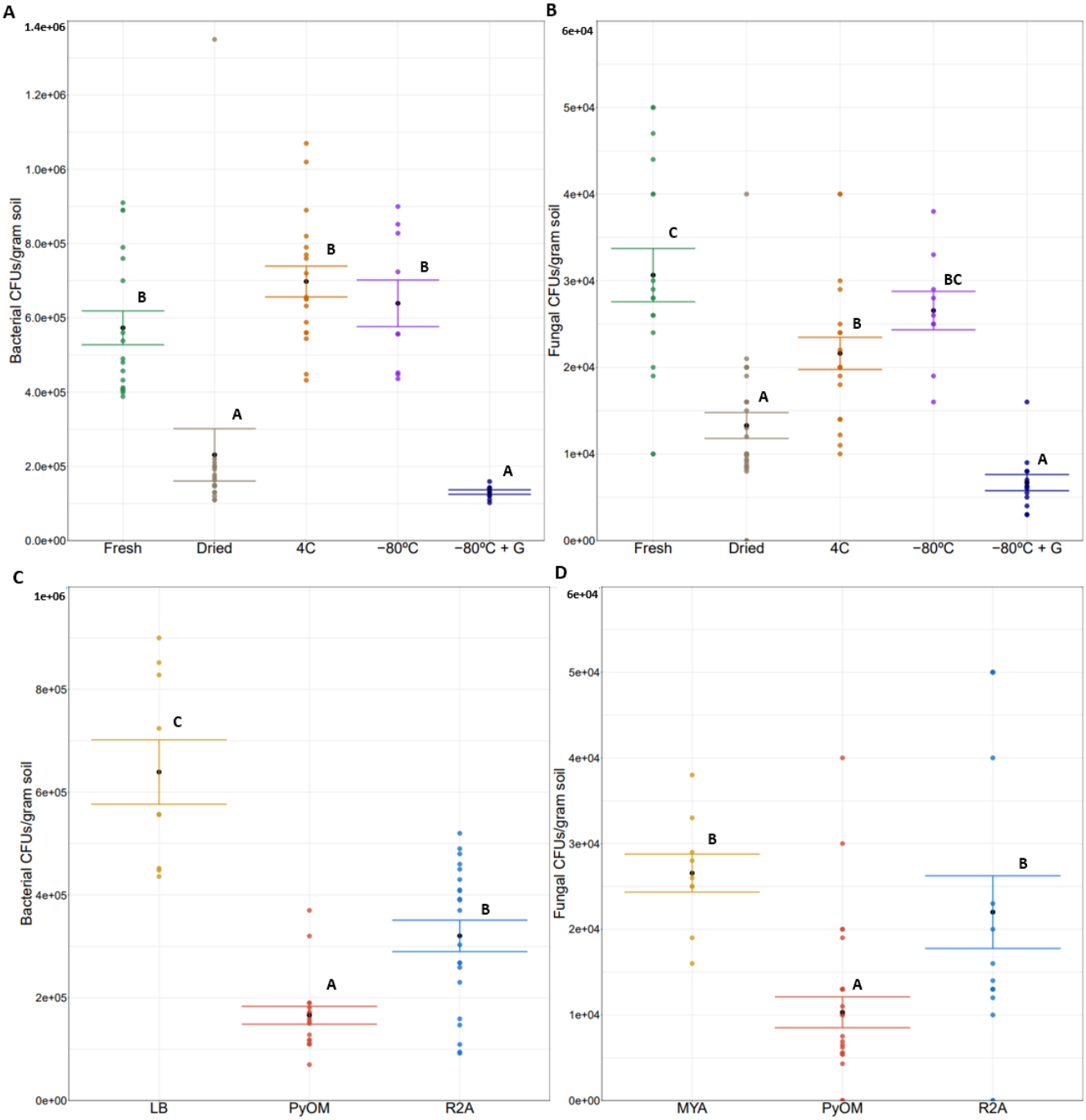

Figure 3: Calculated capturable CFUs per gram soil for A) bacteria and B) fungi among the tested soil storage methods and C) bacteria and D) fungi across tested media types. Points and bars are colored by treatment with bars indicating standard error of themean and black points indicating the mean CFUs per gram soil for each treatment. Significance was tested using ANOVA and Tukey Post-Hoc lettering has been applied. LB = Lysogeny Broth; PyOM = Pyrogenic Organic Matter; R2A = Reasoner's 2 Agar, and G stands for glycerol.

Figure 4:

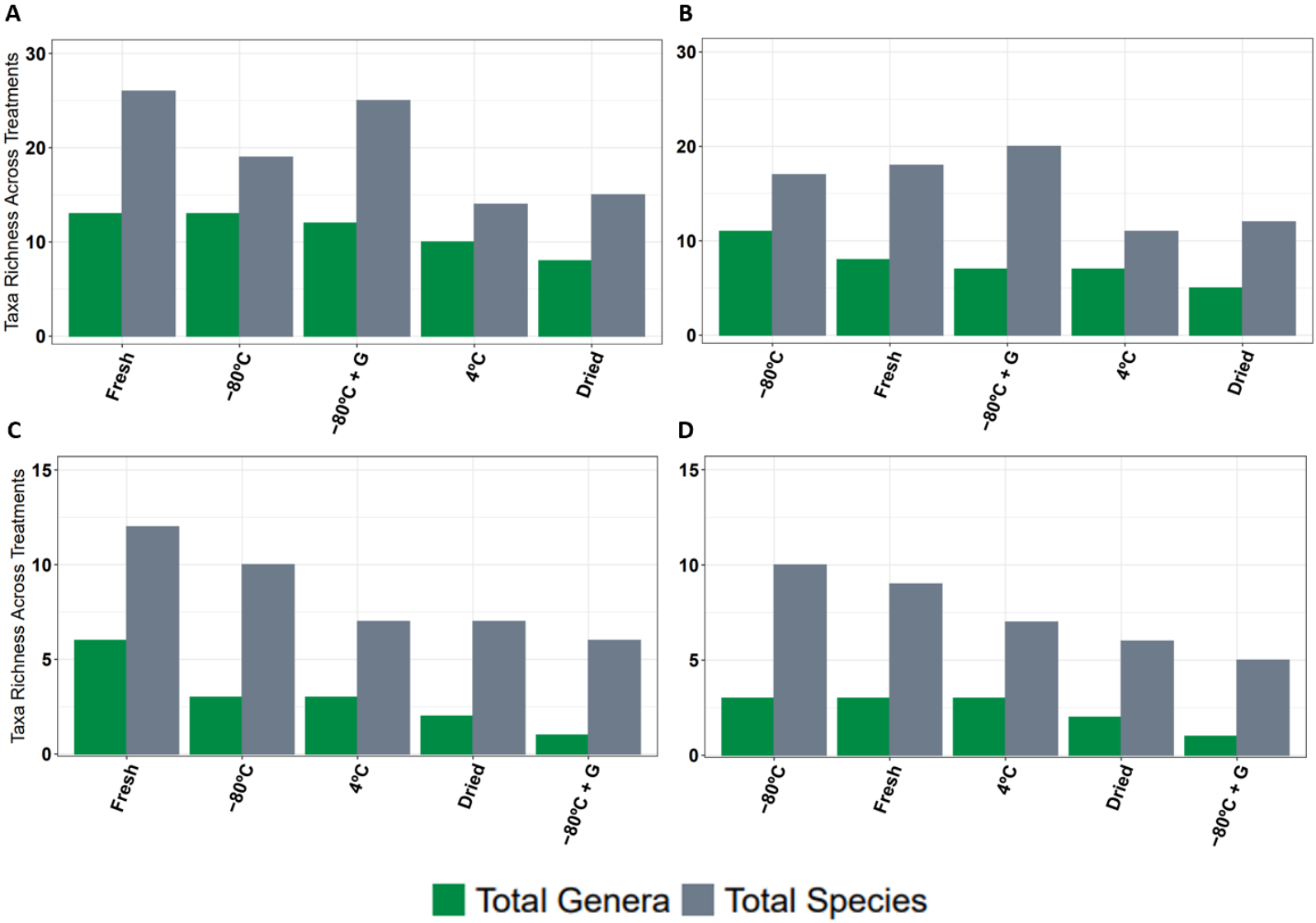

Figure 4: Summarized counts of A) total bacterial, B) pyrophilous bacterial, C) total fungal, and D) pyrophilous fungal genera (green) and species (grey) across four soil storage methods compared to fresh soil when isolated on LB for bacteria or MYA for fungi. Treatments are ordered by highest amount of genera first, then species count second. G = glycerol.

Figure 5:

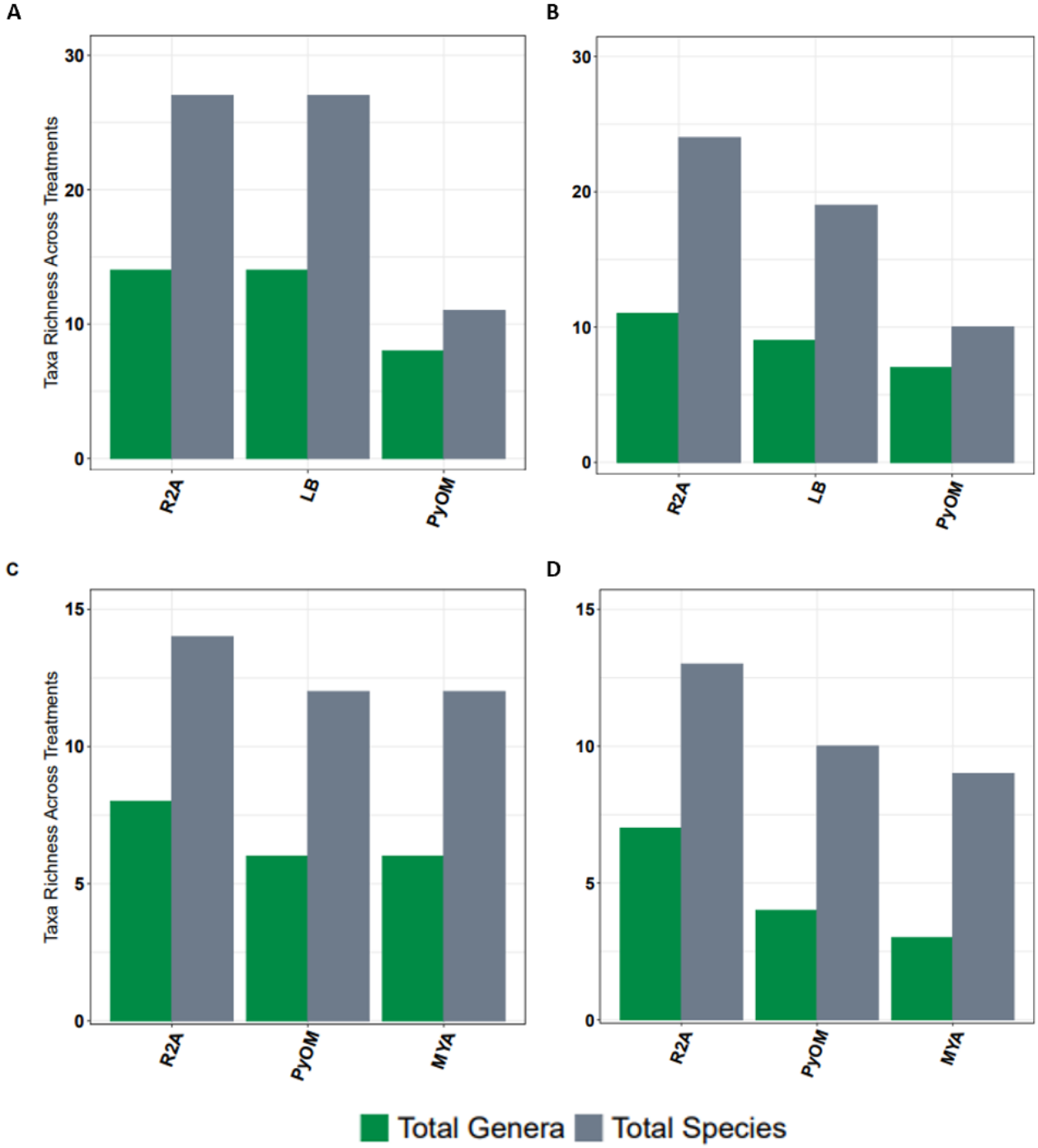

Figure 5: Summarized counts of A) total bacterial, B) pyrophilous bacterial, C) total fungal, and D) pyrophilous fungal genera (green) and species (grey) obtained from fresh soil tested across three media types. Treatments are ordered by highest number of genera first, then species count second. LB = Lysogeny Broth, MYA = Malt Yeast Agar, R2A = Reasoner's 2 Agar, PyOM = Pyrogenic Organic Matter.

Figure 6:

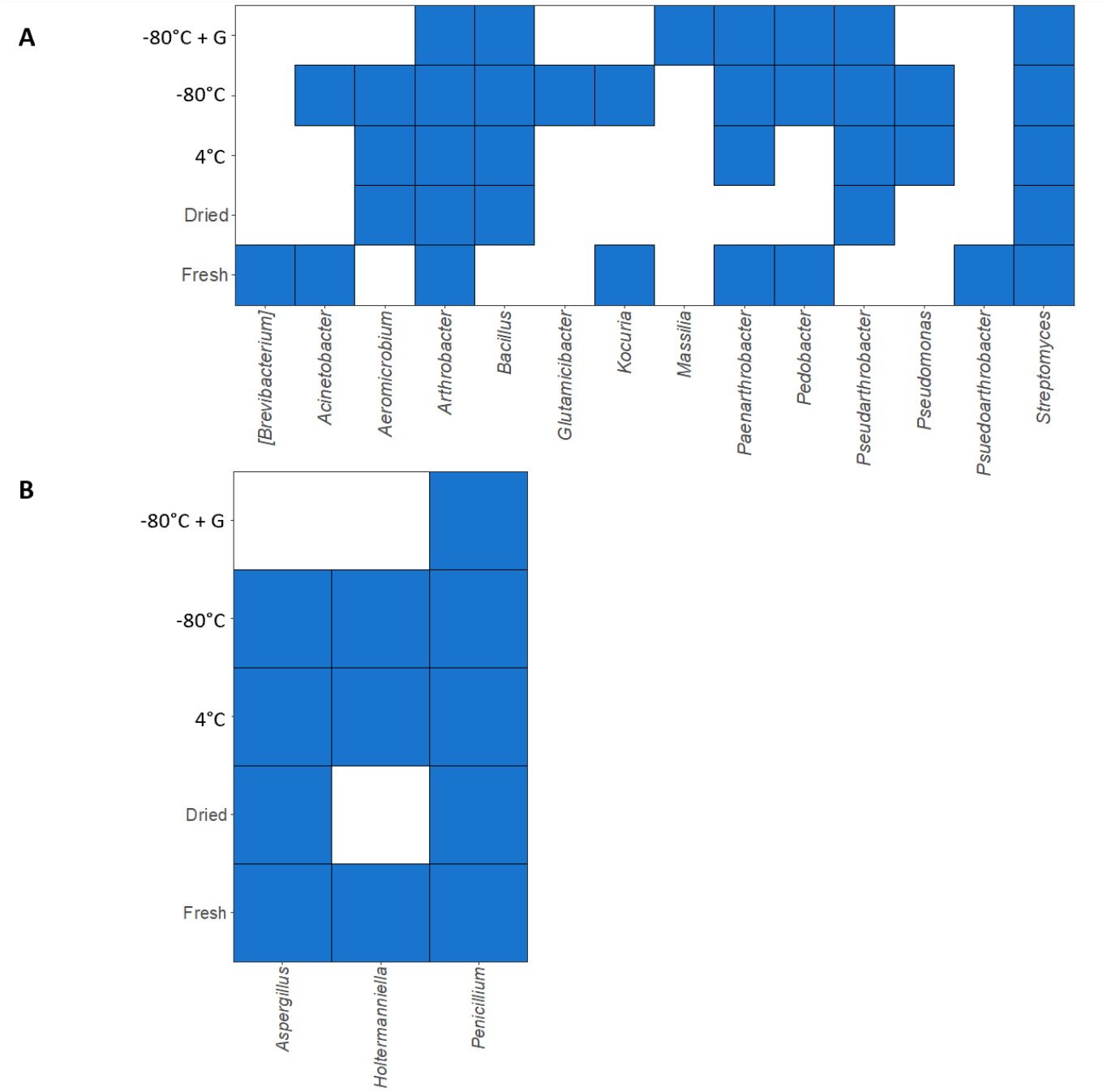

Figure 6: Heatmap-style visualization showing the diversity of pyrophilous A) bacterial and B) fungal genera across the soil storage experiment. A filled blue square indicates the presence of the genus on the x axis in the soil storage method indicated on the y axis. G = glycerol. Genera are ordered in alphabetical order, other than [Brevibacterium] which is in brackets due to controversy over its taxonomy.

Figure 7:

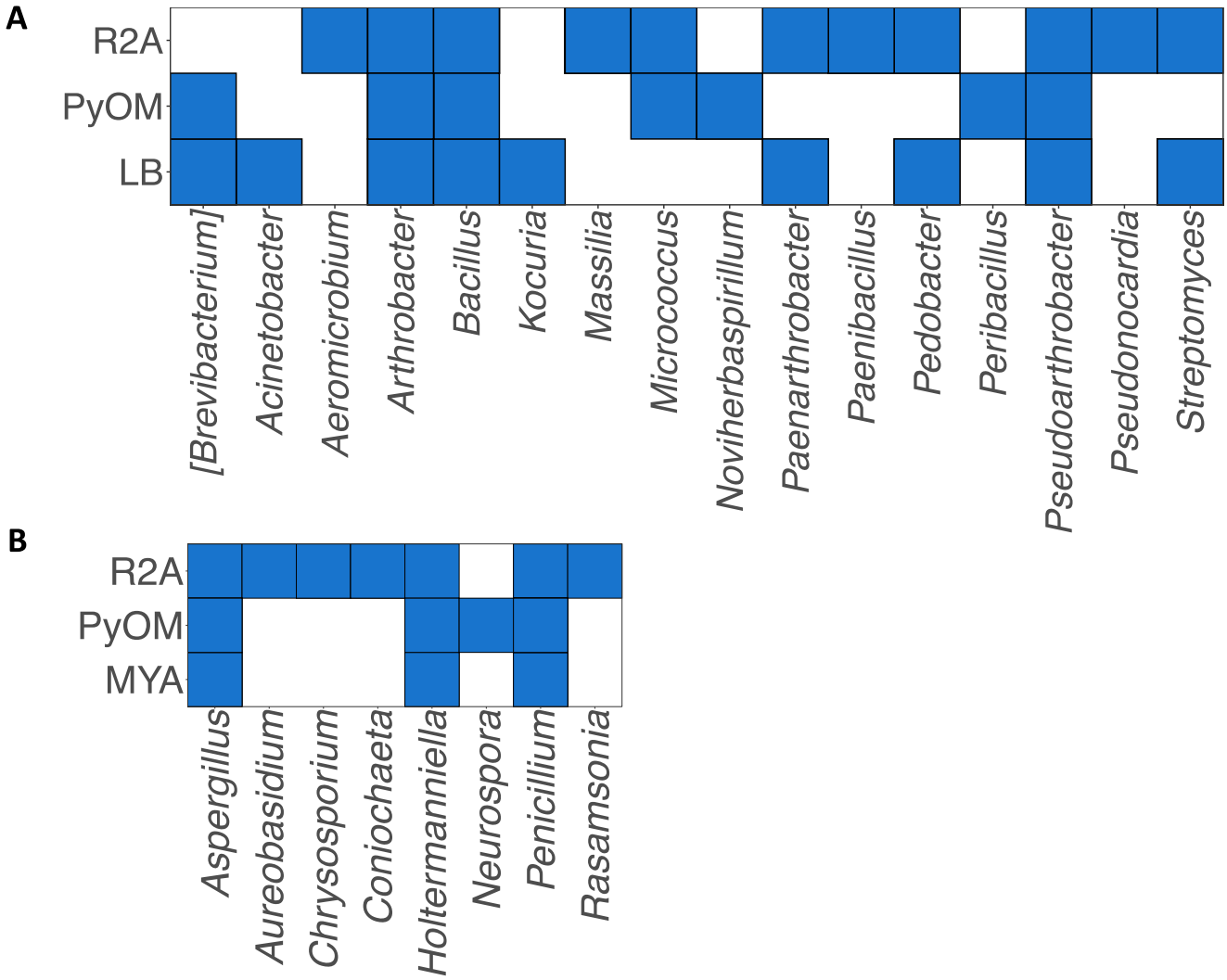

Figure 7: Heatmap-style visualization showing the diversity of pyrophilous A) bacterial and B) fungal genera across the media types experiment. A filled blue square indicates the presence of the genus on the x axis in the media indicated on the y axis. LB = Lysogeny Broth, MYA = Malt Yeast Agar, R2A = Reasoner's 2 Agar, PyOM = Pyrogenic Organic Matter. Genera are ordered in alphabetical order, other than [Brevibacterium] which is in brackets due to controversy over its taxonomy.