**Title:** Bulk and spatially resolved extracellular metabolomics of free-living nitrogen fixation

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**Abstract**

Soil microorganisms drive ecosystem function, but challenges of scale between microbe and ecosystem hinder our ability to accurately quantify and predictively model the soil microbe-ecosystem function relationship. Quantifying this relationship necessitates studies that systematically characterize multi-omics of soil microorganisms and their activity across sampling scales from spatially resolved to bulk measures, and structural complexity, from liquid pure culture to *in situ*. To address this need, we cultured two diazotrophic bacteria in liquid and solid media, with and without nitrogen (N) to quantify differences in extracellular metabolites associated with nitrogen fixation under increasing environmental structural complexity. We also quantified extracellular metabolites across sampling scales including bulk sampling via GC-MS analysis and spatially resolved analysis via MALDI mass spectrometry imaging. We found extracellular production of inorganic and organic N during free-living nitrogen fixation activity, highlighting a key mechanism of terrestrial N contributions from this process. Additionally, our results emphasize the need to consider the structural complexity of the environment and spatial scale when quantifying microbial activity. We found differences in metabolite profiles between culture conditions, supporting previous work indicating environmental structure influences microbial function, and across scales, underscoring the need to quantify microbial scale conditions to accurately interpret microbial function.

**Importance:** Studying soil microorganisms, both who is present and what they are doing, is a challenge because of vast differences in scale between microorganism and ecosystem and because of inherent complexities of the soil system (e.g., opacity, chemical complexity). This makes measuring and predicting important ecosystem processes driven by soil microorganisms,

like free-living nitrogen fixation, difficult. Free-living nitrogen fixing bacteria play a key role in terrestrial nitrogen contributions and may represent a significant, yet overlooked, nitrogen source in agricultural systems like bioenergy crops. However, we still know very little about how free-living nitrogen fixation contributes nitrogen to terrestrial systems. Our work provides key insight by hierarchically increasing structural complexity (liquid vs. solid culture) and scale (spatially resolved vs. bulk) to address the impact of environmental structure and sampling scale on detection of free-living nitrogen fixation and to identify the forms of nitrogen contributed to terrestrial systems by free-living nitrogen bacteria.

## Introduction

Soil microorganisms are a key link between above and belowground ecosystem function, driving energy and nutrient transfer between the atmosphere, biosphere, and pedosphere (1-3). Multi-omic analysis aimed at understanding the structure and function of these microorganisms has become a routine tool for environmental samples. Despite generating large amounts of data, even quantitatively linking ‘omics of a specific function (e.g. functional genes and proteins) to measures of that function is often unsuccessful (2-6) because of inherent challenges of studying soils (5-7) and soil microorganisms. Given these challenges, the importance of linking microbial community structure to function has been questioned (8, 9), however without clearer understanding of how microorganisms relate to observed functions we cannot yet rule out the importance of individual microbial community members. Our limited ability to quantitively link soil microbial communities to ecosystem function hinders our understanding of ecosystem processes, leaving us vulnerable to losing vital ecosystem services provided by our soils, particularly in the face of climate change (10).

92 Quantifying the link between soil microorganisms and ecosystem function requires
93 systematic studies that characterize multi-omics of soil microorganisms and their functions
94 hierarchically across scales of space and complexity (Fig. 1; (4, 11). *In vitro* studies using pure
95 cultures or limited species are an appealing option and have the potential to provide fundamental
96 microbial and ecological knowledge (10, 12, 13). However, culturing conditions are often quite
97 different from those experienced by microorganisms in soil and attachment to surfaces has been
98 shown to impact microbial growth and function (14, 15). Thus, physical structure influences
99 microbial function, and it is therefore essential for studies to systematically characterize multi-
100 omics and function *in vitro* under growth conditions of increasing complexity (e.g.
101 environmental structure) in order to determine how culture work may better inform *in situ*
102 processes (Fig. 1).

103 In this study, we explored two questions surrounding the microbe-ecosystem function
104 relationship: (1) Are there microbial metabolomic signatures associated with target ecosystem
105 processes? (2) How do growth conditions and sampling scale influence metabolomic signatures?
106 We used free-living nitrogen fixation (FLNF), biological nitrogen fixation (BNF) carried out by
107 heterotrophic bacteria (diazotrophs), as a model microbial process and ecosystem function to
108 address these questions. FLNF is carried out by a wide diversity of soil bacteria and occurs in all
109 terrestrial biomes, contributing significantly to terrestrial N (16, 17). These contributions are
110 thought to occur predominately through release of ammonium and organic N sources, like amino
111 acids, as occurs during symbiotic BNF (18-21). Thus, extracellular metabolites may be valuable
112 to understanding FLNF and identifying biosignatures.

113 We examined extracellular metabolites from two diazotrophic bacteria cultured under
114 conditions that promote (N-free) or inhibit (N-rich) FLNF. Additionally, cultures were grown in

115 liquid or on solid media to examine the impact of physical structure on detected metabolites.
116 Lastly, we examined extracellular metabolites across sampling scale from spatially resolved
117 measures using matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI–
118 MSI) to bulk sampling via gas chromatography–mass spectrometry (GC-MS) analysis. We
119 hypothesized: (1) bulk metabolite profiles differ between N-rich and N-free conditions and also
120 between liquid and solid cultures, (2) there are extracellular metabolites associated with FLNF
121 activity and only observed under N-free conditions, (3) these extracellular metabolites are
122 detectable with bulk and spatially resolved sampling, and (4) N-containing compounds are
123 produced during FLNF and are readily detectable in bulk and spatially resolved extracellular
124 metabolites under N-free conditions.

125

126 **Results**

127 *Microbial biomass – total biomass, biomass C, and biomass N*

128 Total microbial biomass, including cells and associated debris such as EPS, was collected
129 from all treatments except for the AV N-rich solid treatment. In this case, microbial colonies had
130 grown into and below the agar surface and it was not possible to collect biomass. Total biomass
131 was highly variable across all treatments (CV across treatments ranged from 4.6% to 100.4%)
132 and there were no significant differences observed with N treatment, culture type, or organism
133 (Fig. 2D). Both biomass C and N differed significantly across treatments (Fig. 2A and 2B,
134 respectively). In general, C and N content were greater in N-rich treatments where N was readily
135 available compared to N-free treatments. There was also a trend towards greater biomass C and
136 N from solid media, but this was mostly observed in the N-rich treatments. These C and N values
137 translated to C:N ratios that predominately differed only between N treatments with the N-free

138 treatment resulting in greater biomass C:N ratios than N-rich conditions, regardless of culture
139 type or organism (Fig. 2C).
140
141 *Extracellular ammonium availability*
142 Extracellular ammonium availability was measured supernatant and rinsate samples and
143 was detected in all treatments regardless of culture type, N treatment, or organism. On a per unit
144 biomass basis, ammonium concentrations differed significantly by organism ($F = 16.390$, $p =$
145 $0.0012$) and by the interaction between culture type and N treatment ($F = 35.411$, $p < 0.0001$).
146 Extracellular ammonium availability per unit biomass was over 8x greater in PP than AV
147 cultures (Fig. 3A). Under N-free conditions, ammonium availability was greater in liquid than in
148 solid culture while in N-rich conditions the opposite was observed (Fig. 3B). Extracellular
149 ammonium availability is of particular interest in N-free treatments as ammonium is
150 hypothesized to be released from cells actively fixing N and thus represents a major form of N
151 contributed by FLNF to terrestrial systems (18, 33). Therefore, we also calculated the percent of
152 fixed N available as extracellular ammonium (Fig. 4). Total fixed N was estimated as total
153 biomass N measured in N-free treatment samples. We find only upwards of 7.5% (± 2.3) of fixed
154 N is readily available as extracellular ammonium. This did not differ significantly between
155 culture types ($F = 2.171$, $p = 0.184$), though ammonium concentrations in solid culture tended to
156 be lower than those in liquid culture.
157
158 *Bulk extracellular metabolites*
159 Across all treatments, 307 metabolites were detected with bulk sampling and of these 93
160 were successfully annotated (>80% confidence). The total number of detected metabolites

161 differed between treatment groups (Supp. Fig. 2) with generally more metabolites detected in N-
162 free treatments, the majority of which were within the unannotated portion of detected
163 metabolites. Distinct metabolite profiles, represented by Bray-Curtis and Jaccard distance based
164 on all detected metabolites, were observed between N treatment, culture type, and their
165 interaction (Fig. 5; Table 1). N treatment and culture type, together with their interaction, explain
166 over 50% of the variance in metabolite profiles based on peak intensity (Fig. 5A) and based on
167 presence-absence (Fig. 5B). Metabolite profiles based on abundance separated predominantly by
168 culture type and then by N treatment (Fig. 5A). Metabolite profiles based on presence-absence
169 show clear separation between culture types for N-rich treatments but have little separation under
170 N-free conditions (Fig. 5B).

171 Because FLNF activity is hypothesized to result in the release of N-containing
172 metabolites (33), we focused on N-containing extracellular metabolites. Of the 93 annotated
173 metabolites detected through bulk sampling, 35 were N-containing. We found significant
174 differences in N-containing compounds across treatments, after correcting for background
175 metabolites, with significant interactions between N treatment, culture type, and organism ($F =$
176 $10.695$, $p = 0.0048$). N-free treatments were richer in N-containing metabolites (Supp. Fig. 3) but
177 had similar or significantly lower total abundances of N-containing metabolites compared to N-
178 rich treatments (Fig. 6). Examining the specific composition of these N-containing compounds,
179 we found a variety of amino acids in N-free samples not well represented in N-rich samples
180 (Supp. Fig. 3), but only a few N-containing metabolites were unique to N-free conditions
181 including pantothenic acid, L-pyroglutamic acid, L-glutamic acid, and 4-pyridoxic acid.

182 *Spatially resolved extracellular metabolites*

183 Across all treatments, METASPACE analysis identified 69 metabolites in spatially
184 resolved samples of which 41 were N-containing. However, only a few potential amino acids
185 were detected at this resolved microbial scale including L-leucine and L-valine. These were only
186 at detectable concentrations within the N-rich treatment (Fig. 7) unlike the diversity of amino
187 acids detected in bulk samples predominately in association with N-free treatments.
188 Observationally, N-free treatments seemed to be characterized by unique presence of organic
189 acids rather than N-containing compounds. However, we did identify a few N-containing
190 compounds unique to N-free treatments at the microbial scale including inosine and 4-pydroxic
191 acid (Fig. 7). Inosine was detected in N-free treatments of both AV and PP and was not at
192 detectable levels in N-rich treatments. Also, much like bulk sampling scale detection, 4-
193 pyridoxic acid was exclusively detected in AV N-free treatment samples.
194
195 **Discussion**
196 We explored the impact of N availability and physical structure on the extracellular
197 metabolomics of diazotrophic bacteria across sampling scales from the spatially resolved to bulk.
198 We find evidence of extracellular organic and inorganic N contributions from FLNF
199 underscoring a key mechanism of terrestrial N contributions from FLNF. In general, we find
200 physical structure and microbial function (e.g. FLNF) alter extracellular metabolite profiles and
201 influence the detection of metabolites at bulk and spatially resolved scales.
202
203 *Nitrogen contributions from FLNF*
204 Products of BNF by symbiotic diazotrophs are well-studied and typically observed as
205 ammonia and ammonium with contested evidence for production of amino acids (18-21). This

206 knowledge of symbiotic BNF is thought to translate directly to FLNF leading to the assumption
207 that free-living diazotrophs also excrete ammonia/ammonium into the surrounding environment
208 during BNF. However, ammonia produced during FLNF is rapidly assimilated through
209 conversion to glutamine or glutamate via the glutamine synthetase (GS) and glutamate
210 synthetase (GOGAT) pathways (34). Thus, excreted ammonium would necessarily be in excess
211 of these assimilation pathways (34). Ammonium excretion has been observed in wild-type
212 *Azotobacter vinelandii* DJ, at concentrations between ~2 and ~25 μM (35, 36), values within
213 range of those measured in this study (Supp. Fig. 4). However, in many cases measurable
214 ammonium excretion was only observed from *Azotobacter vinelandii* cultures genetically altered
215 to disrupt the GS-GOGAT pathways or facilitate constitutive nitrogenase synthesis (37-40).
216 An alternative hypothesis to ammonium excretion is that N contributions occur as organic
217 N, either through direct release of N-rich compounds like amino acids (18, 21) or through
218 turnover of dead biomass (41). Our bulk metabolomics data support this hypothesis with many
219 N-containing organic compounds, including amino acids detected in N-free treatments. In fact,
220 N-free treatments were richer in N-containing compounds than N-rich conditions, particularly
221 when comparing against N-rich solid media which had few N-containing metabolites (Supp. Fig.
222 3). The structure of our study did not allow us to determine whether these organic molecules
223 were directly excreted by active, N-fixing cells or released during cell turnover. However, other
224 metabolites detected in the system suggest cell turnover contributed at least partially to this N
225 release. For example, we detected inosine in both bulk and spatially resolved analysis, and it was
226 unique to N-free treatments in spatially resolved samples. Inosine, a metabolic product of
227 adenine degradation likely indicates salvage activities by the bacterial populations (42, 43) and
228 could indicate freely available nucleotides from cell lysis and turnover. FLNF may therefore

229 contribute available N through increasing microbial biomass and turnover, but this needs to be
230 verified in future studies. Regardless of whether these N-containing compounds are actively
231 excreted or released after cell death, this metabolic exchange with the surrounding environment
232 highlights a key mechanisms of terrestrial N contributions from FLNF.
233
234 *In vitro vs in situ analysis – identification of biosignatures*
235 Through bulk and spatially resolved analysis, we found few N-containing metabolites
236 exclusive to N-free treatments. At bulk scale, these include pantothenic acid, L-pyroglutamic
237 acid, L-glutamic acid, and 4-pyridoxic acid. We similarly find 4-pyridoxic acid at the spatially
238 resolved microbial scale as well as nine other metabolites including inosine. 4-pyridoxic acid
239 was unique to AV N-free treatments at the microbial scale. However, despite being uniquely
240 associated with N-free treatments and therefore microbial populations actively fixing N, it may
241 be difficult to assign these as a signature of FLNF function. Of these compounds, only L-
242 glutamic acid has a direct association with the FLNF pathway. Other metabolites seem more
243 indicative of microbial nutrient needs and function. For example, pantothenic acid, vitamin $B_5$, is
244 involved in the synthesis of coenzyme A and is a coenzyme for many reactions involved in
245 protein and lipid metabolism (44-46). This is particularly important for the processing of organic
246 acids like malate, the main C source provided in this study. Thus, the detection of vitamin $B_5$ is
247 likely indicative of malate metabolism via the TCA cycle and its unique detection in the N-free
248 treatment suggests a higher respiration rate in these N-fixing populations than in the N-rich
249 populations. Increased respiration is a common response among diazotrophs in oxygenated
250 environments as a protection mechanism to prevent or reduce denaturation of nitrogenase via
251 oxygen (17, 47, 48). We also identified 4-pyridoxic acid, a derivative of pyridoxine (vitamin $B_6$).

252 Pyridoxine is a key cofactor in amino acid, fatty acid, and carbohydrate metabolisms, but can
253 also act an oxygen protectant (46). During this redox reaction, pyridoxine degrades and can
254 result in 4-pyridoxic acid. *A. vinelandii* has been observed to produce B vitamins while under
255 diazotrophic conditions and this seems to be a hallmark of FLNF for this organism (46, 49, 50).
256 Though not directly associated with the N-fixation pathway, these vitamins may tangentially
257 indicate bacteria functions surrounding FLNF such as oxygen regulation and highlight the need
258 to analyze bacterial function holistically rather than focusing on single reactions or pathways.
259 Additionally, the limited number of unique extracellular metabolites detected in N-free
260 treatments suggests some microbial functions may not have detectable or unique biosignatures,
261 in the form of extracellular metabolites. This is an important consideration when applying
262 metabolomics to the study of complex soil systems. Soil metabolomics are increasingly being
263 used to study soil microbial ecology and biogeochemical function and have been successfully
264 applied to soil carbon cycling (51-54). However, metabolites are by definition the by-products of
265 and substrates for metabolic function, and turnover rapidly in soils (55, 56). Therefore, typical
266 soil extractions to collect extracellular components (e.g. $K_2SO_4$ extracts, leachate; (57, 58) only
267 capture what is not consumed by the microbial community. This includes metabolites available
268 in dissolved organic matter pools at the time of sampling and metabolites readily exchangeable
269 from mineral surfaces (59). In both cases, metabolites could be temporally separated from their
270 originating processes making it difficult to trace back the associated metabolic pathway. It could
271 be even more challenging to capture metabolic biosignatures from nutrient-limited communities,
272 such as those in bulk soil. Under nutrient-limited conditions, resulting metabolic products are
273 likely to be rapidly assimilated or, in the case of processes like FLNF, not released to the
274 surrounding environment. The potential signature compounds of FLNF found here (e.g. amino

275 acids and B-vitamins) are also not uniquely produced by FLNF processes and would be difficult
276 to directly link to FLNF *in situ*. We acknowledge our study system may provide a biased view on
277 this issue, being a closed incubation system unlike soils where metabolites may diffuse away
278 from microbes and persist in the environment. However, these findings highlight a key need to
279 understand the soil microhabitat (11).

280 Lastly, our work highlights the importance of considering culture conditions and their
281 association to *in situ* conditions as we observed clear differences in metabolite profiles between
282 liquid and solid culture. Interestingly, though culture type strongly influenced metabolite
283 profiles, it played a secondary role to N treatment in influencing the number of detected
284 metabolites. This was particularly notable when N was readily available, where presence-
285 absence based profiles were distinct between liquid and solid culture, but only under N-rich
286 conditions. These differences in metabolite profiles were likely not driven by differences in
287 biomass production as culture type had small and non-significant impacts on microbial metrics,
288 like total biomass, and biomass C and N content. Thus, these responses seem specifically
289 associated with the presence or absence of physical structure in the environment. Additionally,
290 these findings suggest nutrient limitation, as experienced in the N-free treatments, may be a
291 stronger driver of microbial activity than physical structure and simplified liquid culture may be
292 somewhat informative to nutrient-limited *in situ* conditions.

293

294 *Implications scaling from microbial scale to bulk sampling*

295 The combination of techniques in this study allowed us to explore detection of
296 metabolites across scales from spatially resolved, relevant to microorganisms, to bulk, relevant
297 for soil microbial ecology analysis. MALDI MSI allowed us to resolve abundances of

298 extracellular metabolites on solid media at a microbial scale. Using GC-MS, we were able to
299 evaluate detection of extracellular metabolites at the bulk scale. While the detection ranges of
300 these two techniques do not fully overlap (50 – 500 m/z for GC-MS and 92 – 700 m/z for
301 MALDI), many metabolites of interest to this study are measurable with both techniques
302 providing valuable information about metabolite detection and sampling scale. It is also
303 important to note that a lack of detection is not equivalent to metabolite absence but only
304 indicates metabolite concentrations were below detection.

305 Through bulk sampling, we found a wide variety of N-containing compounds in N-free
306 samples, but generally lower abundances of N-containing compounds than in the N-rich
307 treatment. While N-containing compounds are characteristic of N-free samples at a bulk scale,
308 these treatments had fewer N-containing metabolites when spatially resolved at the microbial
309 scale. Interestingly, there was a shift in amino acid detection between spatially resolved and bulk
310 scales where amino acids were commonly detected in N-free samples at bulk scale, but in N-rich
311 samples when spatially resolved. This somewhat counterintuitive result highlights differences in
312 N competition at the microbial scale and its influence on bulk measurements.

313 First, detection of a diverse array of amino acids in the N-free treatment in bulk sample,
314 but not in spatially resolved samples suggests N competition at the microbial scale resulted in
315 rapid uptake of amino acids, while extraction of the bulk metabolite pool likely captured the
316 cumulative low abundance signal of the entire system. Amino acids are shown to have short
317 residence times in soils and experience rapid uptake and turnover (60, 61). In the case of
318 microbial vs. bulk scale, it is likely the spatially resolved pool of extracellular amino acids
319 collected from microbial colonies (~200 μm spatial resolution) was small and often below
320 detection. However, in bulk sampling of millions of cells, a larger pool of amino acids coupled to

321 our sampling method could have allowed amino acids to diffuse away and accumulate to
322 detectable levels.

323 Second, biofilm formation is likely to influence diffusion of metabolites into the
324 surrounding environment (62, 63). Bacteria tend to live in biofilms in their natural environments
325 rather than as individually dispersed cells (64). However, the impact of surrounding
326 environmental conditions, including nutrient availability, on biofilm production is unclear. For
327 example, some studies suggest nutrient limiting conditions may promote greater biofilm
328 formation (65), while others suggest biofilm formation is greater under more favorable growth
329 conditions (66, 67). This is particularly notable for diazotrophs as biofilms can play a role in
330 oxygen protection (47), thus investment in biofilm could be beneficial to FLNF activity. Yet,
331 under severe N limitation imposed by an N-free environment the high energy demands of FLNF
332 may limit investment in biofilm. While not directly measured in this study, we noted solid agar
333 plates of *Azotobacter vinelandii* and *Paenibacillus polymyxa* cultures had visually greater
334 biofilm production under N-rich than N-free conditions. Thus, diffusion of amino acids away
335 from populations would have been more easily achieved in the N-free treatment. This is
336 evidenced by the similarity between metabolite chemistry in liquid and solid culture under N-
337 free treatments (Fig. 5B). Similarly, a small number of amino acids were detected in spatially
338 resolved samples from N-rich treatments, but not in bulk samples for the similar N-rich solid
339 media treatment. This could have resulted from greater biofilm formation under N-rich
340 conditions and limited diffusion of small molecules away from cell populations.

341 The detection of small molecules across scales has important implications for the
342 influence of soil microbial communities on their surrounding environment. In general, our results
343 indicate microbial scale processes drive bulk metabolite availability. The N-rich treatment in this

experiment is an optimal environment and most representative of carbon and nutrient rich soil environments like the rhizosphere or detritusphere. Our findings suggest these conditions would result in production of valuable small molecules, like N-rich amino acids, potentially exchangeable with the immediate environment, but biofilm formation may limit diffusion far into the soil environment. Under limiting conditions of the N-free treatment, similar those of bulk soil, microbial activity produces valuable metabolites, like amino acids, but competition between microbes reduces the exchange of these molecules. Understanding how these differences in microbial scale conditions influence microbial activity and detectability of function is crucial to accurately linking microbe and ecosystem.

## Conclusions

We demonstrate extracellular production of inorganic and organic N during FLNF and reveal the importance of habitat conditions and sampling scale when quantifying microbial activity. Across bulk and spatially resolved sampling scales, we identified metabolites uniquely associated with FLNF activity including several B-vitamins, which may play roles in mitigating oxygen damage to nitrogenase. Despite finding unique metabolites and potential biosignatures, many detected metabolites are not exclusively produced through FLNF related pathways, thus would be difficult to assign to FLNF for *in situ* soil samples. This would likely hold true for other processes under nutrient limited conditions where metabolic products are rapidly assimilated and not captured during sampling. Our findings highlight the need to carefully consider both structural complexity and sampling scale when quantifying microbial function. We found culture conditions to be a key driver of metabolite chemistry under N-rich and N-free conditions, indicating physical structure influences microbial processes. Across scales, our

367 results indicate high N competition at the microbial scale under N-free conditions, while at the
368 bulk scale N appeared readily available within the microbial environment. These differences in
369 environmental conditions across scales could lead to incorrect interpretations of microbial
370 function as immediate conditions surrounding microorganisms will drive their activity and may
371 not necessarily match what is measured through bulk or composite sampling.
372

373 **Materials and Methods**

374 *Culture conditions*

375 Two diazotrophic bacteria with distinct growth strategies (e.g. gram-negative vs gram-
376 positive) and fully sequenced genomes (22, 23)were chosen for this study, *Azotobacter*
377 *vinelandii* DJ (ATCC BAA 1303; hereafter AV) and *Paenibacillus polymyxa* (ATCC 842;
378 hereafter PP). Bacteria were cultured under N-free and N-rich conditions, respectively promoting
379 or inhibiting FLNF. Nfb media, commonly used to isolate diazotrophs (24), was used for N-free
380 treatments, and was supplemented with tryptone for N-rich treatments. Both treatments
381 contained 1.79 g C $L^{-1}$ as malic acid and N-rich media contained ~1.33 g N $L^{-1}$ as tryptone.
382 Cultures were grown in liquid or solid agar media and all media was autoclave sterilized prior to
383 inoculation.

384 Thirty samples were cultured (2 organisms x 2 N treatments x 2 media types x 3
385 replicates, plus controls) for bulk analysis with an additional set of 14 samples (2 organisms x 2
386 N treatments x 3 replicates, plus controls) on solid media for spatially resolved analysis. Cultures
387 were grown in a temperature-controlled incubator at 25˚C to $10^7$ CFU $mL^{-1}$, based on liquid
388 cultures, and then harvested for analysis of extracellular metabolites at two scales – bulk
389 sampling via MPLEx extraction and GC-MS (25) and spatially-resolved sampling via colony

390 analysis with MALDI MSI. Extracellular ammonium availability and microbial biomass,
391 including total biomass, biomass carbon (C) and biomass N, were also measured. Because FLNF
392 activity is necessary for microbial growth under N-free conditions, measures of total biomass and
393 biomass N are used as estimates of FLNF (24).

394 *Sample collection*

395 Extracellular metabolites were collected from liquid culture by centrifuging culture tubes
396 to pellet cells and collecting the resulting supernatant for bulk analysis as described below. Cell
397 pellets were resuspended in autoclave sterilized nanopure water, immediately flash frozen on
398 liquid nitrogen, and stored at -80˚C until further analysis. Extracellular metabolites were
399 collected from solid media for bulk and spatially resolved analysis. Bulk samples were collected
400 by washing culture plate surfaces with autoclave sterilized nanopure water and collecting the
401 resulting rinsate. Samples were collected for spatially resolved analysis as described below.
402 Lastly, microbial colonies from rinsate plates were collected from the surface by gentle scraping,
403 transferred to autoclave sterilized nanopure water, flash frozen on liquid nitrogen, and stored at -
404 80˚C until further processing.

405 *Microbial biomass – total biomass, biomass C, and biomass N*

406 Frozen cell pellets and colonies were lyophilized until completely dry and weighed to
407 obtain total biomass, including cells and associated debris such as exopolysaccharides (EPS).
408 Dried biomass was ground using sterile steel beads and then analyzed for C and N content on a
409 VarioTOC Cube (Elementar, Langenselbold, Germany).

410 *Extracellular ammonium availability*

411 We measured extracellular ammonium concentrations in supernatant and rinsate samples
412 using a high-throughput colorimetric ammonium assay (26). Briefly, samples were pipetted in

413 triplicate into clear 96-well plates and incubated with ammonium salicylate and ammonium
414 cyanurate reagents to facilitate color change via the Berthelot reaction. Plates were read for
415 absorbance at 610 nm on a Synergy H1 plate reader (BioTek Instruments, Inc., Winooski, VT,
416 USA).

417 *Bulk metabolomics – GC-MS*

418 Bulk metabolomics analysis was conducted on 1 ml subsamples of undiluted, supernatant
419 and rinsate samples. Supernatant and rinsate samples were prepared for metabolite analysis via
420 GC-MS following the MPLEx protocol for simultaneous metabolite, protein, and lipid extraction
421 (25). Additionally, 1 ml of supernatant and rinsate from sterile liquid culture and solid culture
422 plates were also extracted via MPLEx as background controls. This extraction method allows
423 simultaneously collection of metabolites, lipids, and proteins; however, lipid fractions were not
424 analyzed in this study. Additionally, protein yields were too low for downstream analysis.
425 Metabolite samples were completely dried under speed-vacuum concentrator and chemically
426 derivatized prior to analysis by GC-MS as reported previously (27). The m/z range of derivatized
427 metabolites scanned was 50 – 550 m/z which can detect organic acids, amino acids, and mono to
428 tri-saccharides. Raw GC-MS data were processed using the PNNL in-house metabolomics
429 database, which can identify metabolites using two dimensional matching factors (fragmented
430 spectrum + retention index (28), and with cross-checking against commercially available NIST
431 20/Wiley 11$^{th}$ GC-MS spectral databases (25, 29).

432 *Spatially resolved metabolomics – MALDI MSI*

433 Samples were prepared for spatially resolved analysis via MALDI-MSI using a
434 previously described workflow (30). Briefly, areas of agar were excised from Petri dishes and
435 placed onto double-sided adhesive copper tape adhered to indium tin oxide (ITO)-coated glass

436 slides (Bruker Daltonics; Supp. Fig 1.). This approach enhanced our sensitivity for analysis in
437 negative ionization mode and improved adhesion of agar onto the MALDI target. Samples were
438 dried at room temperature overnight, then treated with MALDI matrix using a HTX TM-Sprayer
439 (HTX Technologies). For analysis in negative-ion mode, 7 mg $mL^{-1}$ of N-(1-naphthyl)
440 ethylenediamine dihydrochloride (NEDC) in 70% MeOH was sprayed with eight passes at 1,200
441 µL $min^{-1}$, 75°C, a spray spacing of 3 mm, and a spray velocity of 1,200 mm $min^{-1}$. MALDI-MSI
442 was performed on a 15-Tesla Fourier transform ion cyclotron resonance (FTICR)-MS (Bruker
443 Daltonics, Billerica, MA, USA) equipped with SmartBeam II laser source (355 nm) using 200
444 shots $pixel^{-1}$ with a frequency of 2 kHz and a step size of 200 µm. FTICR-MS was operated to
445 collect m/z 92–700, using a 209-ms transient, which translated to a mass resolution of R ~
446 70,000 at 400 m/z. Metabolites in this range can typically be detected to fmol concentrations.
447 *Data Analysis*
448 A factorial ANOVA with N treatment, culture type, organism and their interactions as
449 main effects followed by a Tukey's post hoc test was used to determine treatment differences for
450 measured variables. Prior to statistical analysis, bulk metabolite values were blank corrected by
451 subtracting peak intensities identified in background controls of the associated treatment.
452 Differences in bulk metabolite chemistry were evaluated using distance matrices based on peak
453 intensities (Bray-Curtis) presence-absence (Jaccard) generated from all detected metabolites
454 using R *vegan* (31). Differences between culture type, N treatment, and organism were
455 determined via PERMANOVA using *adonis* in R *vegan*. Spatially resolved metabolite data was
456 acquired using FlexImaging (v 4.1, Bruker Daltonics), and image processing, segmentation, co-
457 localization analysis and visualization were performed using SCiLS (Bruker Daltonics). The list
458 of m/z values that colocalized with the colonies were uploaded to the METLIN

459 (https://metlin.scripps.edu) for putative molecular annotations based only on accurate m/z,
460 secured by using a 3-ppm window during the search. imzML files (created by SCiLS) of our
461 analyses were also uploaded to METASPACE (32) for metabolite annotation based on both
462 accurate m/z and a comprehensive bioinformatics framework that considers the relative
463 intensities and spatial colocalization of isotopic peaks as well as quantifies spatial information
464 with a measure of spatial chaos followed by the estimation of the False Discovery Rate. For this
465 purpose, we used KEGG-v1 and NPA-2019-08 (Natural Product Atlas) databases that are
466 available in METASPACE. METASPACE uses by default 3 ppm window in its annotation
467 engine.
468
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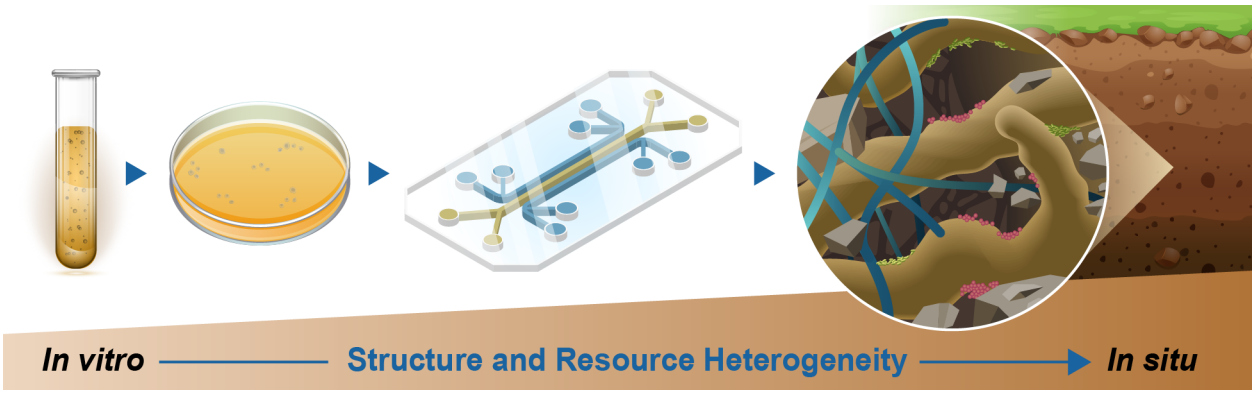
678 **Figure legends**

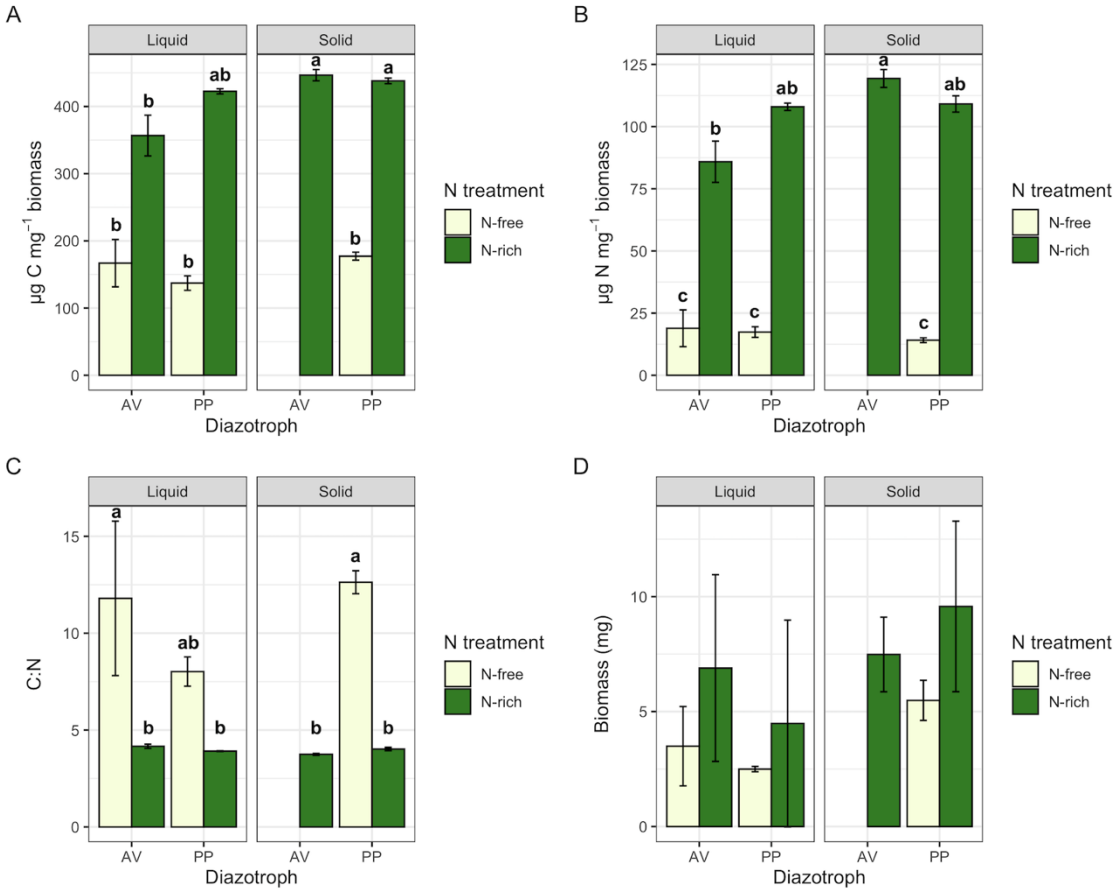



679

680 **Fig. 1**: Depiction of the systematic scaling of system complexity from liquid and solid culture to

681 synthetic soils (represented here as a microfluidic chip) to *in situ* soil conditions. In our study we

682 focus on the first two steps, relating metabolomics in liquid and solid media culture.




683

**Fig. 2:** Microbial (A) C content, (B) N content, (C) C:N ratio, and (D) total biomass. Bars represent average values ± standard error and are colored by nitrogen treatment. Figures are faceted by culture type. Lowercase letters represent significant difference at $p < 0.05$.

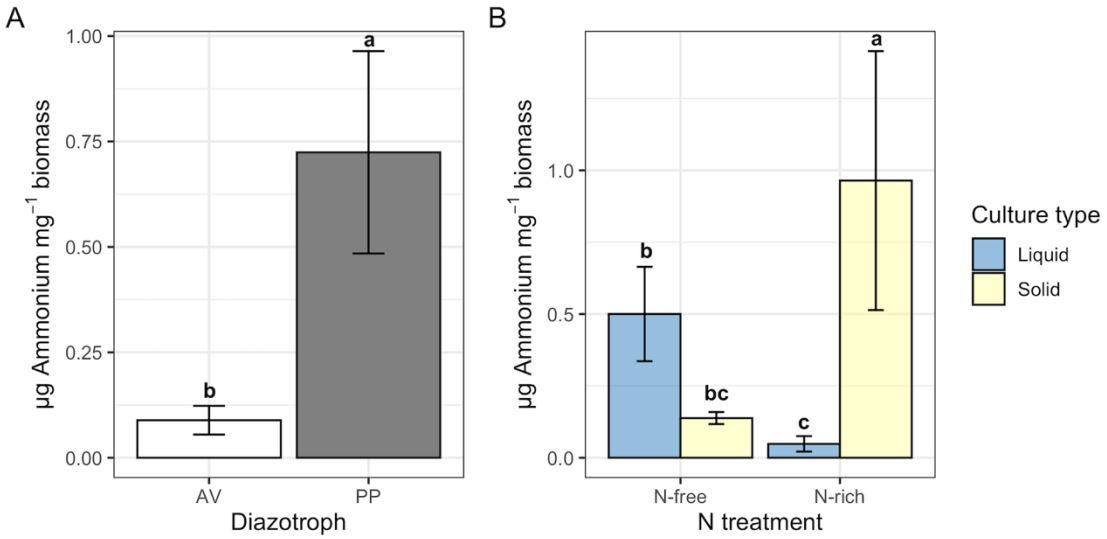



**Fig. 3:** Extracellular ammonium availability per unit biomass shown by (A) diazotrophic organism and by (B) N treatment. Bars represent average values ± standard error. Bars in (B) are colored by culture type. Lowercase letters indicate significant difference at $p < 0.05$.

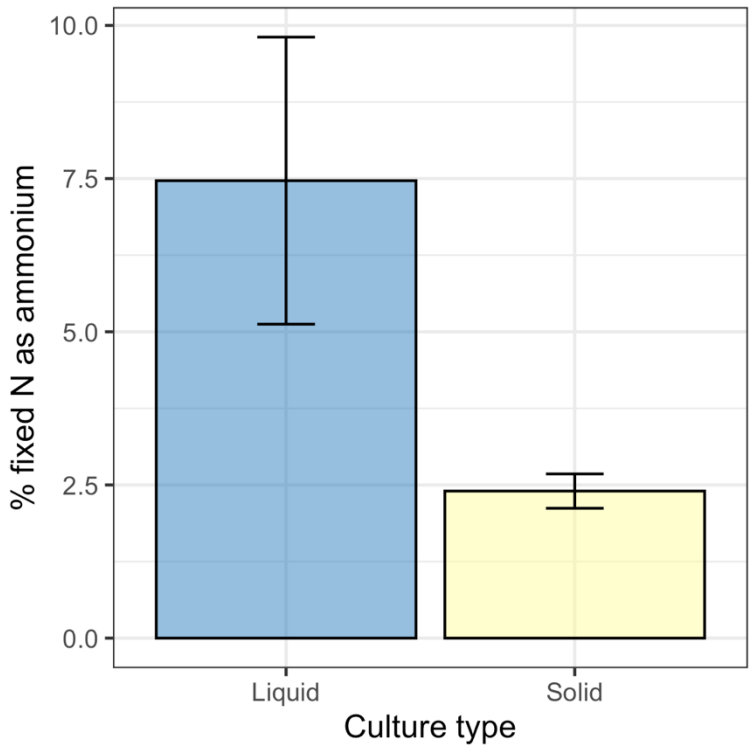
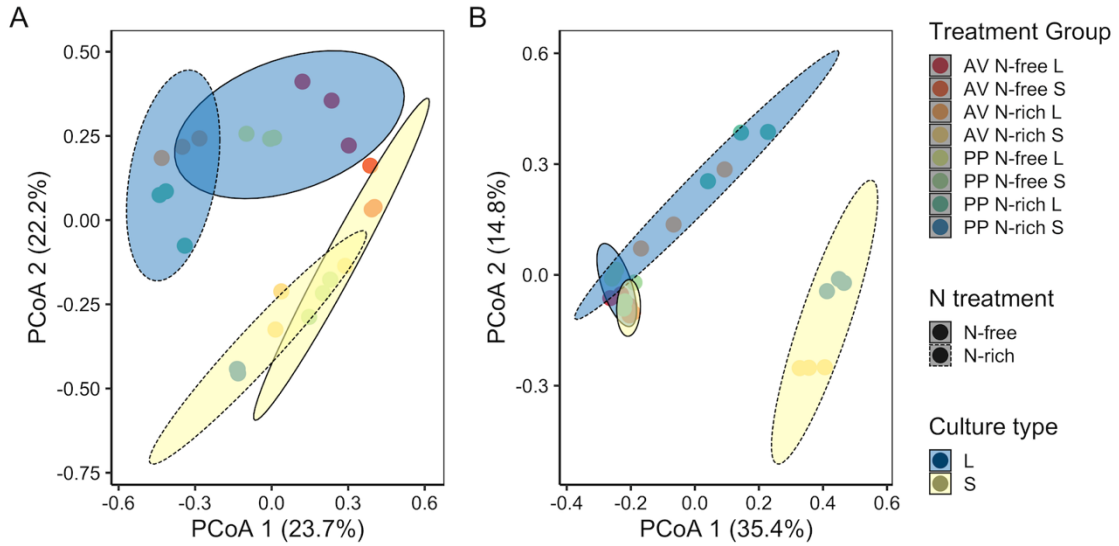
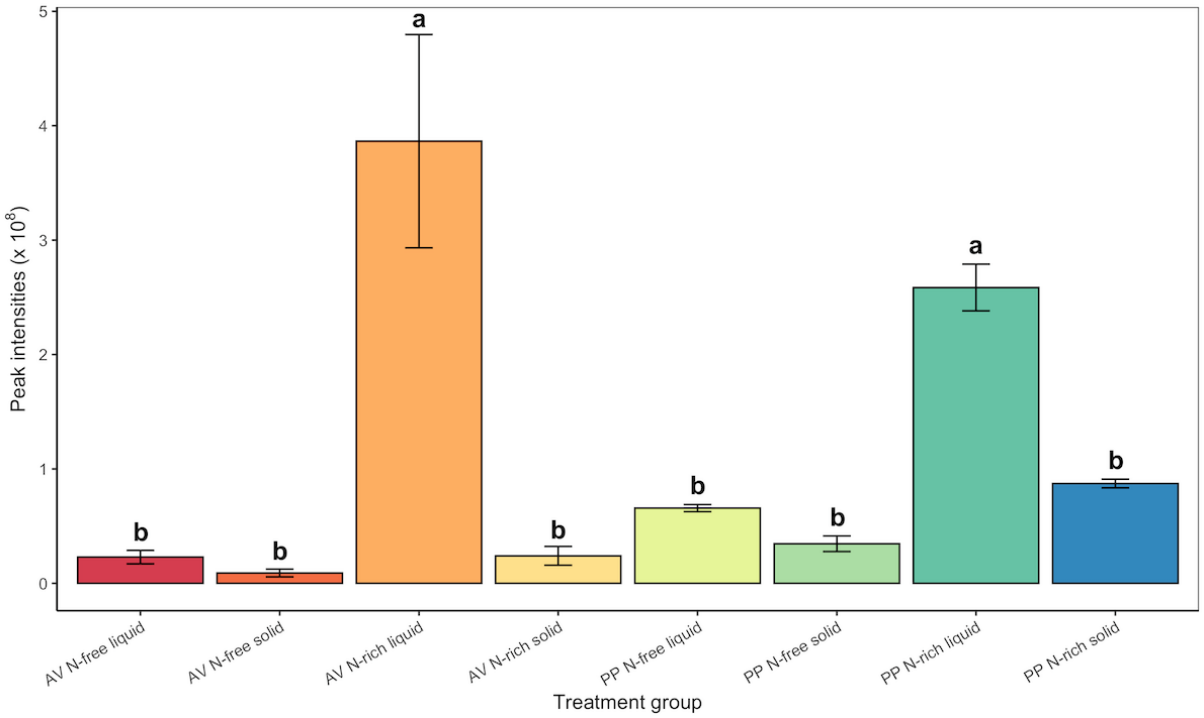



**Fig. 4:** Percent of fixed nitrogen available as extracellular ammonium in N-free treatments. Bars represent average ± standard error. No significant difference was observed between culture types.

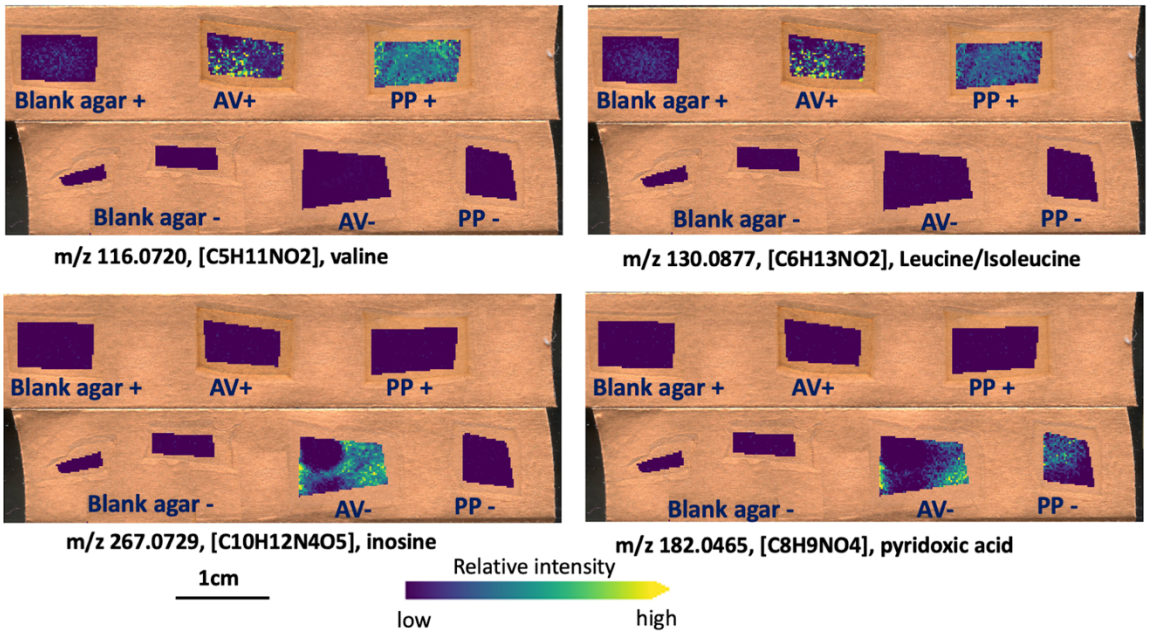


696 **Fig. 5:** Principal coordinates analysis (PCoA) of metabolite chemistry based on (A) Bray-Curtis
697 of peak intensity and (B) Jaccard of presence-absence including all detected metabolites. Each
698 point represents a single sample and are colored by treatment group (organism, N treatment,
699 culture type). 95% confidence ellipses are shown for culture type, represented by color, and N
700 treatment, represented by line type.




701

702 **Fig. 6:** Peak intensities of N-containing metabolites detected across treatment groups. Bars
703 represent average peak intensity ± standard error. Lowercase letters indicate significant
704 difference at $p < 0.05$.




705

706 **Fig. 7:** Examples of the N-containing metabolites detected at the microscale using MALDI MSI.

707 All ions are annotated as [M–H]- adducts. Ion images of individual m/z values were generated on

708 the same color bar scale for visual comparison in terms of relative ion abundance.

709

**Table 1**: PERMANOVA results for Bray-Curtis and Jaccard distance of macroscale metabolite abundance.

| | | Df | Sums of Squares | Mean Squares | F model | R squared | p–value |
|---|---|---|---|---|---|---|---|
| Bray-Curtis | N treatment | 1 | 1.422 | 1.422 | 7.899 | 0.192 | 0.0001 |
| | Culture type | 1 | 1.548 | 1.548 | 8.599 | 0.209 | 0.0001 |
| | Interaction | 1 | 0.841 | 0.842 | 4.676 | 0.114 | 0.0001 |
| | Residual | 20 | 3.601 | 0.180 | | 0.486 | |
| | Total | 23 | 7.413 | | | 1 | |
| Jaccard | N treatment | 1 | 1.350 | 1.350 | 11.547 | 0.279 | 0.0001 |
| | Culture type | 1 | 0.639 | 0.639 | 5.460 | 0.132 | 0.0003 |
| | Interaction | 1 | 0.515 | 0.515 | 4.406 | 0.106 | 0.0016 |
| | Residual | 20 | 2.339 | 0.117 | | 0.483 | |
| | Total | 23 | 4.843 | | | 1 | |

710