Translationally active soil microorganisms during substrate-induced respiration

Nina Rose Camillone,*[1,2] Mary Ann Victoria Bruns,[1,2,3] Raúl Román,[1,2,4] Daniel Wasner,[5] Estelle Couradeau[1,2,3]

[1]Department of Ecosystem Science and Management, The Pennsylvania State University, University Park, PA, USA

[2]One Health Microbiome Center, Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA, USA

[3]Intercollege Graduate Degree Program in Ecology, The Pennsylvania State University, University Park, PA, USA

[4]Instituto Multidisciplinar para el Estudio del Medio Ramon Margalef, Universidad de Alicante, Alicante, Spain

[5]Department of Environmental Systems Science, ETH Zurich, Zurich, Switzerland

*Address correspondence to E. Couradeau, estelle@psu.edu

M. A. Bruns and E. Couradeau are co-senior authors.

Word counts: Abstract 230, Importance 68, Body text 5386

ABSTRACT

Soil microorganisms carry out many processes that are fundamental to soil functions. Among the millions of microbial cells present in a gram of soil, however, less than 2% are commonly estimated to be active at any point in time. Because the respiratory response of a bulk soil to carbon substrate addition would be expected to reflect the number of active cells, we hypothesized a positive correlation between active cells and soil respiration rates during substrate-induced respiration (SIR) assays. To test this, we monitored respiration and active cell counts during 24-h incubations of agricultural soil subsamples after treating with two carbon

substrates or a water-only control. We enumerated active cells with the Bioorthogonal Non-canonical Amino Acid Tagging (BONCAT) method. BONCAT provides a labeled amino acid for active cells to incorporate into newly synthesized proteins, which can then be tagged with a fluorescent dye to enable enumeration by flow cytometry. Both respiration rates and active cell counts increased over time and were positively correlated with each other after 6 h of incubation. After 24 h, increases in active cells were proportionally greater than increases in respiration. Additionally, carbon-amended soils had higher respiration rates than water-only soils with similar active cell counts, suggesting differences in carbon use efficiency. Our study documents for the first time the respiratory response from *in-situ* microbial activation induced by substrate amendment of soil within 6 h, a short enough timescale to exclude most cell replication. This study also demonstrates that the correlation between active cell numbers and respiration is substrate-dependent.

IMPORTANCE

While many critical ecosystem services provided by soil are known to rely on microbial activity, the soil microbial community largely remains a black box. While respiration is a common indicator of bulk soil microbial activity, this study demonstrates that the relationship between respiration and the number of active cells differs based on available carbon substrates. Advancing knowledge in this area will both enable better interpretation of biological soil tests by land managers and inform researchers modeling contributions of soil microbial respiration to global carbon dynamics.

INTRODUCTION

Soil ecosystem services are intrinsically dependent on microbial activity through the many vital processes carried out by soil microbes. These include organic matter decomposition, plant nutrient provisioning, soil aggregate formation, nitrogen fixation, antibiotic production, and more[1], with global implications for nutrient cycling and climate change. Eighty percent of the 3200 Pg carbon in terrestrial ecosystems is stored in soil, with a flux of around 60 Pg per year due to microbial decomposition[2]. Microbial carbon use efficiency (CUE) is considered low when biomass production is accompanied by high carbon mineralization as atmospheric CO_2,

53 whereas high-CUE indicates more soil carbon retention in biomass per unit respiration.
54 Variations in CUE significantly impact global carbon cycling[3].

55 The relationship between the soil microbiome and macroscale nutrient cycles is
56 complicated by the soil's high percentage of cells that are resting, inactive, or dormant[4, 5].
57 Since soil microenvironments are typically low in carbon and energy sources, many microbes
58 remain dormant to survive and reactivate pending resource availability[6]. Early studies
59 enumerating redox-active soil bacterial cells report active fractions of 5-20% or as low as 1%[7,
60 4, 8, 9]. More recently, however, other methods have found over 40%[10], 50%[11], or 90%[12]
61 active bacteria in soil samples. In a laboratory incubation study, transcriptionally active soil
62 bacterial fraction increased from near zero to 15% after 48 h[13]. This highlights that cell activity
63 status is dynamic, with a potentially activatable fraction that may be difficult to classify in a
64 dormant-active binary. Going forward, we define active microbes as those synthesizing new
65 proteins and dormant microbes as those with no detectable translation to focus on potential for
66 metabolic activity while excluding residual extracellular enzymes.

67 In light of the numerous beneficial functions of soil microbes, increased soil microbial
68 activity is often considered desirable overall. For example, bulk soil respiration is a common
69 proxy for potential microbial activity used as an indicator of agricultural soil health [14, 15].
70 Additionally, multiple substrate-induced respiration (MSIR) assays, such as MicroResp©,
71 measure soil microbial responses to diverse C substrates, so that a broader variety of respired
72 substrates indicates a more active and dynamic community [16, 17]. It is well established that
73 carbon amendments initially stimulate soil respiration to a greater extent than cell division[18].
74 Particularly within 6 h, no shifts in community composition were observed to suggest substrate-
75 specific reproductive selection[19]. However, it remains unknown whether reactivation of
76 dormant microbes is an important contributor to SIR. Otherwise, bulk soil respiration may be
77 controlled by a small percentage of already-active cells through fluctuations in their cell-specific
78 respiration. This would indicate a major limitation for whole-community microbiome analysis in
79 understanding and predicting C cycling. Further, experimentally and conceptually decoupling
80 bulk community activity from the number of active microbes allows differentiation of active,

81 efficient community members from and less efficient community members having similar
82 respiration rates.

83 In this study, we investigate whether the SIR response is linked to increases in active cells
84 relative to the total number of cells in the soil. Specifically, we employed bioorthogonal non-
85 canonical amino acid tagging (BONCAT) and flow cytometry to enumerate active cells in tandem
86 with measuring SIR. BONCAT enables the detection of translationally active microbes through
87 the incorporation of a modified amino acid that is later tagged with a fluorescent dye[13, 20].
88 We considered that the relationship between bulk respiration and number of active cells may
89 be dependent on the simplicity of available C substrates. Thus, as in MSIR, we used two carbon
90 substrate treatments (glucose and galactose) to induce different respiration rates along with a
91 water-only treatment. We measured activity after four incubation times up to 24 h. Having
92 simultaneously monitored soil microbial activity at the bulk and cell levels, we calculated
93 respiration rate per active cell. We hypothesized that variation in respiration rate among
94 substrate treatments and across time would reflect changes in the number of active cells and
95 not the number of total cells, suggesting shifts in cellular activity status. This finding would
96 support the consideration of the fluidity of microbial activity status as a factor in modeling and
97 managing soil ecosystem processes. Further, if bursts in respiration are strongly linked with
98 dormant cell activation, respiration tests can be developed as proxies for the potentially active
99 microbial fraction, facilitating future collection and interpretation of soil microbial data.

100 MATERIALS AND METHODS

101 Soil collection and experimental design

102 All soil samples were collected at the Pennsylvania State University's Russell E. Larson
103 Agricultural Research Center near Pennsylvania Furnace, PA (40.72°N, -77.92°W), from a long-
104 term tillage experiment established in 1978[21]. A composite sample of bulk soil was collected
105 in May 2023 from the top 10 cm of a long-term no-till plot planted with wheat, representing the
106 Ap horizon of a Hagerstown silt loam (fine, mixed, semiactive, mesic Typic Hapludalfs in USDA
107 soil taxonomy). The soil was sieved to 2 mm and stored at 4°C for less than 4 months before
108 experimentation. Gravimetric water content was determined to be 16.6% by oven-drying a

109 subsample at 105°C for 24 h. Available water holding capacity was determined to be 62.2% by
110 the funnel method[42]. Incubations of soil subsamples with cycloheximide to inhibit fungi or
111 chloramphenicol to inhibit bacteria for 6 h showed that respiration was around 60% attributable
112 to bacteria and 40% to fungi (Supplemental Table 1)[22].

113 The experimental timeline is summarized in Figure **1**. Each experimental replicate
114 consisted of 1.2 g field-moist soil (1 g oven-dried soil equivalent). Replicates were placed into
115 12-mL glass tubes sealable via screw-on caps with silicone septa (Exetainer®12ml Vial - Flat
116 Bottom 738W, Labco Limited, High Wycombe, UK). The septa also allowed needle syringe access
117 for headspace sampling. For substrate treatments, 100 µL of liquid was added to each tube:
118 either an aqueous substrate solution containing 3.0 mol L^{-1} carbon (in the form of glucose or
119 galactose, Sigma-Aldrich, Saint Louis, MO, USA) or ultrapure water. This resulted in a final
120 concentration equivalent to 30 mg glucose g^{-1} soil water, which is the concentration commonly
121 used with the MicroResp*TM* system[16, 17]. The gravimetric water content of the soil after
122 addition of the substrate treatments was 30%, which was 50% of the available water holding
123 capacity. The tubes were sealed to limit moisture evaporation and incubated in the dark at 22°C
124 for 2, 6, 12, or 24 h. The time intervals reflect previous studies where respiration rates in surface
125 horizon soil microcosms amended with glucose peaked from 5-10 h (depending on glucose
126 concentration) and returned to near initial rates after 24 h[23]. Galactose was selected as a
127 substrate treatment because it stimulates a soil respiration rate intermediate to those of
128 glucose and water-only[16]. Initial pilot experiments included lignin and vanillic acid as
129 additional substrates, but these treatments resulted in very low BONCAT activity levels (on
130 average less than 0.5%) compared to the false positive discovery rate (0.2%) even after 24 h
131 incubation. These substrates were not included, and further research will be necessary to
132 understand why the BONCAT signal was so low in these treatments. Six replicate tubes were
133 included per substrate treatment per incubation length.

134 **Activity monitoring: respiration measurement and cell extraction**

135 Two hours prior to the end of the incubation period (i.e. concurrently with the start of
136 the substrate treatment for the 2-h replicates), the tubes were opened, flushed with ambient

air, and resealed to begin a CO_2 accumulation period of equal length for all replicates. While the tubes were open, 250 µL of 800 µmol L^{-1} L-homopropargylglycine (HPG), a synthetic amino acid homologous to methionine was also added to 5 replicates per substrate treatment per incubation length. The sixth replicate in each group received 250 µL of ultrapure water as a control for the BONCAT dye signal. As a result, the moisture content of all replicates reached 90% of available water holding capacity. The addition of HPG initiated the first stage of the BONCAT active cell enumeration method, where translationally active cells incorporate HPG into their proteins such that they can later be distinguished from HPG-negative inactive cells[13].

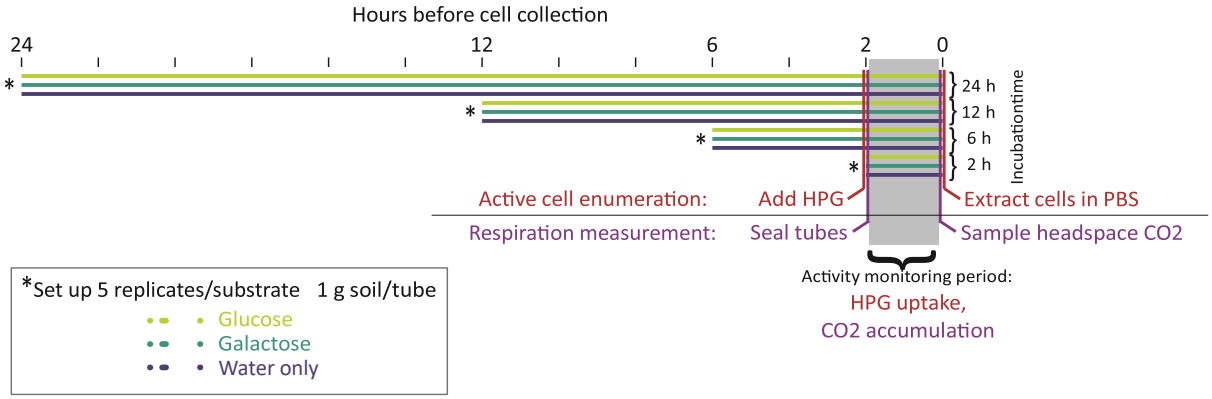


FIG 1 Experimental design as a timeline of substrate incubations, headspace sampling, and cell collection. L-homopropargylglycine (HPG) is a synthetic amino acid homologous to methionine used to label translationally active cells in the BONCAT workflow.

Two hours later at the conclusion of the incubation period, 2 mL of headspace air was collected with a needle syringe from each tube and immediately analyzed for CO_2 content using a LI-7000 CO_2/H_2O Gas Analyzer (LI-COR). A calibration curve based on standards with known CO_2 concentration was used to determine µmol CO_2 in experimental samples. The CO_2 content of the ambient air was recorded and later subtracted from the headspace concentrations to calculate the soil respiration rate. Immediately after headspace sampling, 5 mL 0.02% Tween 20 in phosphate buffer saline (PBS) was added to each replicate, and tubes were shaken horizontally for 5 min at high speed to extract cells from the soil. At this point, a killed soil replicate was added to the workflow. This subsample had been autoclaved three times at 1-day intervals (15 min, 121°C) in order to provide a control for staining with no cells. After shaking,

159 the tubes were centrifuged for 5 min at 30 x g to separate soil particles without pelleting cells.
160 The supernatant cell suspensions were stored at -20°C in 1 mL aliquots to which 500 µL 30%
161 glycerol was added (final concentration 10% glycerol) and were processed within one week.
162 Three replicates (one each from 6-h glucose, 12-h glucose, and 6-h water) had to be removed
163 from analysis because respiration rates could not be quantified due to leaky tube seals.

164 **Active microbial cell labeling**

165 The methods for collecting cells and BONCAT staining with a click chemistry reaction
166 were adapted from previously published methods [13, 24]. After thawing and briefly vortexing
167 the frozen cell suspensions, they were centrifuged at 50 x g for 5 min to pellet any larger
168 remaining soil particles. Previous studies have used much higher centrifuge speeds while
169 maintaining high cell recovery[25], but for this soil we had very low cell yields when higher
170 speeds were used to pellet soil particles, perhaps due to cell clumping. The supernatant was
171 recovered and passed through a 35 µm filter. Then, cells were pelleted from the suspension by
172 centrifuging at 16,000 x g for 5 min. The supernatant was poured off and discarded, and the
173 cells were resuspended in 70.4 µL 1x PBS in 2-mL snap-cap tubes by briefly vortexing. A dye
174 mixture was prepared and added to each tube for a final reaction concentration of 5 µmol L^{-1}
175 amino guanidine hydrochloride, 5 µmol L^{-1} sodium L-ascorbate, 100 µmol L^{-1} copper sulfate
176 pentahydrate (ThermoFisher Scientific, Eugene, OR, USA), 500 µmol L^{-1} tris-
177 hydroxypropyltriazolylmethylamine (THPTA), and 5 µmol L^{-1} FAM picolyl-azide dye in a final
178 volume of 80 µL. Unless otherwise noted, all BONCAT reagents including HPG were purchased
179 from Click Chemistry Tools (Scottsdale, AZ, USA). The tubes were incubated in the dark for 60
180 min to permit the click reaction between the azide dye and the HPG incorporated into the
181 proteins of translationally active cells. Next, samples were washed to remove excess dye by
182 centrifuging at 16,000 x g for 5 min, resuspending in 1 mL 1x PBS by brief vortexing, and
183 repeating these steps twice more. The resulting cell suspension was diluted in 1x PBS and
184 passed through a 35 µm filter (Falcon Round-Bottom Tubes with Cell Strainer Cap, 5 mL). SYTO™
185 59 Red Fluorescent Nucleic Acid Stain (ThermoFisher Scientific, Invitrogen, Eugene, OR, USA)
186 was added as a counterstain to distinguish cells from clay particles. Cell suspensions were
187 incubated in the dark for 15 min.

188 **Flow cytometry**

189 Cell enumeration was performed using the LSR Fortessa flow cytometer (BD Biosciences,
190 Franklin Lakes, NJ, USA) at the Pennsylvania State University Flow Cytometry Core, which is
191 equipped with 405 nm (violet), 488 nm, (blue), 532 nm (green), and 640 nm (red) lasers. The
192 BONCAT dye, FAM-picolyl azide (490 nm excitation, 510 nm emission), was captured in a green
193 channel ("FITC") off the 488 nm blue laser. The DNA counterstain, SYTO59 (622 nm excitation,
194 645 nm emission), was captured in a red channel ("APC") off the 640 nm red laser. Forward and
195 side light scatter were also collected for all samples. All samples were run on the low flow rate
196 setting (which we measured to be 22.6 µL/min for our samples). Data was collected for 120 s.
197 Knowing the flow rate of the instrument enabled calculation of the original concentration of
198 cells in the soil using the number of cells detected, the volume of extract processed, and the
199 mass of soil extracted.

200 As a negative control, a "cell extract" from a soil sample that had been autoclaved 3
201 times at 1-day intervals (killed control) was counterstained with SYTO59 to set the threshold for
202 distinguishing cells from soil mineral particles. Similarly, soil samples incubated with water
203 instead of HPG solution (HPG-negative control) were used to set the threshold for BONCAT
204 positive cells, since they should not retain any of the BONCAT dye.

205 Data analysis was performed using FlowJo Software (Tree Star, Ashland, OR, USA). First,
206 forward and side scatter fluorescence thresholds were set to constrain the size of particles
207 examined. Secondly, a threshold for SYTO positive (SYTO+) particles, which are assumed to be
208 cells rather than clay particles, was determined based on fluorescence intensity in the APC
209 channel from the killed (autoclaved) control samples counterstained with SYTO59. This step
210 accounts for any potential autofluorescence from or staining of soil particles. Finally, a threshold
211 for BONCAT positive (BONCAT+) cells was determined based on the fluorescence intensity in the
212 FITC channel from the SYTO+ population (cells) in the HPG-negative control. To account for
213 electronic noise from the flow cytometer or any potential retention of dyes on soil particles, the
214 number of SYTO+ events detected in the killed control sample was subtracted from the SYTO+
215 count in other samples within each batch. Additionally, the number of BONCAT+ events

216 detected in the HPG-negative control was subtracted from the BONCAT+ population in
217 experimental samples. Any sample where the number of BONCAT+ events was less than or
218 equal to the number of BONCAT+ events in the counterstain-only control was recorded to have
219 zero active cells. Preliminary work using the LIVE/DEAD™ *Bac*Light™ Bacterial Viability and
220 Counting Kit, for flow cytometry (ThermoFisher Scientific, Invitrogen, Eugene, OR, USA)
221 detected negligible percentages of dead microbes recovered using our extraction method
222 (<0.65% on average; see Supplemental Table 2), likely due to our cell extraction method failing
223 to preserve cells without intact membranes, i.e. those deemed dead by the staining kit used.
224 Thus, we report total cells as representative of the living community.

225 Statistical analysis

226 Statistical analysis was conducted using R version 4.2.1[26], and figures were generated
227 using R packages ggplot2[27] and ggbreak[28]. Substrate treatments within each incubation
228 length and incubation lengths within each substrate were compared using one-way ANOVAs.
229 Incubation length was treated as a categorical variable due to the non-linear relationship of
230 response variables and time. Tukey HSD post-hoc tests were performed for variables with
231 significant ANOVAs ($p<0.05$). The rate of change of numbers of active cells in each substrate
232 treatment was calculated per unit time as if it were a growth rate, but we do not refer to this as
233 a "growth rate" since new active cells could be the result either of cell division or activation of
234 dormant cells. Instead, this variable will be referred to simply as the rate of change of numbers
235 of active cells. The cell-specific respiration rate was calculated by dividing the bulk respiration
236 rate by the number of active cells in the same experimental replicate as an indicator of
237 resource-use efficiency. The relationship between respiration rate and log-transformed number
238 of active cells was analyzed using linear regression.

239 RESULTS

240 Cell counts and bulk respiration

241 The total number of microbial cells ranged from 3.1×10^5 to 2.2×10^7 g^{-1} soil throughout
242 the experiment (Figure **2**A). The number of active cells ranged from 1.7×10^3 to 4.4×10^6 g^{-1} soil
243 (bars in Figure **2**B, left y-axis), or 0.1% to 27% of the total (percentages noted below data bars in

Figure 2A). Generally, the number and percentage of active cells increased with time for all treatments. For the glucose treatment, numbers of total and active cells were highest after 12 h of incubation. In galactose, total and active cells were highest at 24 h. For the water treatment, total cells were highest at 12 h while active cells were highest at 24 h. Among the treatments, the glucose treatment had the highest total number of cells throughout the experiment while the total cells in the galactose and water treatments never significantly differed from each other. As for active cells, they likewise were highest in glucose compared to the other treatments at 6 h, 12 h, and 24 h. In water, active cell numbers averaged slightly lower than in galactose at 6 h and 24 h, but they were significantly higher than in galactose at 12 h. Contrastingly, galactose had the most active cells at 2 h, although it was still low at 2% of total cells.

The bulk respiration rate increased after 24 h in all 3 treatments (lines in Figure 2B, 267 right y-axis). The respiration rate of the substrate-amended treatments did not significantly differ from their initial rates until 24 h. Glucose consistently had a higher respiration rate than galactose, and galactose consistently had a higher respiration rate than water-only.

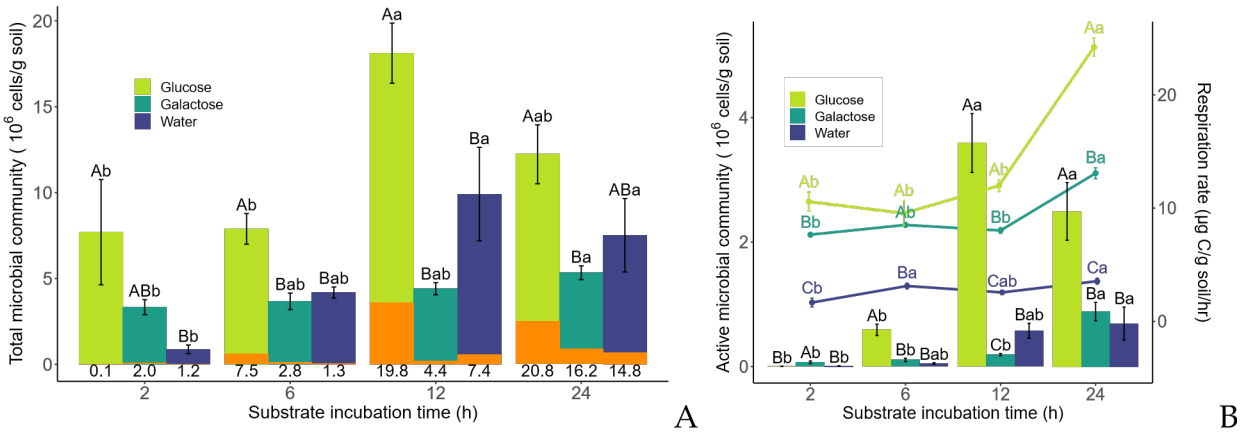


FIG 2 Total cells, active cells, and respiration rate. (A) Total cells over time for different substrate treatments, reported in cells per g dry soil. The lower (orange) portion of each bar represents the active cell fraction. Numbers below each bar indicate the percentage of active cells. (B) Active cells (bars, left y-axis) and respiration rate (lines, right y-axis). Note that 0 for the right y-axis is elevated. For both (A) and (B), uppercase letters represent statistically significant differences found by Tukey's HSD test ($p<0.05$) among

266 different substrate treatments within the same time point, indicated by the x axis.
267 Lowercase letters represent statistically significant differences across time points within the
268 same substrate treatment, indicated by color. The absence of a letter of a particular case
269 above a bar indicates that Tukey's test was not conducted due to an insignificant ANOVA
270 result. Bars indicate standard error.

271 The largest absolute increases in numbers of total and active cells coincided with each
272 other for all three treatments. In glucose and water, they were between 6 and 12 h, but in
273 galactose they were later, between 12 and 24 h. For glucose, the largest increase in respiration
274 rate occurred between 12 and 24 h, after the large increase in number of active cells between 6
275 and 12 h. For galactose, the largest changes in number of active cells and respiration coincided
276 between 12 and 24 h. For water, the largest increase in respiration occurred first, between 2 and
277 6 h, before the increase in active cells at 12 h. Glucose had the largest changes in total and
278 active cells and in respiration rate compared to the other treatments.

279 **Cell-specific respiration rate**

280 Cell-specific respiration was calculated for each replicate by dividing bulk respiration by
281 the number of active cells. In all three treatments, the cell-specific respiration rate was highest
282 after 2 h and decreased thereafter. However, this decrease was only statistically significant for
283 glucose (Figure **3**). At 2 h, the glucose treatment had the highest cell-specific respiration, driven
284 by low active cell counts and a high respiration rate. At 12 h, cell-specific respiration was highest
285 for galactose, caused by lower active cell counts than glucose and higher respiration rates than
286 water. After 6 h and after 24 h, there were no statistically significant differences in cell-specific
287 respiration rate among treatments.

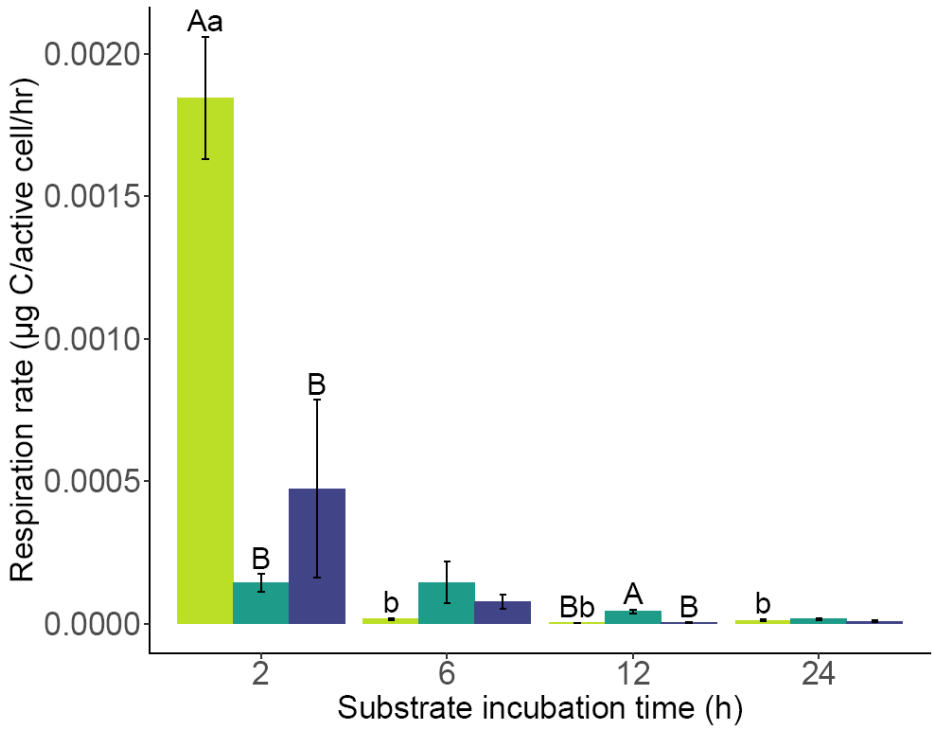


FIG 3 Cell-specific respiration rate, calculated by dividing bulk respiration by the number of active cells. Uppercase letters represent statistically significant differences found by Tukey's HSD test ($p<0.05$) among substrate treatments within the same time point, indicated by the x axis. Lowercase letters represent statistically significant differences across time points within the same substrate treatment, indicated by color. The absence of a letter of a particular case above a bar indicates that Tukey's test was not conducted due to an insignificant ANOVA result. Bars indicate standard error.

DISCUSSION

Methodological insights and comparisons

Our study documents for the first time *in-situ* soil microbial cell activation induced by substrate amendment within 6 h, a short enough timescale to exclude most cell replication. This is possible using the BONCAT method because it detects protein translation rather than cell growth, which has previously been used to monitor soil microbial activity via methods such as SIP[29]. A longer activity labeling period can increase the detection of low-activity cells but does not give a snapshot for time-resolved changes in cell activity status. Other methods for

304 enumeration of active soil microbes involve dyeing active cells post extraction[5, 9, 10, 11],
305 whereas in BONCAT active cells are tagged *in situ* with a synthetic amino acid prior to extraction
306 and subsequently dyed through a click-chemistry reaction. This could lead to discrepancies
307 between detected and actual activity if dormant cells are reawakened or if activity is suppressed
308 through the disturbance of extraction, e.g. aerobic respiration decreasing upon saturation.
309 Differences among soil active microbes labeling methods are summarized in Table **1**.
310 Comparative studies are needed to determine if variation in reported numbers of active cells
311 reflects actual cell physiology (e.g. some cells contain active enzymes but are not currently
312 translating new ones), natural differences in soil samples across time and space, or inefficiencies
313 in some methods compared to others.

314 Some remaining methodological challenges in active cell enumeration are specific to
315 BONCAT, while others apply more broadly. For example, BONCAT does not detect the activity of
316 microbes with access to native methionine (outcompeting analog HPG) or microbes with
317 adequate protein reserves without need for translation. Regardless of staining method,
318 extraction cannot capture all microbes present in the soil, especially filamentous microbes such
319 as fungi. Typically, soils contain 10^7-10^{10} prokaryotic cells per gram[30], compared to our counts
320 of 5×10^6 after 2 h of incubation (Figure **2**A). We confirmed that our cell extracts had very low
321 numbers of dead microbes (Supplemental Figure 1), which may contribute to our lower cell
322 counts considering previous research reporting 40% dead microbial cells in soil[7]. We assumed
323 that our percentages of active cells were representative of the whole live community. In the
324 end, any binary divide between active and inactive cells is inherently a simplification, yet it can
325 still yield insights into overall community status for the purpose of this discussion.

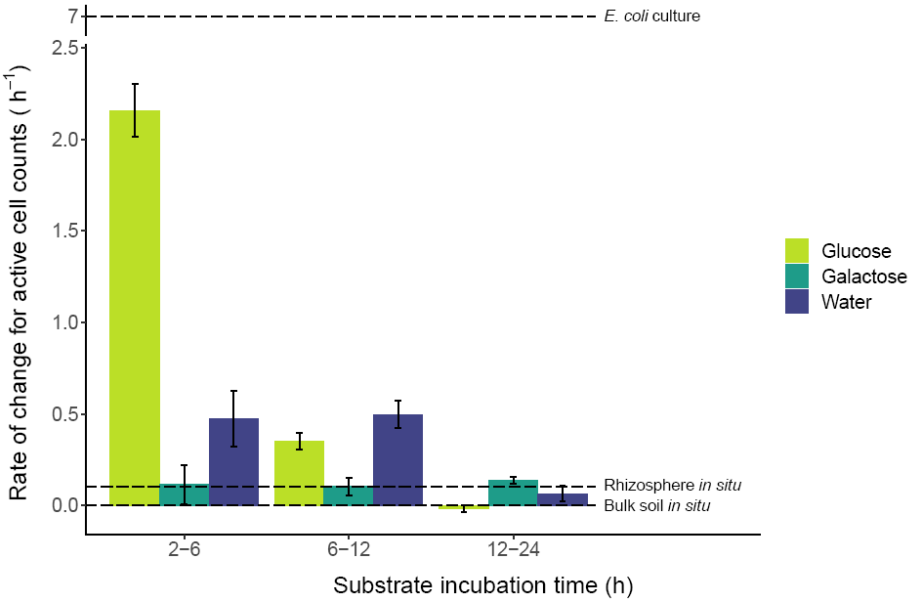
326 **TABLE 1** Comparison of methods to enumerate active microbial enumeration in soil.
327 Abreviations: 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, an artificial electron acceptor),
328 5(6)-carboxyfluorescein diacetate (CFDA, a substrate hydrolyzed by intracellular esterases),
329 fluorescence *in situ* hybridization (FISH), stable-isotope probing (SIP).

Method	**CTC**	**CFDA**	**FISH**	**DNA-SIP**	**BONCAT**
Activity type	Aerobic respiration (redox)	Metabolic enzymes	rRNA content	DNA replication	Protein translation
Time scale	30+ min	30+ min	Fixed cells	1+ days	30+ min
Labeling location	Extracted cells	Extracted cells	Extracted cells	*in situ*	*in situ*
Reported active fraction	2-6%[9] <1%[5]	42%[10]	55%[11]	91%[12]	25%[13]

330 **Glucose amendment reactivated dormant cells**

331 Active cell numbers increased most rapidly from 2-6 h of incubation with glucose (Figure
332 **4**). Notably, this was our only observation of an increase in total cells that was outpaced by a
333 larger increase in active cells. In other words, the number of dormant cells (estimated by
334 subtracting active from the total) decreased, suggesting cell activation. Alternatively, or in
335 addition, this could indicate a death rate among inactive cells outpaced by reproduction among
336 active cells; however, this seems less likely considering known soil microbial growth rates
337 (depicted in Figure **4** as rate of change per h). Assuming no growth or reactivation of dormant
338 cells, active cells would have been doubling every 37 min to achieve the observed increase
339 between 2-6 h for the glucose treatment. This approaches the theoretical maximum growth rate
340 under optimum soil conditions with as a generation time of 0.5 h[31]. For comparison,
341 *Eschedrichia coli* has a generation time around 0.3 h *in vitro*[32]. In actuality, soil microbes have
342 been clocked with doubling times only as low as 7.2 h in the rhizosphere[33] and 14 to 45 days
343 in bulk soil[34] (depicted in Figure **4** as rate of change per h). This suggests that reactivation of

344 dormant cells largely contributed to the increase in active cells. With the same reasoning of a
345 high rate of increase in active cells, dormant microbes were likely reactivating in the water
346 treatment between 2-12 h (Figure **4**). The rate of change in active cell counts in the galactose
347 treatment did not measurably change throughout the experiment and remained comparable to
348 a highly active rhizosphere environment. The growth rates for total cells were generally much
349 lower than active cells' (Supplemental Figure 1).



350

351 **FIG 4** Rate of change in number of active cells over time. Bars indicate standard error,
352 propagated from the standard error in the enumerated community sizes. For comparison,
353 dashed lines indicate several known growth rates: a laboratory culture of *Escherichia coli*
354 (generation time 0.3 h)[32], an active rhizosphere community (generation time 7.2 h)[33],
355 and bulk forest soil (generation time 14.1 days) [34]. Note the break in the y axis between
356 2.5 and 7.

357 **Cell-specific activity differed by treatment**

358 As hypothesized, respiration rates and active cell counts were positively correlated,
359 although the relationship differed by substrate. Regression analysis revealed statistically
360 significant relationships between these variables within each treatment and across all
361 treatments together, although in the glucose treatment it was only significant at the $p<0.06$

362 level (equations reported in Figure **5**A). Differences among the regression models' slopes or
363 intercepts result in the regression line for glucose being higher than that of galactose, which is
364 in turn higher than water's. This pattern reflects overall trends in the treatments' cell-specific
365 respiration across the experiment. Lower respiration and active cell counts in the galactose
366 treatment than the glucose treatment are consistent with the ability of cells to utilize glucose
367 directly. By contrast, galactose requires multiple enzyme-catalyzed reactions before a
368 downstream metabolite enters the glycolytic pathway [35].

369 Without any carbon addition, the water-only treatment has the lowest regression
370 coefficient (Figure **5**A). This indicates that this moist soil environment supported an expanding
371 active community with minimal changes in respiration rate. This finding is consistent with the
372 Microbial Efficiency-Matrix Stabilization (MEMS) framework, which theorizes that respiration is
373 highest where inputs are low quality (excess ratio of C to other nutrients) and have a low
374 capacity to be stabilized in the mineral soil matrix [36], e.g. glucose supports lower CUE than
375 lignin. Active microbes in the water-only, carbon-poor treatment may use carbon more
376 efficiently, i.e. prioritize its assimilation as biomass relative to mineralization. This is also
377 consistent with previous studies that found that soil microbes metabolized complex substrates
378 with a higher carbon use efficiency[37]. (also check decreased CUE with excess C in [38])

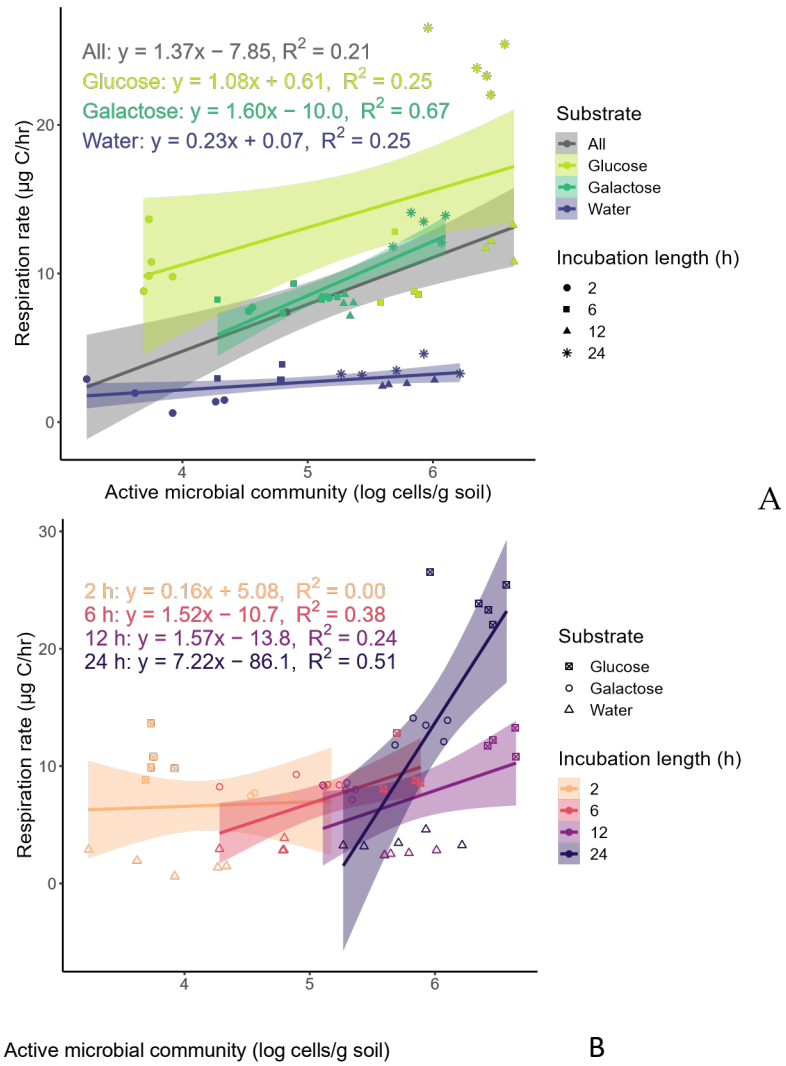


FIG 5 Regressions of bulk respiration onto active cell counts in log scale. The shaded regions show the 95% confidence region for each regression line. (A) Regression lines for all data and for each substrate individually. P-values for coefficients and intercepts respectively are <0.001 and 0.08 for all data, 0.06 and 0.9 for glucose, <0.0001 and 0.009 for galactose, and 0.03 and 0.9 for water. (B) Regression lines for each time point individually. P-values for coefficients and intercepts respectively are 0.9 and 0.6 for 2 h, 0.03 and 0.2 for 6 h, 0.08 and 0.2 for 12 h, and 0.003 and 0.008 for 24 h.

Lag between respiration response and increase in active cells

Within 2 h of substrate addition, the number of active cells did not differ among treatments (Figure 2), while the two carbon-amended treatments already had much higher bulk

391 respiration rates than water-only (Figure **2**B). Thus, our results indicate an initial lag where
392 changes in translational activity are decoupled from the respiration response. As hypothesized,
393 we observed that active cell counts and respiration rates followed similar patterns across
394 treatments when cell-specific respiration was consistent, i.e. after 6 and 24 h of substrate
395 incubation (Figure **3**). Regression models calculated within each time point also indicated
396 positive correlations between respiration and active cell counts with statistically significant
397 regression coefficients at both 6 and 24 h, but not at 2 or 12 h (Figure **5**B). At these time points,
398 by contrast cell-specific respiration differed among treatments, reflecting weak or no correlation
399 between respiration and number of active cells. The glucose treatment had the highest cell-
400 specific respiration at 2 h, and galactose had the highest at 12 h (Figure **3**). These differences
401 suggest that glucose-amended soil microbes began rapidly metabolizing with a burst of
402 respiration per cell within 2 h before the activation of dormant cells by 6 h. Meanwhile,
403 galactose induced increases in respiration and active cell counts at a slower but steadier rate. In
404 fact, the galactose treatment exhibited the least variation in cell-specific respiration and the
405 strongest positive correlation (highest regression R^2) between bulk respiration and active cell
406 counts (Figure **5**A). A low level of respiration from BONCAT-negative cells also would have
407 contributed to low cell-specific respiration and a strong correlation between respiration and
408 active cells. This could be the case if few cells already contained the enzymes necessary for
409 galactose metabolism. Regardless, for all substrate treatments, cell-specific respiration tended
410 to decrease over time, suggesting an initial dip in CUE with excess carbon and a lag in cell
411 activation post substrate addition. In the field, this could contribute to increased carbon
412 emissions from more frequently disturbed soils.

413 **Implications for soil testing**

414 Our results demonstrate the utility of SIR as a proxy for soil microbial activation in an
415 agricultural soil. Soil testing already utilizes SIR for crop yield prediction[39, 40]. Thus, future
416 research in more soils establishing a correlation between SIR and numbers of active microbes
417 would also implicate a correlation with agricultural yields. This may seem intuitive, considering
418 the microbial basis for many critical agricultural soil functions such as decomposition and
419 aggregate formation. However, other factors complicate the relationship between microbial

activity and optimal soil functionality. Frequent tillage disturbance, for example, could over-stimulate microbial activity and cause faster depletion of soil organic matter with time. Additionally, CUE can affect the relationship between respiration and microbial functionality. A high respiration rate in an SIR test can indicate a responsive microbial community or simply a less efficient one.

However, microbial researchers have described activity status not simply as a dichotomy between dead and alive or between active and dormant, but as a spectrum of activity levels, defining potentially active microbes as those that resume activity after 4-12 h of stimulation [7]. While a large active microbial fraction could achieve a high respiration rate and rapidly deplete soil carbon stores, a large *potentially* active fraction would be a better indicator of a responsive and resilient microbiome supporting soil functions. The present study found rapid increases in active cell counts but not total cell counts for 6 h of substrate incubation. Previous MSIR studies have found 6-h incubation time to be optimal for observing the existing community while limiting time for shifts in community composition[16, 17, 19], which our study corroborates. Additionally, our results showed a similar effect of substrate treatment on respiration rate and active cell counts at this time (Figure **2**B), and the earliest correlation between these two indicators (Figure **5**B). These results support the interpretation of SIR tests as measures of potentially active microbes responsive to different substrates.

At the same time, active cell numbers alone could not consistently predict respiration due to differences among substrate treatments. Both carbon substrates induced higher cell-specific respiration than the water-only treatment at different times (Figure **3**), and carbon-amended replicates had higher respiration than the water replicates with similar numbers of active cells (Figure **5**A). Previously, a field study found that tilled soils with incorporation of organic residues had higher respiration rates with lower microbial biomass compared to no-till soils[41], similarly pointing toward higher activity per cell with access to carbon.

Overall, this project demonstrates a relationship between respiration and numbers of active microbes. This provides a basis for interpreting respiration-based soil tests in microbial terms and targeting research efforts on active microbes due to their contribution to essential

soil functions. Future research coupling BONCAT with cell sorting and metagenomic sequencing can reveal which microbes are activated under different soil conditions and amendments. Linking other bulk soil tests to active cell counts can further inform soil management decisions for promoting desired outcomes of microbial activity.

ACKNOWLEDGMENTS

We acknowledge that gas analysis for CO_2 content was conducted in the lab of Jason Kaye with training and support from Brosi Bradley. We would also like to acknowledge that cell enumerations were conducted at the Huck Institutes' Flow Cytometry Core Facility (RRID:SCR_024460) on the BD Fortessa flow cytometer with training and support from multiple staff members.

DATA AVAILABILITY STATEMENT

Original flow cytometry data are available as FCS files at doi.org/10.26208/G8KX-GG32 through Penn State's DataCommons.

FUNDING

This work was funded through a Flower Grant awarded by the Ecology Institute at the Penn State Huck Institutes of Life Sciences to authors Bruns, Camillone, and Couradeau. This work was also supported by the USDA National Institute of Food and Agriculture and Hatch Appropriations under Project #PEN04949 and Accession #7006508.

CONFLICTSOFINTEREST

The authors declare no conflict of interest.

REFERENCES

References

[1] **Hartmann M, Six J**. Nov 2022. Soil structure and microbiome functions in agroecosystems. *Nature Reviews Earth & Environment* 4 (1):4–18. doi: 10.1038/s43017-022-00366-w.

[2] **Lal R**. 2008. Sequestration of atmospheric CO2 in global carbon pools. *Energy & Environmental Science* 1 (1):86. doi:10.1039/b809492f.

[3] **Tao F, Huang Y, Hungate BA, Manzoni S, Frey SD, Schmidt MWI, Reichstein M, Carvalhais N, Ciais P, Jiang L, Lehmann J, Wang YP, Houlton BZ, Ahrens B, Mishra U, Hugelius G, Hocking TD, Lu X, Shi Z, Viatkin K, Vargas R, Yigini Y, Omuto C, Malik AA, Peralta G, Cuevas-Corona R, Di Paolo LE, Luotto I, Liao C, Liang YS, Saynes VS, Huang X, Luo Y**. May 2023. Microbial carbon use efficiency promotes global soil carbon storage. *Nature* 618 (7967):981–985. doi:10.1038/s41586-023-06042-3.

[4] **Lennon JT, Jones SE**. 2 2011. Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nature Reviews Microbiology* 9:119–130. doi: 10.1038/nrmicro2504.

[5] **Salazar A, Lennon JT, Dukes JS**. May 2019. Microbial dormancy improves predictability of soil respiration at the seasonal time scale. *Biogeochemistry* 144(1):103–116. doi:10.1007/s10533-019-00574-5.

[6] **Hobbie JE, Hobbie EA**. 2013. Microbes in nature are limited by carbon and energy: the starving-survival lifestyle in soil and consequences for estimating microbial rates. *Frontiers in Microbiology* 4. doi:10.3389/fmicb.2013.00324.

[7] **Blagodatskaya E, Kuzyakov Y**. 12 2013. Active microorganisms in soil: Critical review of estimation criteria and approaches. *Soil Biology and Biochemistry* 67:192–211. doi: 10.1016/j.soilbio.2013.08.024.

[8] **Wang G, Mayes MA, Gu L, Schadt CW**. Feb 2014. Representation of Dormant and Active Microbial Dynamics for Ecosystem Modeling. *PLoS ONE* 9 (2):e89252. doi:10.1371/journal.pone.0089252.

[9] **Winding A, Binnerup SJ, Sørensen J**. Aug 1994. Viability of Indigenous Soil Bacteria Assayed by Respiratory Activity and Growth. *Applied and Environmental Microbiology* 60 (8):2869–2875. doi:10.1128/aem.60.8.2869-2875.1994.

498 [10] **Espina L**. Aug 2020. An approach to increase the success rate of cultivation of soil bacteria
499 based on fluorescence-activated cell sorting. *PLOS ONE* 15 (8):e0237748.
500 doi:10.1371/journal.pone.0237748.

501 [11] **Bertaux J, Gloger U, Schmid M, Hartmann A, Scheu S**. Jun 2007. Routine fluorescence
502 in situ hybridization in soil. *Journal of Microbiological Methods* 69 (3):451–460.
503 doi:10.1016/j.mimet.2007.02.012.

504 [12] **Ding C, Xu X, Liu Y, Huang X, Xi M, Liu H, Deyett E, Dumont MG, Di H, Hernández M, Xu J,**
505 **Li Y**. Mar 2023. Diversity and assembly of active bacteria and their potential function along soil
506 aggregates in a paddy field. *Science of The Total Environment* 866:161360.
507 doi:10.1016/j.scitotenv.2022.161360.

508 [13] **Couradeau E, Sasse J, Goudeau D, Nath N, Hazen TC, Bowen BP, Chakraborty R,**
509 **Malmstrom RR, Northen TR**. 2019. Probing the active fraction of soil microbiomes using
510 BONCAT-FACS. *Nature Communications* 10:2770. doi:10.1038/s41467-019-10542-0.

511 [14] **Rieke EL, Cappellazzi SB, Cope M, Liptzin D, Bean GM, Greub KLH, Norris CE, Tracy PW,**
512 **Aberle E, Ashworth A, Tavarez OB, Bary AI, Baumhardt RL, Gracia AB, Brainard DC, Brennan JR,**
513 **Reyes DB, Bruhjell D, Carlyle CN, Crawford JJW, Creech CF, Culman SW, Deen B, Dell CJ, Derner**
514 **JD, Ducey TF, Duiker SW, Dyck MF, Ellert BH, Solorio AE, Fonte SJ, Fonteyne S, Fortuna AM,**
515 **Foster JL, Fultz LM, Gamble AV, Geddes CM, Griffin-LaHue D, Grove JH, Hamilton SK, Hao X,**
516 **Hayden ZD, Honsdorf N, Howe JA, Ippolito JA, Johnson GA, Kautz MA, Kitchen NR, Kumar S,**
517 **Kurtz KSM, Larney FJ, Lewis KL, Liebman M, Ramirez AL, Machado S, Maharjan B, Gamiño**
518 **MAM, May WE, McClaran MP, McDaniel MD, Millar N, Mitchell JP, Moore AD, Moore PA,**
519 **Gutiérrez MM, Nelson KA, Omondi EC, Osborne SL, Alcalá LO, Owens P, Pena-Yewtukhiw EM,**
520 **Poffenbarger HJ, Lira BP, Reeve JR, Reinbott TM, Reiter MS, Ritchey EL, Roozeboom KL, Rui Y,**
521 **Sadeghpour A, Sainju UM, Sanford GR, Schillinger WF, Schindelbeck RR, Schipanski ME,**
522 **Schlegel AJ, Scow KM, Sherrod LA, Shober AL, Sidhu SS, Moya ES, Luce MS, Strock JS, Suyker**
523 **AE, Sykes VR, Tao H, Campos AT, Eerd LLV, Verhulst N, Vyn TJ, Wang Y, Watts DB, William BB,**
524 **Wright DL, Zhang T, Morgan CLS, Honeycutt CW**. May 2022. Linking soil microbial community

525 structure to potential carbon mineralization: A continental scale assessment of reduced tillage.
526 *Soil Biology and Biochemistry* 168:108618. doi:10.1016/j.soilbio.2022.108618.

527 [15] **Franzluebbers A**. Jul 1999. Potential C and N mineralization and microbial biomass from
528 intact and increasingly disturbed soils of varying texture. *Soil Biology and Biochemistry* 31
529 (8):1083–1090. doi:10.1016/s0038-0717(99)00022-x.

530 [16] **Creamer RE, Stone D, Berry P, Kuiper I**. 2016. Measuring respiration profiles of soil microbial
531 communities across Europe using MicroResp™ method. *Applied Soil Ecology* 97:36–43.
532 doi:10.1016/j.apsoil.2015.08.004.

533 [17] **Campbell CD, Chapman SJ, Cameron CM, Davidson MS, Potts JM**. Jun 2003. A
534 Rapid Microtiter Plate Method To Measure Carbon Dioxide Evolved from Carbon Substrate
535 Amendments so as To Determine the Physiological Profiles of Soil Microbial Communities by
536 Using Whole Soil. *Applied and Environmental Microbiology* 69 (6):3593–3599.
537 doi:10.1128/aem.69.6.3593-3599.2003.

538 [18] **NGUYEN TT, MARSCHNER P**. Feb 2017. Soil Respiration, Microbial Biomass and Nutrient
539 Availability in Soil After Addition of Residues with Adjusted N and P Concentrations.
540 *Pedosphere* 27 (1):76–85. doi:10.1016/s1002-0160(17)60297-2.

541 [19] **Gao F, Fan H, Chapman SJ, Yao H**. 4 2022. Changes in soil microbial community
542 activity and composition following substrate amendment within the MicroResp™ system.
543 *Journal of Soils and Sediments* 22:1242–1251. doi:10.1007/s11368-022-03143-w.

544 [20] **Hatzenpichler R, Connon SA, Goudeau D, Malmstrom RR, Woyke T, Orphan VJ**.
545 Jun 2016. Visualizing in situ translational activity for identifying and sorting slow-growing
546 archaeal-bacterial consortia. *Proceedings of the National Academy of Sciences* 113 (28).
547 doi:10.1073/pnas.1603757113.

548 [21] **Duiker SW, Beegle DB**. Jul 2006. Soil fertility distributions in long-term no-till, chisel/disk
549 and moldboard plow/disk systems. *Soil and Tillage Research* 88 (1-2):30–41.
550 doi:10.1016/j.still.2005.04.004.

551 [22] **Anderson JPE, Domsch KH**. Mar 1975. Measurement of bacterial and fungal contributions
552 to respiration of selected agricultural and forest soils. *Canadian Journal of Microbiology* 21
553 (3):314–322. doi:10.1139/m75-045.

554 [23] **Drobník J**. 4 1960. Primary oxidation of organic matter in the soil. *Plant and Soil* 12:199–
555 211. doi:10.1007/BF01343649.

556 [24] **Reichart NJ, Jay ZJ, Krukenberg V, Parker AE, Spietz RL, Hatzenpichler R**. 11 2020. Activity-
557 based cell sorting reveals responses of uncultured archaea and bacteria to substrate
558 amendment. *ISME Journal* 14:2851–2861. doi:10.1038/s41396-020-00749-1.

559 [25] **Khalili B, Weihe C, Kimball S, Schmidt KT, Martiny JBH**. Oct 2019. Optimization of a
560 Method To Quantify Soil Bacterial Abundance by Flow Cytometry. *mSphere* 4 (5).
561 doi:10.1128/msphere.00435-19.

562 [26] **R Core Team**. 2022. R: A Language and Environment for Statistical Computing. R Foundation
563 for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

564 [27] **Wickham H**. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.
565 https://ggplot2.tidyverse.org.

566 [28] **Xu S, Chen M, Feng T, Zhan L, Zhou L, Yu G**. Nov 2021. Use ggbreak to Effectively
567 Utilize Plotting Space to Deal With Large Datasets and Outliers. *Frontiers in Genetics* 12.
568 doi:10.3389/fgene.2021.774846.

569 [29] **Papp K, Hungate BA, Schwartz E**. 1 2020. Glucose triggers strong taxon-specific responses
570 in microbial growth and activity: insights from DNA and RNA qSIP. *Ecology* 101:e02887.
571 doi:10.1002/ECY.2887.

572 [30] **Frossard A, Hammes F, Gessner MO**. Jun 2016. Flow Cytometric Assessment of Bacterial
573 Abundance in Soils, Sediments and Sludge. *Frontiers in Microbiology* 7.
574 doi:10.3389/fmicb.2016.00903.

575 [31] **Borer B, Or D**. Feb 2022. Bacterial age distribution in soil – Generational gaps in
576 adjacent hot and cold spots. *PLOS Computational Biology* 18 (2):e1009857.
577 doi:10.1371/journal.pcbi.1009857.

578 [32] **Tuttle AR, Trahan ND, Son MS**. Jan 2021. Growth and Maintenance of *Escherichia coli*
579 Laboratory Strains. *Current Protocols* 1 (1). doi:10.1002/cpz1.20.

580 [33] **Bowen GD, Rovira AD**. Sep 1976. Microbial Colonization of Plant Roots. *Annual Review of*
581 *Phytopathology* 14 (1):121–144. doi:10.1146/annurev.py.14.090176.001005.

582 [34] **Caro TA, McFarlin J, Jech S, Fierer N, Kopf S**. Apr 2023. Hydrogen stable isotope probing of
583 lipids demonstrates slow rates of microbial growth in soil. *Proceedings of the National Academy*
584 *of Sciences* 120 (16). doi:10.1073/pnas.2211625120.

585 [35] **Wang H, Sun T, Zhao Z, Gu S, Liu Q, Wu T, Wang D, Tian C, Li J**. Apr 2021. Transcriptional
586 Profiling of Myceliophthora thermophila on Galactose and Metabolic 580 Engineering for
587 Improved Galactose Utilization. *Frontiers in Microbiology* 12. doi:10.3389/fmicb.2021.664011.

588 [36] **Cotrufo MF, Wallenstein MD, Boot CM, Denef K, Paul E**. Feb 2013. The Microbial Efficiency-
589 Matrix Stabilization (MEMS) framework integrates plant litter decomposition with soil organic
590 matter stabilization: do labile plant inputs form stable soil organic matter? *Global Change*
591 *Biology* 19 (4):988–995. doi:10.1111/gcb.12113.

592 [37] **Bölscher T, Wadsö L, Börjesson G, Herrmann AM**. Feb 2016. Differences in substrate use
593 efficiency: impacts of microbial community composition, land use management, and substrate
594 complexity. *Biology and Fertility of Soils* 52 (4):547–559. doi:10.1007/s00374-016-1097-5.

595 [38] **Manzoni S, Taylor P, Richter A, Porporato A, Ågren GI**. Jul 2012. Environmental and
596 stoichiometric controls on microbial carbon-use efficiency in soils. *New Phytologist* 196 (1):79–
597 91. doi:10.1111/j.1469-8137.2012.04225.x.

598 [39] **Franzluebbers AJ**. Jan 2016. Should Soil Testing Services Measure Soil Biological Activity?
599 *Agricultural I& Environmental Letters* 1 (1). doi:10.2134/ael2015.11.0009.

600 [40] **Stenberg B**. May 1998. Soil attributes as predictors of crop production under
601 standardized conditions. *Biology and Fertility of Soils* 27 (1):104–112.
602 doi:10.1007/s003740050407.

603 [41] **Mangalassery S, Mooney SJ, Sparkes DL, Fraser WT, Sjögersten S**. May 2015. Impacts of
604 zero tillage on soil enzyme activities, microbial characteristics and organic matter functional

chemistry in temperate soils. *European Journal of Soil Biology* 68:9–17. doi:10.1016/j.ejsobi.2015.03.001.

[42] **Nelson, JT, Adjuik, TA, Moore, EB, VanLoocke, AD, Ramirez Reyes, A, & McDaniel, MD.** Dec 2023. A Simple, Affordable, Do-It-Yourself Method for Measuring Soil Maximum Water Holding Capacity. *Communications in Soil Science and Plant Analysis 55*(8):1190–1204. doi:10.1080/00103624.2023.2296988.