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1 **The concentration of heterotrophic growth substrates in culture medium is a crucial**
2 **determinant of the culturability of subsurface soil microbes**
3
4
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15
16 **Abstract**
17 Soil microbial communities have crucial roles in soil fertility and biogeochemistry. While
18 surface soils have been relatively well characterized, we know far less about the microbiology
19 of oligotrophic shallow subsurface soils that often contain microbial taxa that are distinct from
20 surface soils. We hypothesized that the concentration of heterotrophic growth substrates in
21 microbiological growth medium is a critical determinant of the ability to culture subsurface soil
22 microbes. To test this, we conducted dilution-to-extinction experiments with microbial cells
23 separated from subsurface soils collected in a mid-elevation conifer forest in Arizona, USA. We
24 diluted cells into two defined growth media with identical concentrations of inorganic
25 nutrients and vitamins but contained a 100-fold difference in the availability of an amino acid
26 and organic carbon mixture. Across both media formulations, we isolated a total of 133 pure
27 cultures. The concentration of heterotrophic growth substrate in the medium facilitated the
28 isolation of taxonomically distinct microbes. We isolated representative cultures of the most
29 abundant member in the soil community (*Bradyrhizobium* spp.) and representatives of five of
30 the top 10 most abundant *Actinobacteria* phylotypes, including *Nocardioides* spp.,
31 *Mycobacterium* spp., and several other phylogenetically divergent lineages. Flow cytometric
32 analysis of the cultures showed that SYBR green-stained cells isolated on medium with
33 reduced heterotrophic substrate concentrations had significantly lower nucleic-acid
34 fluorescence than those isolated on medium with higher substrate concentrations. These
35 results show that medium substrate concentration can facilitate the isolation of certain
36 microbial lineages and that dilution-to-extinction is an effective method to isolate abundant
37 soil microbes.
38
39 **Importance**
40 Isolating environmental microbes and studying their physiology under controlled conditions is
41 an essential aspect of understanding their ecology. In this study, we use modified high-
42 throughput cultivation methods to isolate subsurface soil microbes. Subsurface ecosystems
43 are typically nutrient-poor environments that harbor diverse microbial communities—the vast
44 majority of which are uncultured. We show that a critical component of whether a microbe is

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45 culturable from subsurface soils is the concentration of growth substrates in the culture
46 medium. Our results offer new insight into technical approaches and growth medium design
47 that can be used to access the vast uncultured diversity of soil microbes.
48

49 **Introduction:**

50 Soil microbial communities are tremendously diverse and mediate crucial aspects of plant
51 fertility, biogeochemistry, pollutant mitigation, and carbon sequestration (1–4). While the
52 diversity and community composition of surface soils have been relatively well described, we
53 know far less about the microbes inhabiting deeper soils, despite their key role in soil
54 formation and mineralization of key plant nutrients. In contrast to surface soils that are
55 typically rich in plant-derived compounds, subsurface soils are often oligotrophic and
56 characterized by lower amounts of organic carbon, mineralizable nitrogen, and phosphorus (5–
57 8). The temperature and soil moisture of subsurface soils also vary less over time than surface
58 environments (9). As depth from the surface increases, the diversity of subsurface microbial
59 communities decreases and the dissimilarity between the subsurface microbial community
60 composition increases relative to surface microbial communities (10, 11). Moreover, the
61 microbial taxa that are abundant in subsurface environments are generally absent from
62 microbial culture and genome databases (10). Thus, we do not understand fundamental
63 biological aspects of a major fraction of subsurface soil microbes (10).

64 Part of the challenge in culturing and studying subsurface microbes is that many of
65 these uncultured lineages likely require low nutrient concentrations for optimal growth. These
66 microbes are termed 'oligotrophs' and are paradoxical to what we understand about most
67 cultivated microbes that tend to grow best when nutrient availability is high. Although
68 oligotrophs dominate most free-living microbial ecosystems (12), the concept of oligotrophy is
69 enigmatic: there is no consensus on what defines oligotrophic metabolism. Several general
70 traits of oligotrophic microbes have emerged from studies of aquatic oligotrophs. For example,
71 oligotrophs are small, slowly growing cells (13–16). The genomes of marine oligotrophs tend to
72 be streamlined often coding for fewer copies of the rRNA gene operon and transcriptional
73 regulators than microbes capable of robust growth at high nutrient availability, suggesting
74 oligotrophs lack the ability to sense and rapidly respond to varying environmental conditions
75 (12, 14, 17). Instead, genomic inventories in oligotrophs point toward a reliance on broad-
76 specificity, high-affinity transport functions and relatively constitutive gene expression (15, 18–
77 20). Because oligotrophs do not attain high yields on low nutrient media, traditional methods
78 of quantifying microbial growth are ineffective (for example, optical density measurements
79 and plating on solid media) (16, 21, 22).

80 While the physiology of aquatic oligotrophs have been fairly well studied, far fewer studies
81 have focused on the physiology of oligotrophic soil microbes. Although many soil cultivation
82 approaches rely on the use of low nutrient media (23–26), we lack a fundamental
83 understanding about why these media facilitate the isolation of distinct taxa and often
84 produce higher culturable counts than nutrient-rich media. We hypothesized that the
85 concentration of heterotrophic growth substrates in a growth medium would constrain the
86 taxa that grow on a defined artificial medium. We tested this by extracting cells from
87 oligotrophic subsurface soils and inoculating high-throughput dilution-to-extinction
88 experiments on a defined medium with differing amounts of heterotrophic substrates. We

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89 isolated several bacteria representing abundant microbial community members. In these
90 experiments, the substrate content significantly influenced which taxa grew in the laboratory.
91 Moreover, we provide evidence that cells isolated on low nutrient medium may contain
92 reduced nucleic acid content relative to those isolated on higher nutrient medium.
93
94 **Results:**
95 We collected shallow subsurface soil (55 cm) from the Oracle Ridge field site in a mid-
96 elevation conifer forest that is part of the Santa Catalina Mountains Critical Zone Observatory
97 in Arizona, USA. We modified high-throughput dilution-to-extinction approaches designed for
98 aquatic microbes (21, 27) to culture shallow subsurface soil microbes from these samples (Fig.
99 1). The primary modification to existing protocols was to add a cell-separation step to detach
100 inoculum cells from mineral soils prior to diluting them to extinction. We diluted the inoculum
101 to an average of 5 cells well^{-1} into deep-well polytetrafluoroethylene 96-well plates containing
102 a defined Artificial Subterranean Medium (ASM), having either low or high concentrations of
103 heterotrophic growth substrates (ASM-low and ASM-high, respectively). The ASM-low and
104 ASM-high media contained identical inorganic mineral and vitamin amendments but a 100-
105 fold difference in the concentration of organic carbon and amino acids (Supplementary Table
106 1). We prepared triplicate 96-well plates for each growth medium formulation. These dilution-
107 to-extinction experiments were screened for growth with flow cytometry after ~4 weeks of
108 incubation and again after ~11 weeks of incubation. Wells displaying growth were sub-cultured
109 into larger volumes and subsequently identified by 16S rRNA gene sequencing.
110 Across both medium types, a total of 214 wells (119 for ASM-low and 95 for ASM-high)
111 exceeded our growth threshold (1.0×10^4 cells ml^{-1}) after 11 weeks of incubation. We
112 successfully propagated 182 (85%) of the cultures from microtiter plates to polycarbonate
113 flasks containing fresh medium. Of the cultures that successfully propagated, we confirmed
114 that 73% (133 cultures) were pure cultures by amplifying and sequencing full-length 16S rRNA
115 gene sequences. The remaining 49 cultures were either mixed (16S rRNA genes did not
116 assemble due to base ambiguities) or did not amplify under several amplification conditions.
117 We defined microbial culturability as the ratio of cells capable of being isolated in a given
118 condition to the total number of cells initially diluted into a cultivation chamber (28). By the
119 end of the experiment, we approached ~20% culturability across both medium formulations
120 (Fig. 2). In general, microbial culturability was higher for ASM-low than for ASM-high, but this
121 effect was only significant after 4 weeks of incubation (Fig. 2; Wilcoxon rank-sum test $P \leq 0.05$
122 at 4 weeks).
123 Cultures isolated on ASM-high were taxonomically distinct (at a genus level) from those
124 isolated on ASM-low (Kruskall-Wallis rank sum, $P=1.28 \times 10^{-5}$). All pure cultures grown on both
125 ASM-low and ASM-high belonged to one of two bacterial phyla: Actinobacteria (110 cultures;
126 83% of the pure cultures) or Proteobacteria (23 cultures; 17% of the pure cultures) (Fig. 3). The
127 Proteobacterial cultures were exclusively Alphaproteobacteria, 57% of which were isolated on
128 ASM-low medium and the remainder (43%) were isolated on ASM-high (Fig. 3 and
129 Supplementary Fig. 1). Cultures that classified as *Bradyrhizobium* spp. were the most frequent
130 alphaproteobacterial isolates (13 isolates), seven of which were isolated on ASM-low medium.
131 Cultures classified as *Reyranella* spp. and *Nordella* spp. were also isolated on both ASM-high

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132 and ASM-low medium. The remaining five alphaproteobacterial cultures were taxonomically
133 distinct—three were isolated on ASM-low and two on ASM-high.
134 The taxonomy of the actinobacterial cultures was split among three classes:
135 Actinobacteria (107 cultures), Thermoleophilia (2 cultures), and Acidimicrobiia (1 culture). Of
136 these Actinobacteria, 65 (59%) were isolated on ASM-low, and 45 (41%) on ASM-high. The
137 actinobacterial cultures were numerically dominated by two genera that were differentially
138 isolated on ASM-low and ASM-high: *Nocardioides* and *Mycobacterium*. *Nocardioides* spp. (46
139 cultures) were exclusively isolated on ASM-low medium (Fig 3 and Supplemental Fig. 2). Other
140 cultures that were isolated on ASM-low included those classified as *Arthrobacter* (3 cultures),
141 *Marmoricola* (2 cultures), *Nakamurella* (2 cultures), *Aeromicrobium* (1 culture), *Blastococcus* (1
142 culture), and *Patulibacter* (1 culture) (Fig 3 and Supplemental Fig 2). While cultures classified as
143 *Mycobacterium* sp. were generally isolated on ASM-high (38 cultures), we did isolate seven
144 mycobacterial cultures on ASM-low medium—five of which form a phylogenetically distinct
145 cluster from those isolated on ASM-high (Fig. 3 and Supplementary Fig. 2). Other
146 actinobacterial cultures isolated on ASM-high included *Jatrophihabitans* (4 cultures),
147 *Conexibacter* (1 culture), and *Amycolatopsis* (1 culture).
148 Interestingly, we isolated what are likely the first members of two novel actinobacterial
149 lineages on ASM-low. The first such culture—Microtrichales sp. str. AZCC_0197—belongs to
150 the Microtrichales order of the Acidimicrobiia class. The best 16S rRNA gene sequence match
151 to an existing isolate is 93.4% identity to *Aquihabitans daechunggensis* str. G128, but str.
152 AZCC_0197 more closely matched numerous 16S rRNA gene sequences from environmental
153 clones of uncultured Acidimicrobiia. The second lineage—Frankiales sp. strains AZCC_0102
154 and AZCC_0072—classified as members of the Frankiales order of the Actinobacteria class
155 with best matches of <97% nucleotide identity to existing Frankiales isolates (29).
156 Several of the cultures we isolated are representative of abundant members of the
157 subsurface soil microbial community at Oracle Ridge. We matched the 16S rRNA gene
158 sequences from our cultures to the operational taxonomic units (OTUs) present in the Oracle
159 Ridge soil sample collected at 55 cm. The cultures isolated from these 55 cm subsamples are
160 representative of 13 OTUs (Fig. 4) that comprise a total of 8.8% of the total amplifiable
161 microbial community in subsamples collected at the same time and depth. For example, our
162 *Bradyrhizobium* isolates match a single *Bradyrhizobium* OTU that was the most abundant OTU
163 at 55 cm (relative abundance of ~5.7%) (Fig. 4). Similarly, we isolated representatives of most
164 of the abundant Actinobacteria (Fig. 4) including: two *Mycobacterium* OTUs (the 11th and 17th
165 most abundant OTUs overall, and the most abundant and sixth most abundant actinobacterial
166 OTUs, respectively); *Nocardioides* (the 13th most abundant OTU overall, and third most
167 abundant actinobacterial OTU), and two *Arthrobacter* OTUs (16th and 1,271st most abundant
168 OTU overall, and the fifth and 143rd most abundant actinobacterial OTUs). The other
169 Actinobacteria cultured in these experiments represent rarer species in bulk soils. The 16S
170 rRNA gene sequences from several of our pure cultures did not match any of the 16S rRNA
171 gene amplicon OTUs amplified from these soils, including *Nakamurella* (2 cultures),
172 *Nocardioides* (5 cultures), *Mycobacterium* (1 culture), *Jatrophihabitans* (1 culture), *Patulibacter* (1
173 culture), *Conexibacter* (1 culture), *Rhizobiales* sp. "GAS113" (1 culture), *Reyranella* (1 culture),
174 and *Microtrichales* sp. str. AZCC_0197.

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175 Cultures isolated on ASM-low exhibited significantly lower mean nucleic acid fluorescence
176 than those isolated on ASM-high (Fig. 5; Kruskal-Wallis Rank sum $P=6.3 \times 10^{-7}$). The overall
177 mean fluorescence was not significantly different across phyla (Kruskal-Wallis Rank sum
178 $P=0.647$) but was significant across assignable genera (Kruskal-Wallis Rank sum $P=3.0 \times 10^{-6}$).
179 Moreover, the mean nucleic acid fluorescence values within a given genera were generally
180 similar (Supplementary Fig. 3). For example, *Mycobacterium* isolates had relatively high nucleic
181 acid fluorescence, regardless of which medium they were isolated on (Supplementary Fig. 3).
182 In contrast, *Nocardioides* (ASM-low) and *Jatrophihabitans* (ASM-high) displayed relatively low
183 nucleic acid fluorescence. Interestingly, when cultured on ASM-high, we observed a clear
184 nucleic acid fluorescence dichotomy across the *Bradyrhizobium* isolates (Supplementary Fig. 3).
185
186 **Discussion:**
187 We designed a proof-of-concept workflow to determine the feasibility of high-throughput
188 dilution-to-extinction cultivation for the isolation of soil microbes. The method was
189 conceptually based on an improved high-throughput culturing workflow that was designed to
190 isolate oligotrophic marine microbes in inert plastic microtiter plates using automated flow
191 cytometry to detect microbial growth rapidly and at relatively low cell densities (21). However,
192 unlike aquatic samples, microbial cells in soils are heterogeneously dispersed within, or
193 attached to, a complex matrix comprised of non-cellular organic matter and minerals. The
194 inherent complexity of the soil matrix complicates accurate enumeration of viable cells—
195 especially of small oligotrophic microbes—because mineral and organic matter can interfere
196 with flow cytometry. To circumvent these issues, we separated cells by gently shaking soils in a
197 cell extraction buffer containing a dispersing agent and a nonionic surfactant. Cells were
198 separated from this slurry by density gradient centrifugation (Fig. 1). This procedure allows
199 cells to be 'floated' on top of a solution of Nycodenz while allowing minerals to pellet through
200 the Nycodenz (30).
201 We expected 168 pure cultures across all experiments, based on the proportion of
202 cultivation chambers displaying measurable growth (214 chambers) and the number of
203 chambers inoculated (576 chambers). The number of pure cultures we obtained (133) was
204 within 38% of this value. However, this result does not account for the cultures that were
205 initially scored as positive for growth but did not successfully subculture. Some of the cultures
206 that failed to propagate from microtiter plates to larger volumes might have been false
207 positives, where flow cytometer instrument noise or well-to-well carryover was mistaken for a
208 low-density culture. The mean culturability we observed for a given experiment (1.4%-11%;
209 Fig. 2) is comparable to dilution-to-extinction cultivation studies of marine microbes, which
210 report 0.5%-14.3% culturability (27). Similar to previous observations for soil microbes (25), we
211 observed the culturability increased by lengthening the incubation time (Fig. 2).
212 The concentration of heterotrophic growth substrates in the isolation medium was a
213 determinant of the ability to isolate certain microbial lineages (Fig. 3). Carbon type and
214 availability are crucial for heterotrophic soil microbes since carbon acts as both a source of
215 electrons for respiration and carbon for biomass. To accommodate this requirement, many
216 common microbial growth medium formulations for heterotrophic microbes supply diverse
217 growth substrates (yeast extract or casein digests, for example), usually at concentrations
218 much higher than are normally available *in situ*. Two key assumptions made with these

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219 common media formulations are that: 1) microbes will use only the relevant constituents and
220 any remaining compounds will have minor or no effect on microbial growth; and 2) microbes
221 grow optimally when nutrient availability is much greater than their half saturation constant
222 (31). While many microbes have the capacity to grow on these commonly used complex, high-
223 nutrient formulations, environmental nucleic acid data informs us that the vast majority of
224 Earth's microbes cannot (32).
225 Numerous studies have demonstrated that dilute growth media is superior to substrate
226 rich growth media for the isolation of novel soil microbes (25, 26, 33, 34). However, the
227 physiological explanation as to why low-nutrient media facilitates the growth of diverse
228 microbes, or high nutrient concentrations inhibit the growth of some taxa, are unclear. One
229 possible explanation for these concentration-dependent effects may be that growth medium
230 formulations applied at high concentrations contain high amounts of inhibitory substances—
231 substances that are reduced to non-inhibitory levels in dilute medium formulations. For
232 example, a key amino acid transporter in *Chlamydia trachomatis* can be blocked by non-
233 essential amino acids, preventing the transport of required amino acids, resulting in growth
234 inhibition (35). A similar phenomenon was demonstrated in the marine oligotroph '*Candidatus*
235 Pelagibacter ubique', where alanine was conditionally required for cell division but abolished
236 growth at higher concentrations (36). Furthermore, reactive oxygen species can be produced
237 during the autoclaving of nutrient-rich medium that either directly inhibit growth or combine
238 with organics in the medium to form inhibitory compounds (37, 38). Finally, growth inhibition
239 may be the result of misbalanced regulation of growth or accumulation of nutrient storage
240 structures (poly-β-hydroxybutyrate, for example) ultimately leading to cell lysis (39). A better
241 understanding of the mechanism(s) that enable growth on low nutrient medium—or prevent
242 growth on high-nutrient medium—may help us design better strategies for isolating
243 uncultivated lineages. Critically, having a collection of cultures that were isolated on medium
244 with identical constituents applied at different concentrations is a first step towards an
245 experimental method capable of addressing these questions.
246 While several of the taxa we isolated were abundant microbial members of the shallow
247 subsurface microbial community (Fig. 4), other isolates were rare or not identified in the
248 cultivation-independent soil microbial community at all. Although the cultivation of microbial
249 phylotypes that are not observed in cultivation-independent analyses has been observed (40),
250 dilution-to-extinction favors the cultivation of abundant microbes in a given sample (28), such
251 that the appearance of rarer taxa or taxa that were not observed in the original sample is
252 unusual. There are several possible explanations for this observation. First, the absence of a
253 particular taxon in a soil microbiome analysis may be the result of insufficient sequencing
254 depth (40). Similarly, the 'universal' primers used in the soil microbiome analysis (41) may have
255 been biased against some of the divergent lineages we obtained in pure culture, resulting in
256 either underrepresentation of these phylotypes in the original community or no amplification
257 at all. Finally, the taxa isolated in these experiments may have had similar buoyancies and
258 migrated to similar locations in the density gradient during cell extraction effectively skewing
259 the proportions of microbes that were diluted into microtiter plate wells. At this point, we do
260 not have sufficient evidence pointing toward one of these scenarios over the other.
261 Additionally, as is true in any microbial cultivation experiment, there are many taxa that we did
262 not isolate. In particular, these soils contained high relative abundances of Verrucomicrobia

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263 related to '*Ca*. Udaeobacter copiosus' (42), and Acidobacteria (Subgroup 6), which belong to
264 highly sought-after lineages of uncultured microbes (22). The reasons for not culturing these
265 (and other) lineages are numerous but may be the result of inappropriate medium composition
266 (43, 44), toxic compounds in the cell separation constituents, long doubling times (> ~6 days),
267 or dormancy (reviewed in (45)).
268 Similar to aquatic oligotrophs (15, 46), we provide evidence that soil oligotrophs contain
269 reduced nucleic acid content relative to other soil microbes that prefer higher nutrient
270 concentrations (Fig. 5). Depending on the taxa in question and the effective population size,
271 microbial genome reduction can be driven by genetic drift or streamlining selection (reviewed
272 in (47)). Genome streamlining is strongly linked with oligotrophy in free-living aquatic microbes
273 as a mechanism to reduce the overhead cost of replication in periodically nutrient-limited
274 environments (reviewed in (14)). However, direct evidence for streamlined genomes in
275 terrestrial oligotrophs has been elusive. Metagenome-assembled genomes suggest that some
276 lineages of abundant and ubiquitous uncultured Verrucomicrobia may contain reduced
277 genome sizes (42), and a more recent study showed that fire-affected warm soils selected for
278 groups of microbes with significantly smaller genomes than cooler soils (48). However, there
279 are few definitive ways to identify these taxa as oligotrophs short of culturing them. The
280 appearance of reduced nucleic acid content in cultures isolated on ASM-low is also an
281 indication that genome reduction may be a successful life strategy for soil oligotrophs. We
282 speculate that "streamlined" genome sizes in soil oligotrophs may be larger than those
283 observed in marine habitats, since success in the complex soil matrix necessitates a greater
284 variety of gene function to succeed (14).
285 We were surprised to identify taxa with such distinct growth substrate concentration
286 preferences from highly oligotrophic soils (0.5% organic carbon). For example, while the
287 environmental distribution of *Nocardioides* sp.—which were isolated on ASM-low—indicate a
288 preference for deeper, more oligotrophic soils (Fig. 4), we also isolated numerous
289 *Mycobacterium* spp. from the same samples. The majority of these *Mycobacterium* isolates
290 were isolated on ASM-high medium and did not show a clear preference for subsurface soils
291 (Fig. 4). Interestingly, we observed that the *Nocardioides* sp. appeared sooner (within 4 weeks)
292 in the dilution-to-extinction screens, and *Mycobacterium* sp. growth was only apparent
293 between 4 and 11 weeks of incubation. These observations suggest that the oligotrophs from
294 these soil samples may grow relatively rapidly compared to the microbes requiring higher
295 nutrient concentrations. This finding is contrary to the perception that oligotrophic microbes
296 grow more slowly than those microbes capable of growth on higher nutrient concentrations
297 (16), suggesting that oligotrophy in shallow subsurface soils may be characterized by traits that
298 are distinct from those described for aquatic microbes.
299 The development of cultivation techniques emphasizing the high-throughput and sensitive
300 detection of microbial growth on low-nutrient medium has revolutionized the field of aquatic
301 microbial ecology in that these methods enable the cultivation of microbes that were
302 previously inaccessible using standard techniques (21, 27, 49–51). Here we show that similar
303 principles facilitate the cultivation of abundant soil oligotrophs. Moreover, we demonstrate
304 that in addition to considering the nutritional composition of a given growth medium, another
305 obstacle to culturing some lineages is the concentration of growth substrates in the growth
306 medium. Although we do not yet understand the mechanism of substrate-induced growth

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307 inhibition, there is evidence that this phenomenon is widespread and results in the inability to
308 culture some microbial lineages in the laboratory (12). Future mechanistic studies aimed at
309 deciphering the physiology of substrate-induced growth inhibition will likely lead to new
310 cultivation approaches that will allow us to isolate abundant free-living oligotrophic microbes.
311
312 **Materials and Methods:**
313 *Soil source:* Fresh soil samples were collected from a soil pit on August 16, 2017 at the
314 Oracle Ridge site in the Catalina Jemez Critical Zone Observatory (coordinates: 32.45 N, -
315 110.74 W, elevation 2,103 m). We collected ~300 g subsamples from 0-5 cm, 10 cm, 20 cm, 30
316 cm, 40 cm and 55 cm depths. We analyzed the microbial community composition at each
317 depth (see *'Soil microbial community analysis,'* below) and conducted cultivation experiments
318 from the 55 cm soil sample. Soils were kept cool with ice packs for <4 h while in transit to the
319 laboratory. At the laboratory, the soils were sieved to 2 mm and kept at 4ºC for 50 days at
320 which point cells were separated from mineral soil.
321 *Soil microbial community analysis:* Replicate 1.0 g subsamples were weighed into MoBio
322 PowerSoil DNA extraction kits and extracted according to the manufacturer's instructions. We
323 amplified 16S rRNA gene fragments using 515F-Y (5'-
324 TATGGTAATTGTGTGYCAGCMGCCGCGGTAA-3') and 926R (5'-
325 AGTCAGTCAGGGCCGYCAATTCMTTTRAGT-3') (52). PCR products were purified using the
326 QIAquick PCR purification kit (Qiagen, Germantown, MD) per manufacturer's specification.
327 Cleaned products were quantified using Tecan fluorometric methods (Tecan Group,
328 Mannedorf, Switzerland), normalized, and pooled for Illumina MiSeq sequencing using custom
329 sequencing primers and the MiSeq Reagent v2 500 cycle Kit (Roche, Branford, CT) following
330 the manufacturer's protocols. Operational taxonomic units (OTUs) were generated *de novo*
331 from raw Illumina sequence reads using the UPARSE pipeline (53). Paired-end reads were
332 trimmed of adapter sequences, barcodes and primers prior to assembly. Sequences of low-
333 quality, and singletons were discarded, and the remaining sequences were subjected to a
334 dereplication step and abundances were determined. Chimera filtering of the sequences was
335 completed during clustering while taxonomy was assigned to the OTUs with mothur (54) using
336 version 123 of the SILVA 16S ribosomal RNA database (55) as the reference. OTUs and
337 corresponding taxonomy assignment tables were generated and used in subsequent analyses.
338
339 *Cell separation:* Cells were separated from sieved soils using density gradient centrifugation
340 with Nycodenz. Briefly, we added 0.5 g wet soil to 44.8 ml of cell extraction buffer (137.5 mM
341 NaCl, 26.78 mM tetrasodium pyrophosphate, and 0.27% (v/v) Tween 80). The soil-buffer slurry
342 was vortexed for 30 seconds and shaken horizontally on a platform shaker for 2 hours at 4°C.
343 We layered 15 ml aliquots of this soil-buffer slurry over 10.0 ml of 80% (w/v) Nycodenz solution
344 in 50 mM tetrasodium pyrophosphate. We used 50 ml Nalgene Oak Ridge high-speed
345 polycarbonate centrifuge tubes for density gradient centrifugations. Tubes containing the soil-
346 buffer solution with Nycodenz were centrifuged at 17,000 × *g* for 30 minutes at 16ºC. We
347 extracted 3 × 0.5 mL aliquots from the resulting density gradient at a location of ~25 mm above
348 the bottom of the tube (coincident with the approximate level of the top of the Nycodenz
349 solution) to sterile microcentrifuge tubes containing 1.0 ml 137.5 mM NaCl. The
350 microcentrifuge tubes containing Nycodenz/NaCl were vortexed and centrifuged for 20 min at

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351 17,000 × *g*. The resulting faint cell pellets were resuspended in 137.5 mM NaCl, pooled and
352 stored at 4°C.
353
354 *Dilution-to-extinction:* An aliquot of cells extracted from the Nycodenz gradient were fixed
355 with 1.75% (final v/v) formaldehyde and stained with SYBR Green I (final stain concentration
356 was a 1:4,000 dilution of commercial stock) for 3.5 h at room temperature in the dark. Cells
357 were enumerated using a Millipore Guava flow cytometer, as described elsewhere (36). We
358 diluted cells into Artificial Subterranean Medium (ASM) -high or ASM-low nutrient medium
359 (Supplementary Table 1) to a density of 5 cells mL^{-1} and aliquoted 1.0 ml of the dilute cell
360 suspension into the wells of 2 ml polytetrafluoroethylene 96-well microtiter plates (Cowie
361 Technology, New Castle, DE) so that on average each well contains 5 cells. Plates were covered
362 with plastic lids that allow air circulation and incubated at 16ºC in the dark. We screened the
363 dilution-to-extinction plates for growth by fixing (1.75% formaldehyde) and staining (1:4,000
364 dilution of commercial SYBR Green I stock) aliquots for 18h in the dark at room temperature
365 and counting by flow cytometry (EMD-Millipore Guava EasyCyte), as described previously (21).
366 We screened plates for growth at 4 and 11 weeks after inoculation. Positive cultures were
367 defined as cultures that exceeded 1.0×10^4 cells ml^{-1}.
368
369 *Culturability estimates and estimates of the number of pure cultures:* Culturability estimates
370 were determined by the equation: $V = -\ln(1-p)/X$, where V is the estimated culturability, p is the
371 proportion of inoculated cultivation chambers that displayed measurable growth (number of
372 chambers positive for growth ÷ total number of chambers inoculated), and X is the number of
373 cells added to each cultivation chamber (28). The number of pure cultures (u) was estimated as
374 follows: $u=-n(1-p)\ln(1-p)$, where n is the number of inoculated growth chambers (28).
375
376 *Culture transfer and storage:* We subcultured positive growth chambers into 25 ml of the
377 respective growth medium (ASM-high or ASM-low) in acid-washed, sterile polycarbonate
378 flasks and incubated them at 16ºC. At the time of transfer, we assigned cultures a unique
379 Arizona Culture Collection (AZCC) number. Flasks were monitored for growth every other
380 week for 2 months. Flasks displaying growth within two months were cryopreserved in 10%
381 glycerol and stored at -80ºC. If no growth appeared within two months, the cultures were
382 discarded and the assigned AZCC number was retired.
383
384 *Mean fluorescence calculations:* We calculated the mean fluorescence of each culture from
385 the subcultures grown in 25 ml volumes 12-15 weeks after inoculating. Culture aliquots were
386 fixed and stained for 15-18 h as described above in 'Dilution-to-extinction.' We manually gated
387 histograms of the intensity of SYBR Green I fluorescence (in arbitrary units) and extracted the
388 mean fluorescence of the gated peak for each culture.
389
390 *Culture identification*: Cultures were identified by full length 16S rRNA gene sequencing.
391 Briefly, we filtered 5-10 ml of cell biomass from 25 mL cultures on to 0.2 µm-pore size Supor
392 filters and extracted DNA using a Qiagen PowerSoil DNA extraction kit following the
393 manufacturer's instructions. We amplified full-length 16S rRNA genes from the resulting DNA
394 using the 27F-1492R primer set (27F: 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492R: 5'-

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395 ACCTTGTTACGACTT-3' (56)). The reaction mix consisted of Promega's GoTaq HotStart 2x
396 PCR master mix with final concentrations of 0.4 µM 27F and 0.4µM 1492R primers, and 1-11.5
397 µl of template DNA, in a total reaction volume of 25 µL. The thermocycling profile was 1 × 94°C
398 for 10 min followed by 36 cycles of: 94°C for 45 s, 50°C for 90 s, 72°C for 90 s; and a single 72°C
399 extension for 10 min. The resulting amplicons were cleaned and Sanger sequenced from either
400 the 27F or 1492R primer by Eurofins Genomics (Louisville, KY, USA) using their standard
401 techniques. Sequences were curated using 4Peaks (57) and Geneious Prime v2019.0.1 (58).
402 Reads were trimmed and assembled using the moderate setting in Geneious. Those reads that
403 failed to assemble with these metrics were considered "mixed" cultures and not analyzed
404 further.
405
406 *Culture taxonomy and determination of taxonomic differences across growth medium*
407 *formulations:* High-quality full length 16S rRNA gene sequences from the cultures were used to
408 assign taxonomy and reconstruct phylogenetic relationships. We assigned taxonomy to all
409 assembled 16S rRNA gene sequences using the SILVA database SINA aligner v128 (59). A
410 Shapiro-Wilk test of normality was conducted in base R (60) on the distribution of taxa
411 recovered from both media types. After concluding the data were non-parametric, we
412 performed a Kruskal-Wallis test in R (Taxa ~ Media Type).
413
414 *Taxonomic selection for phylogenetic reconstruction*: To reconstruct a phylogeny of full
415 length 16S rRNA genes, our culture sequences were compared to NCBI's Microbial Genomes
416 and environmental sequences using web-blast (61). The top five hits for each sequence, from
417 either NCBI database, were chosen based on the highest percent coverage and lowest e-value
418 score. *E. coli* K-12 was used as the outgroup of the Alphaproteobacterial phylogeny, and
419 *Bacillus subtilis* was used as the outgroup for the Actinobacterial tree. These sequences aligned
420 with MAFFT (62) with turn checking enabled to conform sequence orientation to the outgroup.
421 The alignment was then trimmed using TrimAl (63) with the automated1 setting to optimize
422 sequence trimming for maximum likelihood phylogenetic analyses. We reconstructed
423 phylogenetic relationships from this trimmed alignment in the CIPRES Gateway (64).
424 Maximum-Likelihood (ML) trees were constructed using IQ-TREE with 10,000 ultrafast
425 bootstrap trees and Bayesian Information Criterion to select the best fit nucleic acid
426 substitution model (65, 66). After an initial round of ML trees, sequence alignments were
427 heuristically curated with IQ-TREE to eliminate duplicitous sequences. Finalized ML trees were
428 then imported into the ARB environment (67), where any duplicate sequences from our AZCC
429 cultures were added to the ML trees through ARB's quick add parsimony function. Final trees
430 were visualized with FigTree (68).
431
432 *Environmental contextualization of AZCC isolates:* We matched the AZCC isolate full length
433 16S rRNA gene sequences against a database of the clustered OTUs from the shallow soil
434 depth profile samples (see *'soil microbial community analysis,'* above) using the usearch_global
435 command (69) at a stringency of ≥97% identity, in both strand orientations, with maxaccepts=1
436 and maxrejects=0.
437

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438 *Data Availability:* Full length Sanger Sequenced 16S PCR product sequences are
439 available on NCBI GenBank, accession numbers: MK875836 - MK875967. Illumina data are
440 available on the NCBI SRA, accession numbers: SRR9172130-SRR9172198.
441
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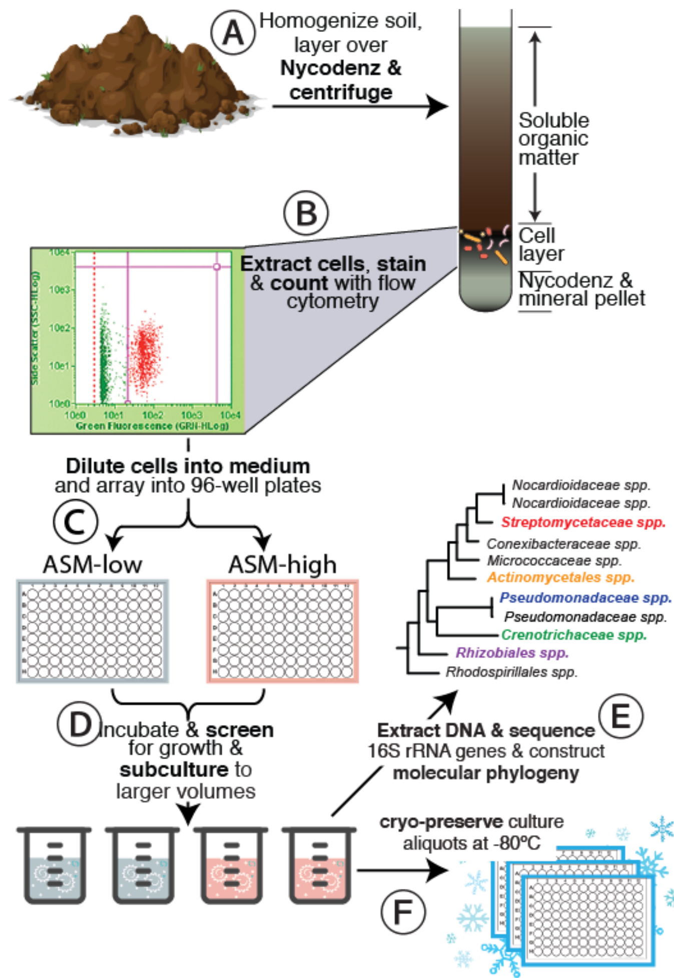

634
635

636 **Figure 1: Dilution-to-extinction workflow.** Soils are collected and brought to the lab where
637 they are homogenized in cell extraction buffer, layered over a Nycodenz solution, and
638 centrifuged (A). The cell layer is extracted from the Nycodenz solution and counted with flow
639 cytometry (B). Counted cells are diluted into growth medium in 96-well microtiter plates to an
640 average density of 5 cells well^{-1} (C). After incubation, the 96-well microtiter plates are screened
641 for growth with flow cytometry, and wells displaying growth are subcultured into larger
642 volumes (D). After incubating the subcultures, flasks displaying growth are identified by 16S
643 rRNA gene sequencing and molecular phylogeny (E). Aliquots of these identified subcultures
644 are cryopreserved at -80ºC (F).
645

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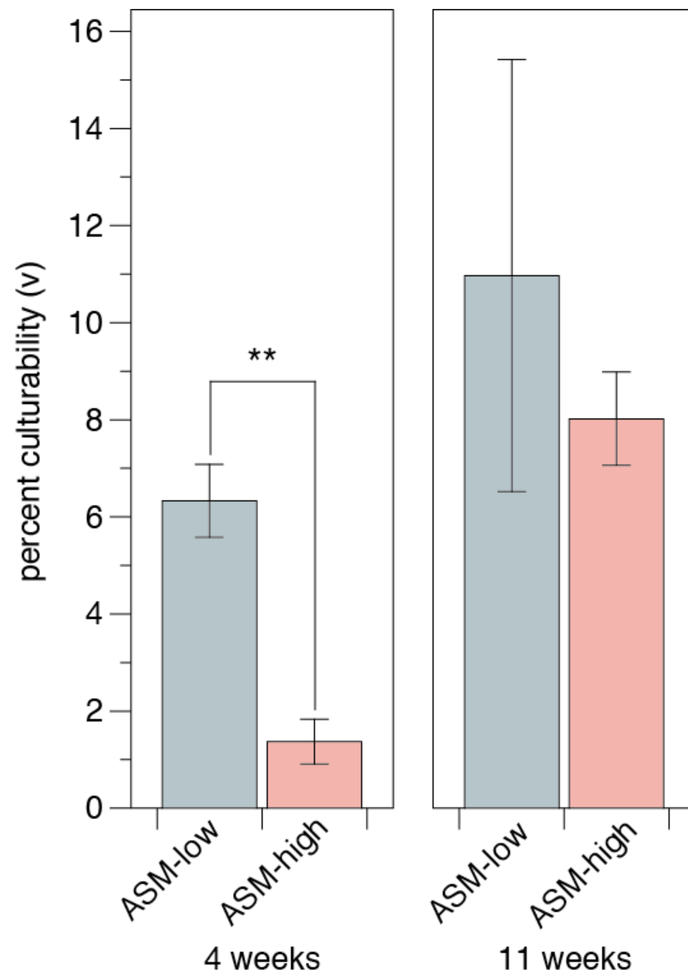

646
647 **Figure 2: Microbial percent culturability (v) is greater on ASM-low than on ASM-high.** Bar
648 heights are the mean culturability ± standard deviation in 96 well microtiter plates (n=3) as
649 calculated from the initial cell inoculum and the proportion of wells positive for growth. Double
650 asterisks indicate Wilcoxon rank sum test P values ≤ 0.05.

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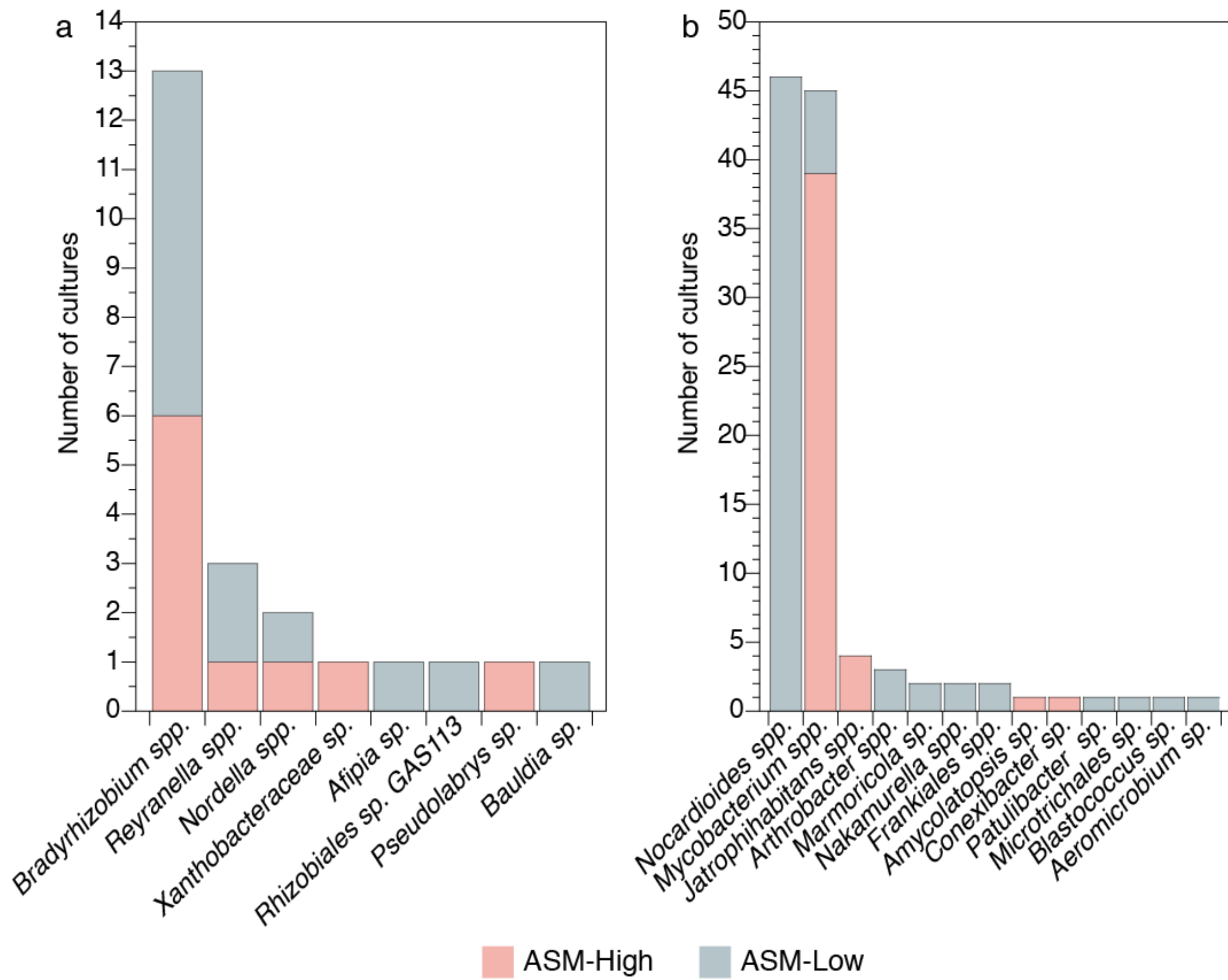

651
652 **Figure 3: ASM-low and ASM-high cultured distinct alphaproteobacterial (a) and**
653 **actinobacterial (b) microbial taxa.** Bar heights are the number of cultures obtained for each
654 taxon and are colored by the medium type on which they were isolated.
655

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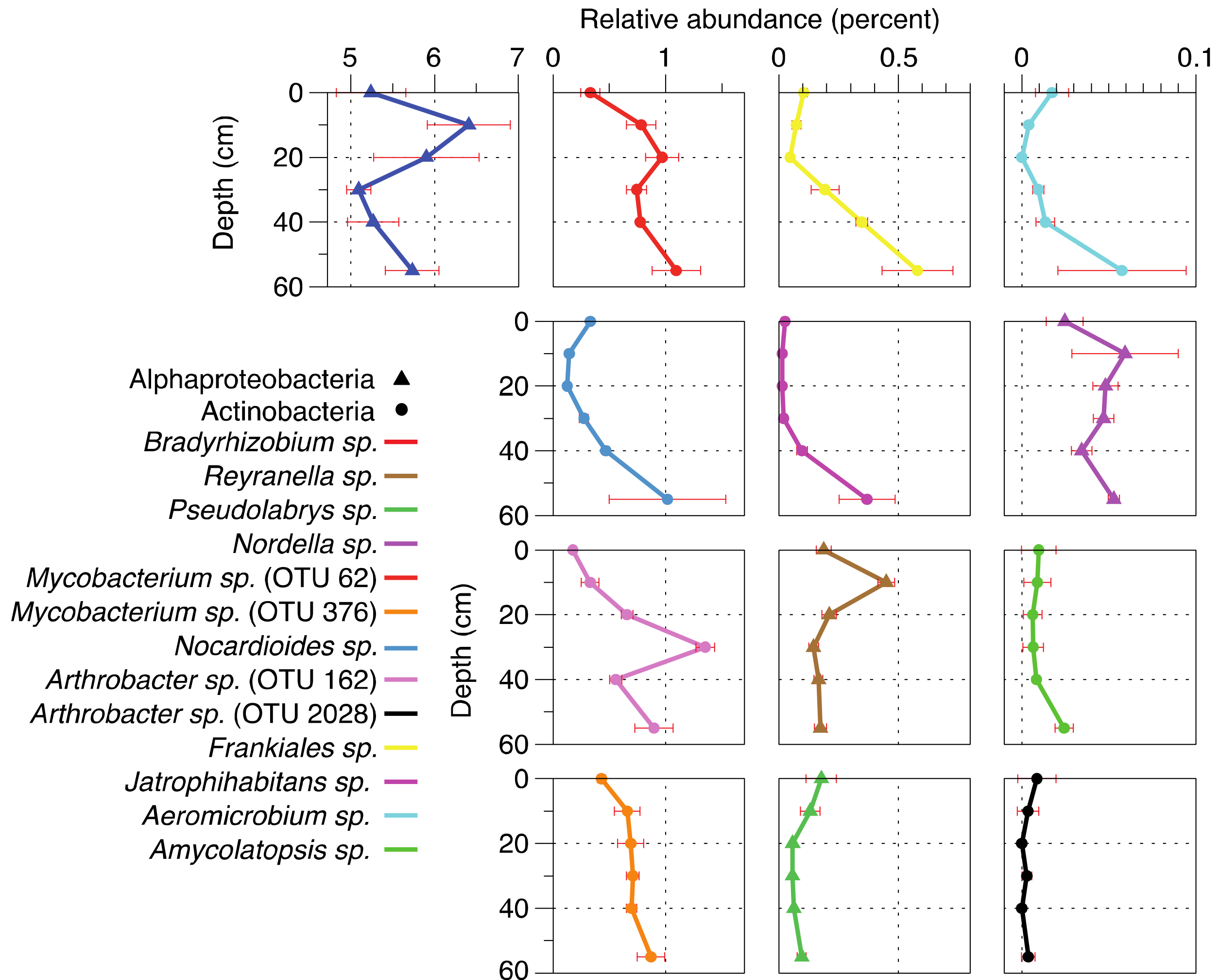

656
657 **Figure 4: The cultures isolated in this study are representative of several abundant soil**
658 **lineages that show dynamic depth distributions in Oracle Ridge soils.** Points are the mean
659 relative abundances ± standard deviation (n=3) of 16S rRNA gene sequence OTUs that
660 matched cultured isolates at ≥97% identity. Error bars that are not visible are located behind
661 the symbol. *Mycobacterium* and *Arthrobacter* cultures best-matched more than one OTU in the
662 cultivation-independent surveys. The best-matching OTU number is designated in
663 parentheses.
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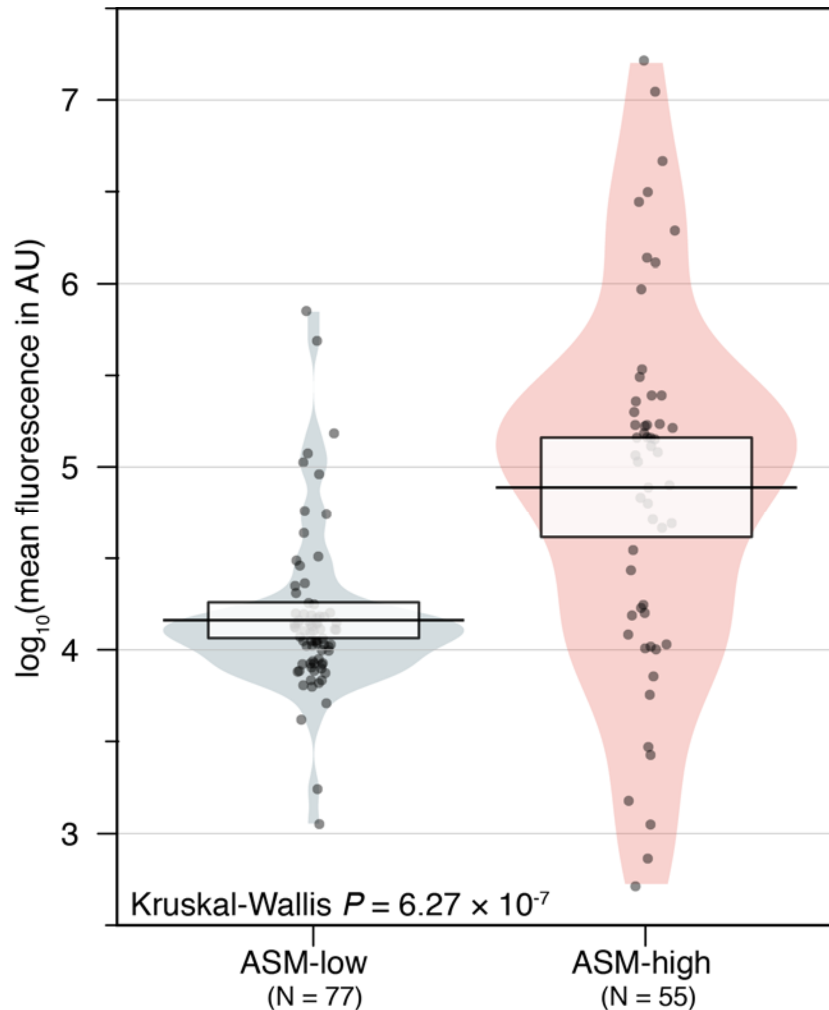

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667 **Figure 5: The mean nucleic acid fluorescence of taxa isolated on ASM-low was significantly**
668 **lower than for those microbes isolated on ASM-high.** Points are the mean $\log_{10}$ fluorescence
669 (in arbitrary units [AU]) of fixed and SYBR green I-stained stationary phase cultures. The mean
670 fluorescence value was obtained from manually-gated histogram plots of fluorescence within
671 the Guava EasyCyte software. Only those cultures that were defined as pure cultures are
672 plotted. The horizontal line in each plot is the mean fluorescence value and the box
673 surrounding the mean is a 95% confidence interval. Shading illustrates the relative distribution
674 of fluorescence values within each medium type.
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