Positive relationship between substrate-induced respiration rate and translationally active bacteria count in soil

Nina Rose Camillone,*[1,2] Mary Ann 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 218, Importance 68, Body text 4612

ABSTRACT

Soil microorganisms carry out many biological functions that are fundamental to soil ecosystems; however, less than 2% of soil bacterial cells are commonly estimated to be active at any point in time. Substrate-induced respiration (SIR) measures

community-level microbial activity as the bulk soil response to carbon substrate addition. We hypothesized that this bulk respiration rate is dependent on changes in the number of active cells. We incubated subsamples of an agricultural soil with 2 carbon substrate treatments and a third water-only treatment, and we periodically monitored respiration and active cell counts for 24 h. To enumerate active bacterial cells, we applied the novel Bioorthogonal Non-canonical Amino Acid Tagging (BONCAT) method. BONCAT provides a labeled amino acid for active cells to incorporate into newly synthesized proteins, and then a fluorescent dye enables enumeration of labeled cells 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, however, increases in active cells were more than proportional to 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 results indicate that number of translationally active bacteria is an important but not singular contributor to observed bulk soil respiration.

IMPORTANCE

While many critical ecosystem services provided by soil are known to fundamentally rely on microbial activity, the soil microbial community largely remains a black box. This study demonstrates that substrate-induced respiration is linked to the number of active cells. 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, aggregate formation, nitrogen fixation, antibiotic production, and more[1]. Recent advances in genetic sequencing technologies

and -omics analyses provide exciting opportunities for taxonomic identification and functional profiling of soil microbial communities, 99% of which are unculturable under conventional laboratory conditions[2]. To date, DNA-based studies have identified dominant bacterial taxa common in global soils across environments[3] and have revealed the effect of agriculturally relevant soil properties, particularly pH, on microbial diversity[4]. Mechanisms and causal relationships linking microbial activity with macroscale soil functions have been demonstrated in studies focused on particular genes, such as those involved in nitrogen cycling[5]. However, other soil functions such as carbon sequestration or maintaining stable structure are too complex to be narrowed down to a specific microbial group or gene of interest. In these cases, the identities or theoretical capabilities of the microbial community may not be as important as their abundance and actual activity. As agricultural intensification and climate change continue to threaten soil microbial diversity, modeling work and mitigation efforts lack fundamental insights about the microbial actors and interactions underlying vital soil functions[6, 7].

Relationships between soil microbiome composition and soil ecosystem services may be obfuscated by the soil's high percentage of cells that are resting, inactive, or dormant cells[8]. Since soil microenvironments are typically low in carbon and energy sources, many microbes remain dormant to survive but readily resume activity when resources become available[9]. At any point in time, 80% of soil bacterial cells may be dormant while 5-20% or possibly as few as 1% are active, with estimates varying among studies[10, 8, 11]. This high level of inactivity complicates efforts to link microbial community composition with bulk soil functions. Novel applications of in-situ approaches to microbial physiology are needed for a causal and predictive understanding of native microbial activity [12]. Recently, bioorthogonal non-canonical amino acid tagging (BONCAT) has enabled the isolation of translationally active microbes when coupled with fluorescence-activated cell sorting (FACS)[13]. In soils, this approach revealed a distinct phylogenetic composition of translationally active microbes compared to the overall community[14]. Another approach, quantitative stable-isotope probing (qSIP) has allowed investigation of soil microbial groups actively involved in organic

matter decomposition[15] and revealed higher activity of motile soil microbes under increased precipitation[16]. Various definitions of active cells and corresponding methodologies for their quantification exist[17, 18], posing opportunities to pinpoint microbial actors contributing to soil ecosystem functions. Here, we define active microbes as those synthesizing new proteins and dormant microbes as those with no present protein translation.

At the bulk or total community level, soil respiration is used as a proxy for soil microbial activity. Multiple substrate induced respiration (MSIR), also known as the community-level physiological profile (CLPP), is a fingerprinting tool for the soil microbial community's potential activity[19, 20]. In this method, the soil's respiration responses to individual addition of multiple carbon substrates are recorded. The ability of the soil to produce CO_2 from a wider diversity of substrates may be an indicator of an active and dynamic microbial community. It remains unknown weather respiration rates across MSIR assays are controlled mainly by numbers of active cells or by activity rates per cell.

In this study, we investigate whether substrate induced respiration (SIR) reflects the activation of dormant cells. Specifically, we employed BONCAT[14] and flow cytometry to enumerate active microbes in tandem with measuring SIR. Since we simultaneously monitored soil microbial activity at the bulk and cell levels, we calculated respiration rate per active cell. As in MSIR, we used two carbon substrate treatments (glucose and galactose) with different respiration responses along with a water-only treatment. We measured activity after 4 incubation times up to 24 h. We hypothesized that variation in bulk soil respiration rate among substrate treatments would reflect numbers of active microbes. Further, we hypothesized that changes in respiration rate over time within a specific substrate treatment would correlate with changes in the number of active soil microbes.

MATERIALS AND METHODS

Soil collection and experimental design

All soil samples were collected at the Pennsylvania State University's Russell E. Larson Agricultural Research Center near Pennsylvania Furnace, PA (40.72°N, -77.92°W), from a long-term tillage experiment established in 1978[21]. A composite sample of bulk soil was collected in May 2023 from the top 10 cm of a long-term no-till plot planted with wheat, representing the Ap horizon of a Hagerstown silt loam (fine, mixed, semiactive, mesic Typic Hapludalfs in USDA soil taxonomy). After being transported to the lab in a cooler, the soil was sieved to 2 mm and stored at 4°C for less than 4 months before experimentation was completed. Gravimetric water content was determined to be 16.6% by oven-drying a subsample at 105°C for 24 h. Available water holding capaciy was determined to be 62.2% by the capilary method: a subsample was air-dried at room temperature for 3 days, placed in a pre-weighed setup of a wetted filter paper in a funnel, and allowed to wick up water for 1 hr or until the entire surface of the soil glistened. Incubations of soil subsamples with cycloheximide to inhibit fungi or chloramphenicol to inhibit bacteria for 6 h showed that respiration was around 60% attributable to bacteria and 40% to fungi (Supplemental Table 1)[22].

The experimental timeline is summarized in Figure **1**. Each experimental replicate consisted of 1.2 g field-moist soil (corresponding to 1 g oven-dried soil). Replicates were placed into 12-mL glass tubes sealable via screw-on caps with silicone septa (Exetainer®12ml Vial - Flat Bottom 738W, Labco Limited, High Wycombe, UK). The septa also allowed needle syringe access for headspace sampling. For substrate treatments, 100 µL of liquid was added to each tube: either an aqueous substrate solution containing 3.0 mol L^{-1} carbon (in the form of glucose or galactose, Sigma-Aldrich, Saint Louis, MO, USA) or ultrapure water. This resulted in a final concentration equivalent to 30 mg glucose g^{-1} soil water, which is the concentration commonly used with the MicroResp^{TM} system[19, 20]. The gravimetric water content of the soil after addition of the substrate treatments was 30%, which was 50% of the available water holding capacity. The tubes were sealed to limit moisture evaporation and incubated in the dark at 22°C for 2, 6, 12, or

24 h. The time intervals reflect previous studies where respiration rates in surface horizon soil microcosms amended with glucose peaked from 5-10 h (depending on glucose concentration) and returned to near initial rates after 24 h[23]. Galactose was selected as a substrate treatment because it stimulates a soil respiration rate intermediate to glucose and water-only[19]. Initial pilot experiments included lignin and vanillic acid as additional substrates. However, these treatments resulted in very low BONCAT activity signals, on average less than 0.5% (compared to the false positive discovery rate of 0.2%) even after 24 h incubation. These substrates were not included and further research will be necessary to understand why the BONCAT signal was so low in these treatments. Six replicate tubes were included per substrate treatment per incubation length.

Activity monitoring: respiration measurement and cell extraction

Two hours prior to the end of the incubation period (i.e. concurrently with the start of 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[14].

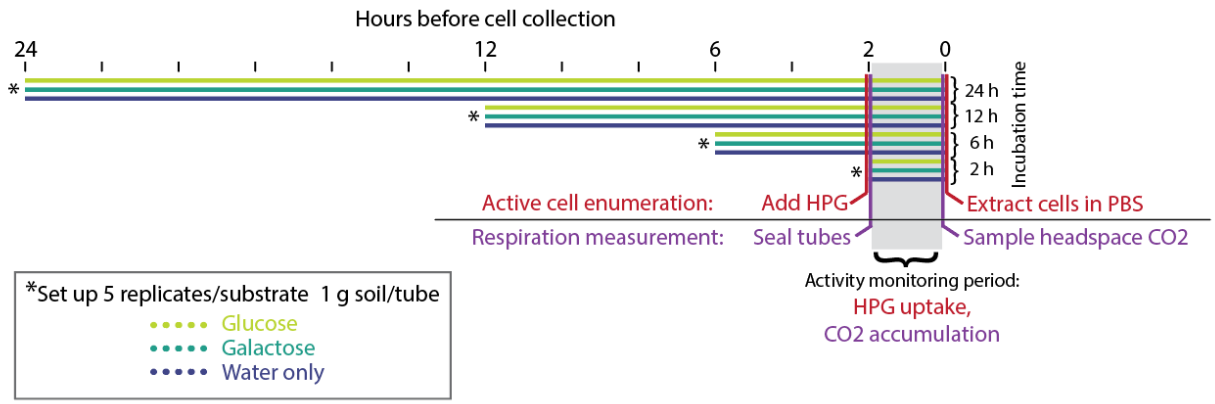

FIG 1 Experimental design as a timeline of substrate incubations, headspace sampling, and cell collection. L-homopropargylglycine (HPG) is a synthetic amino acid homologous to methionine used to label translationally active cells in the BONCAT workflow.

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

Active microbial cell labeling

The methods for collecting cells and BONCAT staining with a click chemistry reaction were adapted from previously published methods [14, 24]. After thawing and briefly vortexing the frozen cell suspensions, they were centrifuged at 50 x g for 5 min to pellet any larger remaining soil particles. Previous studies have used much higher centrifuge speeds while maintaining high cell recovery[25], but for this soil we had very low cell yields when higher speeds were used to pellet soil particles, perhaps due to cell clumping. The supernatant was recovered and passed through a 35 µm filter. Then, cells were pelleted from the suspension by centrifuging at 16,000 x g for 5 min. The supernatant was poured off and discarded, and the cells were resuspended in 70.4 µL 1x PBS in 2-mL snap-cap tubes by briefly vortexing. A dye mixture was prepared and added to each tube for a final reaction concentration of 5 µmol L^{-1} amino guanidine hydrochloride, 5 µmol L^{-1} sodium L-ascorbate, 100 µmol L^{-1} copper sulfate pentahydrate (ThermoFisher Scientific, Eugene, OR, USA), 500 µmol L^{-1} tris-hydroxypropyltriazolylmethylamine (THPTA), and 5 µmol L^{-1} FAM picolyl-azide dye in a final volume of 80 µL. Unless otherwise noted, all BONCAT reagents including HPG were purchased from Click Chemistry Tools (Scottsdale, AZ, USA). The tubes were incubated in the dark for 60 min to permit the click reaction between the azide dye and the HPG incorporated into the proteins of translationally active cells. Next, samples were washed to remove excess dye by centrifuging at 16,000 x g for 5 min, resuspending in 1 mL 1x PBS by brief vortexing, and repeating these steps twice more. The resulting cell suspension was diluted in 1x PBS and passed through a 35 µm filter (Falcon Round-Bottom Tubes with Cell Strainer Cap, 5 mL). SYTO™ 59 Red Fluorescent Nucleic Acid Stain (ThermoFisher Scientific, Invitrogen, Eugene, OR, USA) was added as a counterstain to distinguish cells from clay particles. Cell suspensions were incubated in the dark for 15 min.

Flow cytometry

Cell enumeration was performed using the LSR Fortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) at the Pennsylvania State University Flow

201 Cytometry Core, which is equipped with 405 nm (violet), 488 nm, (blue), 532 nm (green),
202 and 640 nm (red) lasers. The BONCAT dye, FAM-picolyl azide (490 nm excitation, 510 nm
203 emission), was captured in a green channel ("FITC") off the 488 nm blue laser. The DNA
204 counterstain, SYTO59 (622 nm excitation, 645 nm emission), was captured in a red
205 channel ("APC") off the 640 nm red laser. Forward and side light scatter were also
206 collected for all samples. All samples were run on the lowest flow rate. Data was collected
207 for 120 s. Knowing the flow rate of the instrument enabled calculation of the original
208 concentration of cells in the soil using the number of cells detected, the volume of extract
209 processed, and the mass of soil extracted.

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

216 Data analysis was performed using FlowJo Software (Tree Star, Ashland, OR, USA).
217 First, forward and side scatter fluorescence thresholds were set to constrain the size of
218 particles examined. Secondly, a threshold for SYTO positive (SYTO+) particles, which are
219 assumed to be cells rather than clay particles, was determined based on fluorescence
220 intensity in the APC channel from the killed (autoclaved) control samples counterstained
221 with SYTO59. This step accounts for any potential autofluorecense from or staining of soil
222 particles. Finally, a threshold for BONCAT positive (BONCAT+) cells was determined
223 based on the fluorescence intensity in the FITC channel from the SYTO+ population (cells)
224 in the HPG-negative control. To account for electronic noise from the flow cytometer or
225 any potential retention of dyes on soil particles, the number of SYTO+ events detected in
226 the killed control sample was subtracted from the SYTO+ count in other samples within
227 each batch. Additionally, the number of BONCAT+ events detected in the HPG-negative
228 control was subtracted from the BONCAT+ population in experimental samples. Any
229 sample where the number of BONCAT+ events was less than or equal to the number of
230 BONCAT+ events in the counterstain-only control was recorded to have zero active cells.

Preliminary work using the LIVE/DEAD™ *Bac*Light™ Bacterial Viability and Counting Kit, for flow cytometry (ThermoFisher Scientific, Invitrogen, Eugene, OR, USA), detected negligible percentages of dead microbes recovered using our extraction method (<0.65% on average; see Supplemental Table 2), likely due to our cell extraction method failing to preserve cells without intact membranes, i.e. those deemed dead by the staining kit used. Thus, we report total cells as representative of the living community.

Statistical analysis

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

RESULTS

Numbers of total and active cells

The total number of microbial cells ranged between $3.14x10^5$ and $2.2x10^7$ cells g^{-1} soil throughout the experiment (Figure 2A). Generally, the number and percentage of active cells increased with time for all treatments (Figure 2). Throughout the experiment,

numbers of total and active cells were highest for the glucose treatment at 12 h of incubation time. For the galactose treatment, total and active cells peaked at 24 h. For the water treatment, total cells peaked at 12 h while active cells peaked at 24 h. During the first 6 h of the experiment, the glucose treatment had the highest total number of cells while the water treatment had the least. The number and percentage of active cells showed this same pattern at 6 h. At 12 h and 24 h, the pattern was different: the glucose treatment had the most total and active cells, and galactose had the least.

The largest absolute increases in numbers of total and active cells were between 6 and 12 h for the glucose treatment (Figure 2A). The largest rate of change of number of active cells was in the glucose treatment between 2 and 6 h (Figure 3). For the galactose treatment, the largest absolute increase in number of total cells was later, between 12 and 24 h. The rate of change did not measurably change for this treatment throughout the experiment. For the water-only treatment, the largest absolute increase in number of active cells was between 6 and 12 h like glucose. Unlike glucose, this coincided with the greatest rate of change in number of active cells.

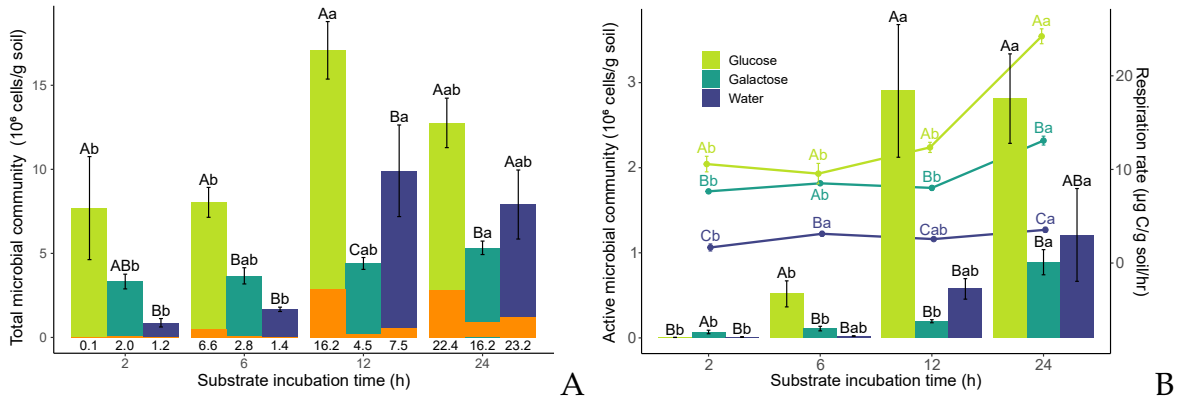

FIG 2 Total cells, active cells, and respiration rate. (A) Total cells over time for different substrate treatments, reported in cells per g dry soil. The lower (orange) portion of each bar represents the active cell fraction. Numbers below each bar indicate the percentage of active cells. (B) Active 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 different 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.

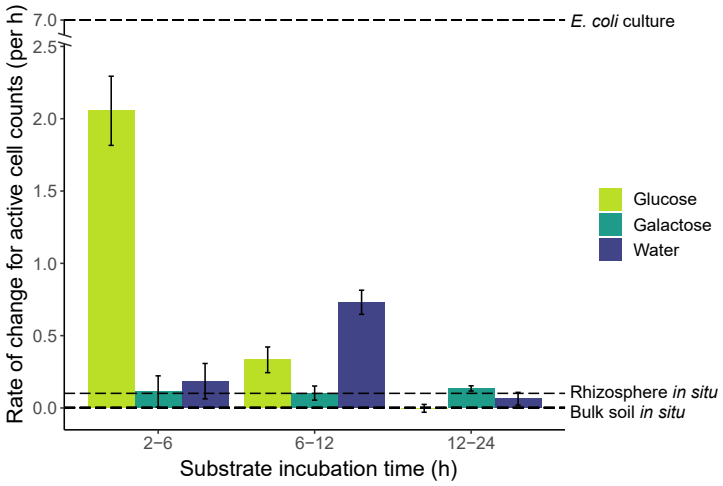

FIG 3 Rate of change in number of active cells over time. Bars indicate standard error, propagated from the standard error in the enumerated community sizes. For comparison, dashed lines indicate several known growth rates: a laboratory culture of *Escherichia coli*,[29] an active rhizosphere community [30], and bulk forest soil [31].

Bulk and cell-specific respiration rate

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

In all three treatments, the cell-specific respiration rate was highest after 2 h and decreased thereafter. However, this decrease was only statistically significant for glucose (Figure 4). While the galactose treatment had the lowest cell-specific respiration after 2 h, it had the higheest cell-specific respiration after 12 and 24 h. After 6 h, there were no statistically significant differences in cell-specific respiration rate among the treatments.

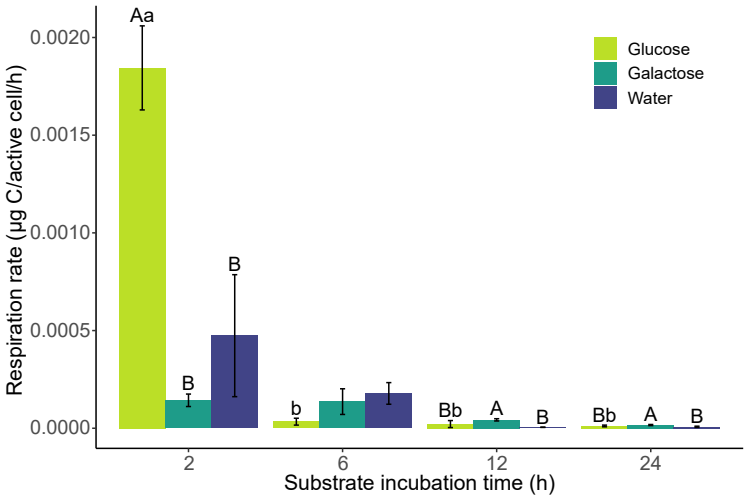

FIG 4 Cell-specific respiration rate, calculated by dividing bulk respiration by the number of active cells. Uppercase letters represent statistically significant differences found by Tukey's HSD test ($p<0.05$) among substrate treatments within the same time point, indicated by the x axis. Lowercase 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

Reactivation of dormant microbes

This study sought to determine whether increases in bulk respiration rate due to substrate addition are best explained by increasing respiration per active cell or increasing numbers of active cells. Within 2 h of substrate addition, the number of active cells among treatments did not differ by a large margin, if at all. Yet, the two carbon-amended treatments had much higher bulk respiration rates (Figure2B). Thus, for the first 2 h of the experiment, the data do not support our hypothesis that activation of dormant cells drives differences in bulk respiration. The observed differences in cell-specific respiration are discussed further below.

295 Active cell numbers increased most rapidly after 2 h of incubation with glucose
296 (Figure 3). Notably, this was the only instance of an increase in total cells that was
297 outpaced by a larger increase in active cells. In other words, the number of dormant cells
298 (estimated by subtacting active from the total) decreased, suggesting cell activation.
299 Alternatively, or in addition, this could indicate a death rate among inactive cells
300 outpaced by reproduction among active cells; however, this seems less likely considering
301 known soil microbial growth rates as illustrated in Figure 3. Assuming no growth or
302 reactivation of dormant cells, active cells would have been doubling every 37 min to
303 achieve the observed rate of increase between 2 and 6 h for the glucose treatment. This
304 would have approached the theoretical maximum growth rate under optimum conditions
305 reported as a generation time of 0.5 h [32]. An abundant supply of glucose could support
306 such an optimal environment for microbial reproduction. Similarly, it would also support
307 an optimal environment for dormant microbe reactivation. In actuality, soil microbes have
308 been clocked with doubling times only as low as 7.2 h in the rhizosphere[30] and 14 to 45
309 days in the bulk soil[31]. This suggests that reactivation of dormant cells must also have
310 contributed to the increase in active cells. The growth rate for total cells in our study was
311 generally much lower than that of active cells (Supplemental Figure 1).

312 Cell-specific activity differed among treatments

313 As hypothesized, respiration rates and active cell counts were positively correlated.
314 Their relationship was statistically significant within each treatment and across all
315 treatments together (Figure 5A). The lower respiration rates and numbers of active cells in
316 the galactose treatment compared to the glucose treatment are consistent with the ability
317 of cells to harvest energy directly from glucose. By contrast, galactose requires multiple
318 enzyme-catalyzed reactions before a downstream metabolite enters the glycolytic
319 pathway [33]. Assuming a consistent cell-specific respiration rate, we hypothesized that
320 active cell counts and respiration rates would follow similar patterns within treatments.
321 We observed this at 6 h of substrate incubation, the only time point with no statistically
322 significant difference in cell-specific respiration among treatments (Figure 4): active cell
323 counts and respiration rate were both highest for glucose and lowest for water (Figure 2B).

At this time point, respiration and active cell counts were positively correlated across treatments, with a statistically significant regression coefficient (Figure 5B).

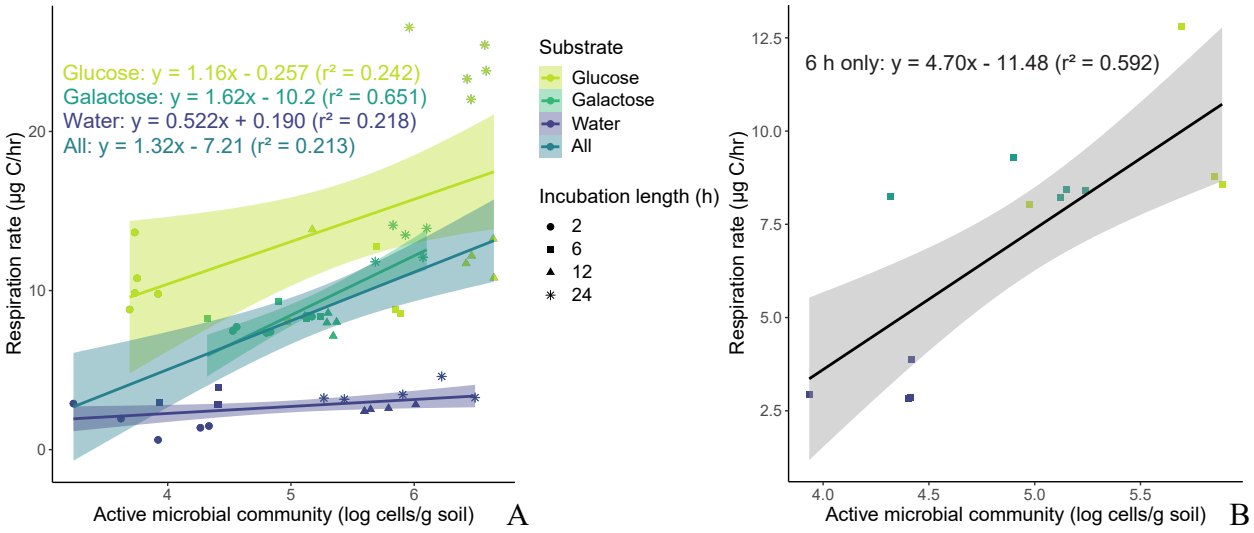

FIG 5 Regressions of bulk respiration onto active cell counts in log scale. The shaded regions show the 95% confidence region for each regression line. (A) Regression lines for all data and for each substrate individually. P-values for coefficients and intercepts respectively are 0.0322 and 0.968 for glucose, <0.0001 and 0.00739 for galactose, 0.0438 and 0.6155 for water, and <0.001 and 0.0898 for all data. (B) Regression line for data from 6-h incubation only. P-values are 0.0021 for the coefficient and 0.0327 for the intercept.

However, the other incubation lengths demonstrate a more complex and substrate-dependent relationship between respiration and active cell numbers. Besides the 6-h time point, cell-specific respiration differed among treatments (Figure 4). At 2 h, it was highest for the glucose treatment, driven by low active cell counts and a high respiration rate. At 12 and 24 h, cell-specific respiration was highest for galactose, caused by lower active cell counts than glucose and higher respiration rates than water. These differences over time between the two carbon substrates suggest that soil microbes rapidly began to metabolize available glucose, with a burst of respiration per cell within 2 h and then the activation of dormant cells by 6 h. Meanwhile, galactose induced increases in respiration and active cell counts at a slower but more steady rate. The galactose treatment exhibited the least variation in cell-specific respiration and the strongest

338 positive correlation (highest regression r^2) between bulk respiration and active cell counts
339 (Figure 5). A low level of respiration from BONCAT-negative cells also would have
340 contributed to low cell-specific respiration and a strong correlation between respiration
341 and active cells. This could be the case if few cells already contained the enzymes
342 necessary for galactose metabolism.

343 Without any carbon addition, the water-only treatment has the lowest regression
344 coefficient (Figure 5). This indicates that this moist soil environment supported an
345 expanding active community with minimal changes in respiration rate. This finding is
346 consistent with the Microbial Efficiency-Matrix Stabilization (MEMS) framework, which
347 hypothesizes that respiration is highest where inputs are low quality (excess ratio of C to
348 other nutrients) and have a low capacity to be stabilized in the mineral soil matrix [34].
349 With a lower abundance of simple carbon sources, active microbes in the water-only
350 community may use carbon more efficiently, i.e. prioritize its assimilation as biomass over
351 mineralization to harvest energy, compared to the carbon-rich substrate treatments. This
352 is also consistent with a previous study that found that soil microbes metabolized
353 complex substrates with a higher carbon use efficiency (CUE), defined as respiration rate
354 per carbon assimilated into biomass[35].

355 Methodological limitations

356 BONCAT offers novel insights into soil microbial activity and its relationship with
357 bulk functions. However, we note that some inherent aspects may impact data
358 interpretation. For example, extraction cannot capture all microbes present in the soil.
359 Typically, soils contain 10^7-10^{10} bacterial cells per gram[36] while most of our counts after
360 2 h of incubation were below $5x10^6$ (Figure 2A). We confirmed that our cell extracts had
361 very low numbers of dead microbes defined by loss of membrane integrity (Supplemental
362 Figure 1). In contrast, a review of previous studies reports findings of 40% dead microbial
363 cells in soil[10], which may contribute to our lower cell counts. We assumed that the
364 percentages of active cells determined for our extracts were representative of the whole
365 live community.

Another consideration is the definition of an active cell. This paper defines activity as protein translation. However, inactive cells under this definition (i.e. BONCAT-negative cells) may still be respiring. Since glucose (unlike galactose) can directly enter glycolysis, it is possible that many cells already contained the requisite proteins for glucose metabolism and did not require novel protein synthesis to contribute to the glucose treatment's initial high respiration rate. Thus, the high cell-specific activity for glucose (Figure 4) could be partly explained by more activity from BONCAT-negative cells. Additionally, cells that used any methionine already present in the soil rather than HPG would be BONCAT-negative despite being translationally active. Other activity assays may be more directly associated with respiration, such as redox-sensitive dyes or stable isotope analysis[10, 17]. In the end, respiration is a continuous variable that cannot be fully explained by the simplistic binary of active or inactive cells.

Implications for soil testing

As a proxy for soil microbial activity, SIR has a practical usage in soil testing for crop yield prediction[37, 38]. Establishing a correlation between SIR and numbers of active microbes would support an inference that more active microbes are also correlated with agricultural yields. This may seem intuitive, considering the microbial basis for many critical agricultural soil functions such as decomposition and 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 a 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 [10]. 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, which is the length of time used in previous MSIR studies to observe the existing community while limiting time for shifts in community composition[19, 20, 39]. Additionally, our results showed the most similar effect of substrate treatment on respiration rate and active cell counts (Figure **2**B) and the strongest correlation between these two indicators (Figure **5**B) after the 6 h incubation. 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. We generally observed higher cell-specific respiration in carbon-amended treatments (Figure **4**), and carbon-amended replicates had higher respiration than the water replicates with similar numbers of active cells **5**. 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[40], similarly pointing toward higher activity per cell with access to carbon.

On the whole, 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 processes. 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

Gas analysis for CO_2 content was conducted in the lab of Jason Kaye with training and support from Brosi Bradley. Cell enumerations were conducted at the Huck Institutes'

Flow Cytometry Core Facility on the BD Fortessa flow cytometer with training and support from multiple staff members.

DATA AVAILABILITY STATEMENT

Data are available from Penn State's DataCommons at doi.org/10.26208/G8KX-GG32.

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.

CONFLICTS OF INTEREST

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] **Pham VHT, Kim J**. Sep 2012. Cultivation of unculturable soil bacteria. *Trends in Biotechnology* 30 (9):475–484. doi:10.1016/j.tibtech.2012.05.007.

[3] **Delgado-Baquerizo M, Oliverio AM, Brewer TE, Benavent-González A, Eldridge DJ, Bardgett RD, Maestre FT, Singh BK, Fierer N**. Jan 2018. A global atlas of the

dominant bacteria found in soil. *Science* 359 (6373):320–325. doi: 10.1126/science.aap9516.

[4] **Fierer N, Leff JW, Adams BJ, Nielsen UN, Bates ST, Lauber CL, Owens S, Gilbert JA, Wall DH, Caporaso JG**. Dec 2012. Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proceedings of the National Academy of Sciences* 109 (52):21390–21395. doi:10.1073/pnas.1215210110.

[5] **Saha D, Kaye JP, Bhowmik A, Bruns MA, Wallace JM, Kemanian AR**. Aug 2021. Organic fertility inputs synergistically increase denitrification-derived nitrous oxide emissions in agroecosystems. *Ecological Applications* 31 (7). doi:10.1002/eap.2403.

[6] **Jansson JK, Hofmockel KS**. Oct 2019. Soil microbiomes and climate change. *Nature Reviews Microbiology* 18 (1):35–46. doi:10.1038/s41579-019-0265-7.

[7] **Mukhtar H, Wunderlich RF, Muzaffar A, Ansari A, Shipin OV, Cao TND, Lin YP**. Jul 2023. Soil microbiome feedback to climate change and options for mitigation. *Science of The Total Environment* 882:163412. doi:10.1016/j.scitotenv.2023.163412.

[8] **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.

[9] **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.

[10] **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.

[11] **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.

[12] **Hatzenpichler R, Krukenberg V, Spietz RL, Jay ZJ**. Feb 2020. Next-generation physiology approaches to study microbiome function at single cell level. *Nature Reviews Microbiology* 18 (4):241–256. doi:10.1038/s41579-020-0323-1.

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

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

[15] **Dong W, Li X, Wang E, Liu X, Wang M, Song A, Yin H, Fan F**. Oct 2021. Linking microbial taxa and the effect of mineral nitrogen forms on residue decomposition at the early stage in arable soil by DNA-qSIP. *Geoderma* 400:115127. doi: 10.1016/j.geoderma.2021.115127.

[16] **Greenlon A, Sieradzki E, Zablocki O, Koch BJ, Foley MM, Kimbrel JA, Hungate BA, Blazewicz SJ, Nuccio EE, Sun CL, Chew A, Mancilla CJ, Sullivan MB, Firestone M, Pett-Ridge J, Banfield JF**. Dec 2022. Quantitative Stable-Isotope Probing (qSIP) with Metagenomics Links Microbial Physiology and Activity to Soil Moisture in Mediterranean-Climate Grassland Ecosystems. *mSystems* 7 (6). doi: 10.1128/msystems.00417-22.

[17] **Emerson JB, Adams RI, Román CMB, Brooks B, Coil DA, Dahlhausen K, Ganz HH, Hartmann EM, Hsu T, Justice NB, Paulino-Lima IG, Luongo JC, Lymperopoulou DS, Gomez-Silvan C, Rothschild-Mancinelli B, Balk M, Huttenhower C, Nocker A, Vaishampayan P, Rothschild LJ**. Aug 2017. Schrödinger's microbes: Tools for distinguishing the living from the dead in microbial ecosystems. *Microbiome* 5 (1). doi:10.1186/s40168-017-0285-3.

[18] **Singer E, Wagner M, Woyke T**. Jun 2017. Capturing the genetic makeup of the active microbiome in situ. *The ISME Journal* 11 (9):1949–1963. doi:10.1038/ismej.2017.59.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

[40] **Mangalassery S, Mooney SJ, Sparkes DL, Fraser WT, Sjögersten S**. May 2015. Impacts of 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.