Quantitative Microbial Ecology Through Stable Isotope Probing

Bruce A. Hungate[a,b,#], Rebecca L. Mau[a], Egbert Schwartz[a,b], J. Gregory Caporaso[a,b,c], Paul Dijkstra[a,b], Natasja van Gestel[a], Benjamin J. Koch[a], Cindy M. Liu[d,e], Theresa A. McHugh[a*], Jane C. Marks[a,b], Ember Morrissey[a,b], Lance B. Price[d,f]

[a]Center for Ecosystem Science and Society, Northern Arizona University, Flagstaff, Arizona, USA; [b]Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona, USA; [c]Center for Microbial Genetics and Genomics, Northern Arizona University, Flagstaff, Arizona, USA; [d]Translational Genomics Research Center, Flagstaff Arizona, USA; [e]Department of Pathology, Johns Hopkins School of Medicine, Baltimore, Maryland, USA; [f]Department of Environmental and Occupational Health, Milken Institute School of Public Health, George Washington University, Washington, District of Columbia, USA

#Address correspondence to Bruce Hungate, Bruce.Hungate@nau.edu

[*]Present address: U.S. Geological Survey, Southwest Biological Science Center, Moab, Utah, USA

Running head: Quantitative Stable Isotope Probing

Abstract

Bacteria grow and transform elements at different rates, yet quantifying this variation in the environment is difficult. Determining isotope enrichment with fine taxonomic resolution after exposure to isotope tracers could help, but there are few suitable techniques. We propose a modification to Stable Isotope Probing (SIP) that enables determining the isotopic composition of DNA from individual bacterial taxa after exposure to isotope tracers. In our modification, after isopycnic centrifugation, DNA is collected in multiple density fractions, and each fraction is sequenced separately. Taxon specific density curves are produced for labeled and non-labeled treatments, from which the shift in density for each individual taxon in response to isotope labeling is calculated. Expressing each taxon's density shift relative to that taxon's density measured without isotope enrichment accounts for the influence of nucleic acid composition on density and isolates the influence of isotope tracer assimilation. The shift in density translates quantitatively to isotopic enrichment. Because this revision to SIP allows quantitative measurements of isotope enrichment, we propose to call it quantitative Stable Isotope Probing (qSIP). We demonstrate qSIP using soil incubations, in which soil bacteria exhibited strong taxonomic variation in ^{18}O and ^{13}C composition after exposure to ^{18}O-H_2O or ^{13}C-glucose. Addition of glucose increased assimilation of ^{18}O into DNA from ^{18}O-H_2O. However, the increase in ^{18}O assimilation was greater than expected based on utilization of glucose-derived carbon alone, because glucose addition indirectly stimulated bacteria to utilize other substrates for growth. This example illustrates the benefit of a quantitative approach to stable isotope probing.

Keywords: stable isotope probing, oxygen-18, ^{18}O-H_2O, biodiversity, ecosystem functioning, soil carbon cycle

48 **Introduction**

49 The types of organisms present in an ecosystem profoundly influence its functioning, an
50 idea well established for plants and animals, formalized in the state factor theory of
51 ecosystem science (1), and illustrated through the impacts of plant and animal invasions
52 on ecosystem processes (2). The physiological and taxonomic diversity of
53 microorganisms far exceeds that of plants and animals combined (3). Yet, despite
54 progress applying molecular tools to analyze microbial diversity of intact assemblages (4-
55 6), our understanding of how individual microbial taxa affect ecosystem processes like
56 element cycling remains weak. When applied to intact microbial assemblages, stable
57 isotope probing (SIP) partly addresses this challenge, in that it links physically the fluxes
58 of elements to an organism's genome. In conventional SIP, organisms that utilize
59 isotopically labeled substrates incorporate the heavy isotope into their nucleic acids,
60 increasing the density of those nucleic acids which then migrate further along a cesium
61 chloride density gradient formed during isopycnic centrifugation. This enables
62 identifying organisms that utilized the labeled compound for growth (7). Conventional
63 SIP applications use a qualitative approach that identifies visually the separation caused
64 by isotope incorporation (7). Nucleic acids in density regions defined as "heavy" or
65 "light" are then sequenced. Organisms disproportionately represented in the "heavy"
66 region are interpreted as having utilized the labeled substrate for growth (8-11).
67
68 SIP is a robust technique to identify microbial populations that assimilate a labeled
69 substrate, but it does not provide quantitative measures of assimilation rates, for three
70 reasons. First, the distinction between labeled and unlabeled organisms is binary, defined

by the density regions selected by the investigator, limiting the resolution of taxon-specific responses to labeled or unlabeled. Second, the distribution of DNA along the density gradient reflects the influences of both isotope incorporation and GC (guanine plus cytosine) content because the density of DNA increases with its GC content (12). Any comparison of density regions will reflect both influences, challenging inferences about quantitative isotope incorporation. Third, in conventional SIP there are no assurances that the identification of the labeled community is complete. Low GC content organisms that incorporated the isotope label may not have shifted sufficiently in density to be part of the "labeled" density fraction, and high GC content organisms that did not incorporate the label may be erroneously inferred to be part of the labeled community. This could result in incomplete coverage when discrete, non-contiguous, density intervals representing "heavy" and "light" fractions (13, 14) are selected for sequencing, omitting information about the microbial assemblage contained in the DNA at intermediate densities. In other cases, only the "heavy" fractions in both labeled and unlabeled treatments were sequenced and compared: any new organisms that appeared in the heavy fraction of the labeled treatment were inferred to have taken up enough of the isotope tracer to have shifted the density of their DNA (15). This approach could have excluded organisms that incorporated the isotope tracer, but did not shift sufficiently to be represented in the "heavy" fraction, because of their low GC content. In these ways, SIP as typically practiced is a qualitative technique capable of identifying some of the organisms that utilize a substrate, not a quantitative one capable of exploring the full range of variation in isotope incorporation among microbial taxa.

Applied and Environmental Microbiology

Here, we describe modifications to SIP that enable quantifying isotopic incorporation into the genomes of individual taxa. We developed an approach that quantifies the baseline density of the DNA of individual taxa without exposure to isotope tracers, and then quantifies the change in DNA density of each taxon caused by isotope incorporation. Using a model of isotope substitution in DNA, we convert the observed change in density to isotope composition. We show how qSIP applies in soil incubations using a specific carbon source (^{13}C-glucose) and using a universal substrate for growing organisms (^{18}O-H_2O). We also show how combining these tracers provides insight into the microbial ecology of a biogeochemical phenomenon widely observed in soil, the priming effect (16). The "priming effect" is the phenomenon where there occurs "extra decomposition of native soil organic matter in a soil receiving an organic amendment" (17), first documented over 80 years ago (18-20). The opposite can also be found, where substrate addition suppresses organic matter mineralization (21). Some hypotheses to explain priming invoke microbial biodiversity (22), yet those controls remain cryptic, in part because of the difficulty of identifying organisms that respond indirectly to substrate addition by increasing decomposition of native soil organic matter. Quantitative SIP has the potential to address these phenomena, by parsing out the contributions of specific microorganisms to decomposition of the added substrate, labeled with ^{13}C, and to the decomposition of native soil organic matter, which an ^{18}O-H_2O label can detect. Furthermore, the determination of taxon-specific isotope enrichment for each element in qSIP lays the foundation for ascribing rates of element fluxes to particular organisms, which could help explain C fluxes in priming, typically measured on a soil mass basis (e.g., µg C g soil^{-1} d^{-1}). In this way, this example illustrates the potential of qSIP to

advance microbial ecology as a quantitative field, relating microbial biodiversity to element cycling at the ecosystem scale.

Methods

Soil incubations and DNA extractions

Our sample processing scheme, from soil collection, nucleic acid extraction, centrifugation, to data analysis, is summarized in Figure 1. Soil (0-15 cm) was collected in November 2012 from a ponderosa pine forest meadow, located on the C. Hart Merriam Elevation Gradient in Northern Arizona, USA (35.42N, -111.67W; http://nau.edu/ecoss/what-we-do/future-ecosystems/elevation-gradient-experiment/). Soil was sieved (2 mm mesh), left to air-dry for 96 hours, then stored at 4°C before the experiment started. One gram of soil was added to 15 mL Falcon tubes and adjusted to 60% water holding capacity, incubated for one week, and then allowed to air dry for 48 hours prior to isotope additions. Samples were incubated for 7 days.

During the incubation, samples received 200 µL of water g^{-1} soil or a glucose solution at a concentration of 500 µg C g^{-1} soil in the following isotope and substrate treatments (each with n=3): 1) water at natural abundance $\delta^{18}O$; 2) ^{18}O-enriched water (atom fraction 97%); 3) glucose and water at natural abundance $\delta^{13}C$ and $\delta^{18}O$; 4) ^{13}C-enriched glucose (atom fraction 99%) and water at natural abundance $\delta^{18}O$; 5) glucose at natural abundance $\delta^{13}C$ and ^{18}O-enriched water (atom fraction 97%). These treatments were selected in order to evaluate the effects of isotope addition on the density and isotopic composition of DNA. We assessed (I) the effect of ^{18}O in the absence of supplemental glucose as the difference between treatment 2 and 1, (II) the effect of ^{13}C in the presence of supplemental glucose as the difference between treatments 4 and 3, and (III) the effect of ^{18}O with supplemental glucose as the difference between treatments 5 and 3. In each

142 case, these comparisons isolate the effect of the presence of an isotope tracer. The
143 specific equations quantifying these comparisons are presented below.
144
145 After the incubation, samples were frozen and stored at -40°C. DNA was extracted from
146 approximately 0.5 g soil using a FastDNA® Spin Kit for Soil (MP Biomedicals, Santa
147 Ana, CA, USA) following the manufacturer's directions. Extracted DNA was quantified
148 using the Qubit® dsDNA High-Sensitivity Assay Kit and a Qubit® 2.0 Fluorometer
149 (Invitrogen, Eugene, OR, USA).
150
151 *Density Centrifugation and Fraction Collection*
152 To separate DNA by density, 5 µg of DNA was added to approximately 2.6 mL of a
153 saturated CsCl and gradient buffer (200 mM Tris, 200 mM KCl, 2 mM EDTA) solution
154 in a 3.3 mL OptiSeal™ Ultracentrifuge tube (Beckman Coulter, Fullerton, CA, USA).
155 The final density of the solution was 1.73 g cm^{-3}. The samples were spun in an Optima™
156 MAX benchtop ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) using a
157 Beckman TLN-100 rotor at 127,000 x *g* for 72 hours at 18°C. After centrifugation, the
158 density gradient was divided into fractions of 150 µL each using a fraction recovery
159 system (Beckman Coulter Inc, Palo Alto, CA, USA). The density of each fraction was
160 subsequently measured with a Reichert AR200 digital refractometer (Reichert Analytical
161 Instruments, Depew, NY, USA). We did not include DNA standards of known GC
162 content in each ultracentrifuge tube. Such standards are traditionally included when
163 computing GC content based on density is the primary goal (e.g., 12, 23), but are not
164 typically included in SIP studies (24).

DNA was separated from the CsCl solution using isopropanol precipitation, resuspended in 50 µL sterile deionized water, and quantified for each density fraction. We determined total number of bacterial 16S rRNA gene copies in each density fraction by qPCR using a pan-bacterial broad-coverage quantitative PCR technique (25). All fractions were analyzed in triplicate in 10 µl reactions that included 1 µl of DNA template and 9 µl of reaction mix containing 1.8 µM of forward (5'-CCTACGGG**D**GGC**W**GCA-3') and reverse (5'-GGACTAC**HV**GGGT**M**TCTAATC-3') primers (bold letters denote degenerate bases), 225 nM of the TaqMan® minor groove-binding probe (6FAM) 5'-CAGCAGCCGCGGTA-3' (MGBNFQ), 1x *Platinum*® Quantitative *PCR SuperMix-UDG* (Life Technologies, Grand Island, NY), and molecular-grade water. Amplification and real-time fluorescence detection were performed on the 7900HT Real Time PCR System (Applied Biosystems). We provide the qPCR data for all density fractions in the supplementary online material.

Data analysis of total 16S rRNA gene copy number

Based on the qPCR data, we produced a conventional SIP density curve by graphing the proportion of total 16S rRNA gene copies as a function of density, an approach often used to visualize the effect of isotope incorporation on the distribution of densities across the bacterial assemblage, delineating "heavy" and "light" regions for sequencing (9-11). We also calculated the average DNA density for each tube as a weighted average of the density of each fraction in which 16S rRNA gene copies were detected, weighted by the proportional abundance of total 16S rRNA gene copies measured in that fraction for each

tube. This provided an estimate of the average DNA density for each tube, enabling testing via bootstrapping whether isotope addition increased the density of DNA.

Sequencing 16S rRNA genes

We sequenced the 16S rRNA gene in every density fraction that contained DNA (9-15 fractions per centrifuge tube) by a dual-indexing amplicon-based sequencing on the Illumina MiSeq (Illumina Inc, San Diego, CA, USA) following (26). For each density fraction, the 16S rRNA gene V3-V4 hypervariable region was amplified in 25 µl reactions that included 5 µl of gDNA in a 20 µl of reaction mix containing 12.5 µl Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs Inc., Ipswich, MA, USA), 0.75 µl DMSO, and 1.75 µl of sterile water and 0.2 µM of each forward (5'-ACTCCTACGGGAGGCAGCAG-3') and reverse (5'-GGACTAC**HV**GGGT**W**TC-TAAT-3') primers, each concatenated to a linker sequence, a 12bp barcode, and a "heterogeneity spacer" of 0-7bp in size. The following thermocyling condition was used: an initial denaturation at 98°C for 30s, followed by 30 cycles of denaturation at 98°C for 30s, annealing at 62°C for 30s, and amplification/ extension at 72°C for 30s. The resultant amplicons were normalized and pooled using the SequelPrep Normalization Kit (Life Technologies, Carlsbad, CA, USA), then purified using the AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and sequenced in combination with ~20% of PhiX control library (v3) (Illumina) on 300bp paired-end MiSeq runs.

Data-analysis

Applied and Environmental Microbiology

Subsequent sequence processing and quality filtering were also performed as described in Fadrosh et al, 2014 (26). Each read was assigned to the original sample based on the 24bp dual-index barcode formed by concatenating the 12bp barcodes from each paired-end read. After trimming the primer sequences, the original V3-V4 amplicon was reconstituted by stitching the paired-end reads without preliminary quality filtering using FLASH (27), as FLASH includes error correction. We obtained 9,378,878 high-quality stitched reads that were subsequently processed at a median length of 410 bp.

The stitched reads were clustered using the uclust-based (28) open reference OTU picking protocol (29) described in QIIME (v1.8.0-dev) (30) against the Greengenes 13_8 reference database (31). Representative sequences for each OTU were chosen as the cluster centroid sequences. OTUs with representative sequences that could not be aligned with PyNAST and OTUs with a total count of less than 2 across all samples (i.e., singleton OTUs) were excluded from subsequent analyses, leaving a total of 76,710 OTUs composed of 9,127,632 reads.

All taxonomic assignments used throughout this study were generated by QIIME's uclust-consensus taxonomy assigner (default parameters, 32) against the Greengenes 13_8 97% reference OTUs (33). The taxonomic abundances for each sample-taxa combination using the uclust-consensus assigner were compared with taxonomic assignments made with the RDP classifier (confidence = 0.5, as recommended in 34) using a non-parametric Pearson correlation test with 999 iterations. For each sample-taxa combination, taxonomic abundances were compared for the two assignment methods

Applied and Environmental Microbiology

(i.e., using QIIME's `compare_taxa_summaries.py` script). The resulting p-values were significant ($p<0.001$) at all taxonomic levels, and the Pearson r-values were high (>0.96, Supplemental Material Table S1), indicating that the taxonomic profiles generated by the different methods were nearly identical. Analyses here focused on taxonomic classification to the level of genus, of which the uclust consensus assignment yielded a total of 790 genera. Genera included for analysis here were the 379 that occurred in all replicate tubes; these were also the most abundant taxa, representing 99.531% of the total 16S rRNA gene copies across the dataset. All QIIME commands used in this analysis are provided in Supplementary Information. All sequence data have been deposited at MG-RAST (35) project ID 14151.

Overview of quantitative taxon-specific isotope incorporation

In the following, we describe the calculations required to determine the isotopic composition of individual taxa after exposure to isotopically labeled substrates. In this approach, the taxon-specific density of DNA in the treatment with the isotopically labeled substrate is computed and compared to the density of DNA for the same taxon in the treatment with no added isotope tracer. For a particular element and isotope, the density of DNA will reach a maximum value when all atoms of that element in the DNA molecule are labeled with the isotope tracer. Smaller shifts in density reflect intermediate degrees of tracer incorporation; the scaling between density shift and isotope incorporation is linear after accounting for the effect of GC content on the elemental composition of DNA. The incorporation of the isotope tracer is expressed as atom fraction excess, which is the increase above natural abundance isotopic composition, and

ranges from a minimum of 0 to a maximum of 1 minus the natural abundance background for a given isotope-element combination. Variables, calculated quantities, and indices are defined in Table 1.

Calculating taxon-specific changes in density

Taxon specific changes in density caused by isotope incorporation were calculated as shown in equations 1-12, below. Calculations at the scale of individual density fractions (Eq. 1) and of individual replicate tubes (Eqs. 2, 3) were conducted for each density fraction and each tube independently. Other calculated quantities compared tubes with and without isotopes (Eqs. 4, and 10-12), where we used means across replicates to estimate the mean difference, and resampling with replacement (bootstrapping) to determine confidence intervals, as described below. In all cases, the independence of true replicates was preserved.

As described above, we determined the total number of 16S rRNA gene copies (f_k) using the universal 16S rRNA primer for qPCR for each fraction (k) in each replicate density gradient (j). Also as described above, we used sequencing to determine the proportional abundance of each taxon (i) within each density fraction (k), again for each replicate density gradient (j). This proportional abundance of each individual taxon within an individual density gradient from a particular replicate tube is abbreviated p_{ijk}. We calculated the total number of 16S rRNA gene copies per µL (y_{ijk}) for bacterial taxon i in density fraction k of replicate j as:

$$y_{ijk} = p_{ijk} \cdot f_{jk} \quad (1)$$

279 The total number of 16S rRNA gene copies (y_{ij}) for bacterial taxon *i* in replicate *j* is
280 summed across all *K* density fractions:

281
$$y_{ij} = \sum_{k=1}^{K} y_{ijk} \quad (2)$$

282

283 The density (W_{ij}) for bacterial taxon *i* of replicate *j* was computed as a weighted average,
284 summing across all *K* density fractions the density (x_{jk}) of each individual fraction times
285 the total number of 16S rRNA gene copies (y_{ijk}) in that fraction expressed as a proportion
286 of the total 16S rRNA gene copies (y_{ij}) for taxon *i* in replicate *j*:

287
$$W_{ij} = \sum_{k=1}^{K} x_{jk} \cdot \left(\frac{y_{ijk}}{y_{ij}} \right) \quad (3)$$

288 For a given taxon, we calculated the difference in density caused by isotope incorporation
289 (Z_i):

290
$$Z_i = W_{LABi} - W_{LIGHTi} \quad (4)$$

291 where W_{LABi} is the mean, across all replicates, of the isotope-enriched treatment (labeled,
292 LAB; n=3) and W_{LIGHTi} is the mean, across all replicates, of the unlabeled treatment
293 (unlabeled, LIGHT; n=6). Because our experiment had multiple treatments without heavy
294 isotopes, we included data from all replicate tubes in those unlabeled treatments (i.e.,
295 unlabeled treatments with and without added carbon; n=6) to estimate the unlabeled
296 average density (W_{LIGHTi}) for each taxon *i*.

297

298 *Calculating taxon-specific GC content and molecular weight*

We calculated the GC content (G_i) of each bacterial taxon using the mean density for the unlabeled (W_{LIGHTi}) treatments (n=6). We derived the relationship between GC content and buoyant density using DNA from pure cultures of three microbial species with known but strongly differing GC content (see below). For these cultures, the linear relationship between GC content (G_i, expressed as a proportion) and unlabeled buoyant density (W_{LIGHTi}) on a CsCl gradient was:

$$G_i = \frac{1}{0.083506} \cdot \left(W_{LIGHTi} - 1.646057 \right) \quad (5)$$

This relationship differs from the established relationship between GC content and density (12). As noted above, our method of determining density relied on direct measurements of refraction on individual density fractions, as is the typical practice for SIP studies (24). Possibly, including DNA standards of known GC content in each ultracentrifuge would yield results more consistent with the established relationship. Practitioners should include specific measures to calibrate their laboratory techniques to this relationship.

The natural abundance molecular weight of DNA is a function of GC content, based on the atomic composition of the four DNA nucleotides. Single stranded DNA made of pure adenine (A) and thymine (T) has an average molecular weight of 307.691 g mol^{-1}. The corresponding average molecular weight for DNA comprising only guanine (G) and cytosine (C) is 308.187 g mol^{-1}. When GC content is known, the average molecular weight of a single strand of DNA can be calculated using:

$$M_{LIGHTi} = 0.496 G_i + 307.691 \quad (6)$$

Percent change in molecular weight associated with isotope incorporation

There are 12 oxygen atoms per DNA nucleotide pair, regardless of GC content: there are 6 for each G and C, and 7 for T, 5 for A). These atoms contain ^{18}O at natural abundance, which we assume to be 0.002000429 atom fraction for ^{18}O (36). The maximum labeling is achieved when all oxygen atoms are replaced by ^{18}O. Therefore, given the molecular weight of each additional neutron (1.008665 g mol^{-1}; 37), the maximal increase in molecular weight (corresponding to 1 atom fraction ^{18}O, or 100% atom percent ^{18}O) is 12.07747 g mol^{-1}. The theoretical maximum molecular weight ($M_{HEAVYMAXi}$) of fully ^{18}O-labeled DNA for taxon *i* is then:

$$M_{HEAVYMAXi} = 12.07747 + M_{LIGHTi} \quad (7)$$

In contrast, the number of carbon atoms per DNA nucleotide varies with GC content. There are 10 carbon atoms in G, A, and T, but only 9 in C. The average number of carbon atoms per DNA nucleotide ($H_{CARBONi}$) for taxon *i* can therefore be expressed as:

$$H_{CARBONi} = -0.5G_i + 10 \quad (8)$$

We assume these atoms are ^{13}C-labeled at natural abundance (0.01111233 atom fraction ^{13}C; (36)). The maximal labeling is achieved when all carbon atoms are replaced by ^{13}C. Complete replacement of carbon atoms with ^{13}C increases the molecular weight by 9.974564 g mol^{-1} for G, A, and T, and by 8.977107 g mol^{-1} for C. Using equation 8, the theoretical maximum molecular weight ($M_{HEAVYMAXi}$) of fully ^{13}C-labeled DNA can be calculated as follows, with GC content (G_i) expressed as a proportion:

$$M_{HEAVYMAXi} = -0.4987282Gi + 9.974564 + M_{LIGHTi} \quad (9)$$

Calculating isotope enrichment from density shifts

We calculated the proportional increase in density (Z_i) relative to the density of the unlabeled treatments (W_{LIGHTi}), and calculated molecular weight of DNA for taxon *i* in the labeled treatment (M_{LABi}) as:

$$M_{LABi} = \left(\frac{Z_i}{W_{LIGHTi}} + 1 \right) \cdot M_{LIGHTi} \quad (10)$$

The atom fraction excess of ^{18}O for taxon *i* ($A_{OXYGENi}$), accounting for the background fractional abundance of ^{18}O (0.002000429 (36)) is then calculated as:

$$A_{OXYGENi} = \frac{M_{LABi} - M_{LIGHTi}}{M_{HEAVYMAXi} - M_{LIGHTi}} \cdot (1 - 0.002000429) \quad (11)$$

. We used the results from a pure culture study with *E. coli*, grown with variable ^{18}O-enriched water (natural abundance, 5, 25, 50, and 70% atom fraction ^{18}O; see below) to compare to the theoretical calculations of atom fraction excess ^{18}O derived above.

Similarly, the atom fraction excess ^{13}C for taxon *i* ($A_{CARBONi}$) accounting for the background fractional abundance of ^{13}C (0.01111233, 36), is calculated as:

$$A_{CARBONi} = \frac{M_{LABi} - M_{LIGHTi}}{M_{HEAVYMAXi} - M_{LIGHTi}} \cdot (1 - 0.01111233) \quad (12)$$

Pure culture studies

To verify the predicted relationship between increased density and atom fraction excess we conducted experiments with a pure *Escherichia coli* culture. *E. coli* (strain HB101, GC content 50.8%) was shaken at 100 rpm, 37 °C for 8 h in Luria-Bertani (LB) broth that was prepared with a mixture of natural abundance and ^{18}O-water to achieve five ^{18}O-enrichment levels (natural abundance, 5, 25, 50, and 70% atom fraction ^{18}O). Genomic DNA was extracted in triplicate using PowerLyzer UltraClean Microbial DNA Isolation Kit according to the manufacturer's instructions (MO BIO Laboratories, Inc., Carlsbad,

CA). We also grew pure cultures of two additional strains of bacteria selected for low GC content (*Staphylococcus epidermidis*, ATTC# 49461, 32.1%) and high GC content (*Micrococcus leuteus*, ATTC# 49732, 73%). *S. epidermidis* was grown for 24 h on Brain Heart Infusion Agar at 37 °C, and *M. leuteus* was grown with LB agar at 23 °C. These cultures were grown with substrates and water at natural abundance stable isotope composition.

For each culture, genomic DNA was extracted in triplicate. Approximately 800 ng of each DNA extract was used for isopycnic centrifugation, density quantification, and DNA isotope analysis. The ^{18}O composition of the *E. coli* DNA was determined with a PyroCube (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the UC Davis Stable Isotope Facility (Davis, CA). Samples were prepared by diluting the *E. coli* DNA with natural abundance salmon sperm DNA to achieve enrichment levels below 100 ‰ $\delta^{18}O$ for isotope analysis. Densities of DNA from the cultures grown at natural abundance isotope composition were used to determine the relationship between the density of DNA and its GC content, yielding the relationship described in Eq. 5 (r^2 = 0.912, $P < 0.001$).

Statistical analysis

We used linear regression to examine the relationships between ^{18}O-H_2O composition of the growth medium and the ^{18}O composition of *E. coli* DNA, and also between the ^{18}O composition of *E. coli* DNA and its density.

Applied and Environmental Microbiology

387

388 Following the equations above, we computed the difference in densities, Z_i, between
389 treatments with and without isotope tracers, and the corresponding values of isotope
390 composition, A_{OXYGEN} and A_{CARBON}. Each calculated quantity was determined for each
391 replicate sample. We then used bootstrap resampling (with replacement, 1000 iterations)
392 of replicates within each treatment to estimate taxon-specific 90% confidence intervals
393 for the change in density (Eq. 4) and the corresponding value of atom fraction excess
394 isotope composition (Eq. 11 for oxygen, Eq. 12 for carbon). For each bootstrap iteration,
395 three samples (with replacement) were drawn from the isotope added treatment, and six
396 samples were drawn from the no-isotope controls. All calculations were performed in R
397 (38).
398 Density fractionation separates organisms according to GC content (12) as well as isotope
399 incorporation, so traditional SIP may be biased toward identifying high-GC content
400 organisms as growing or utilizing a substrate (39, 40). To test whether qSIP exhibited any
401 such bias, we used density without isotope addition as a proxy for GC content, and tested
402 whether the densities of organisms identified as assimilating (90% confidence intervals
403 did not include 0 for A_{CARBON} or A_{OXYGEN}) differed in density from organisms where
404 assimilation was not detected.
405
406 Our focus was on the magnitude of variation in Z_i, A_{OXYGEN}, and A_{CARBON}, because the
407 goal of our work was to establish a means to discern from SIP experiments quantitative
408 estimates of isotope tracer uptake. These values lie along a continuum from no uptake to
409 complete isotope replacement, and our approach estimates the values and places

confidence limits on those estimates. We did not use null hypothesis significance testing for assessing density shifts and isotope tracer uptake, because our priority was on estimation rather than determining statistical significance. For this reason, we selected bootstrap resampling rather than, for example, t-tests or ANOVAs. Parametric tests could of course be applied in future applications of this technique, and may be appropriate, for example, for statistical comparisons of treatments postulated to alter isotope tracer uptake. In such cases, correcting for multiple comparisons may be appropriate, depending on the nature of the question and the balance between type I and type II error rates. We note that, in typical SIP experiments, an organism is considered to be growing or utilizing a substrate if it exhibits a change in relative abundance when comparing the heavy fraction of the labeled versus control (e.g., 10) or comparing the heavy fraction versus the light fraction (e.g., 41), yet assessments of variation in these estimates are not typically presented. Our approach assesses both the quantitative values of isotope uptake and the variation associated with those estimates.

Results

In the pure culture experiments, the ^{18}O composition of *E. coli* DNA was strongly related to the ^{18}O composition of water in the growth medium, supporting the notion that oxygen from water is quantitatively incorporated into the DNA of growing organisms ($P<0.001$, $r^2 = 0.976$, Figure 2A). The slope of the relationship, 0.334 ± 0.017 (n=15), indicates that 33% of oxygen in *E. coli* DNA was derived from water. The shift in density of *E. coli* DNA with ^{18}O incorporation matched well the theoretical prediction of the model of isotope substitution in the DNA molecule (Equations 10 and 11, Figure 2B). These results confirm that ultracentrifugation in CsCl can serve as a quantitative mass separation procedure, resolving variation in isotope tracer incorporation into DNA. These results also support our model of the relationship between the density of nucleic acids and isotopic substitution in the DNA molecule.

In soil incubations, DNA density averaged across the entire community tended to increase in response to isotope addition (Figure 3). Addition of ^{13}C-glucose (Figure 3A) increased the density of DNA by 0.0043 g cm^{-3}, but the 90% confidence interval for this increase overlapped zero (-0.002 to 0.0091 g cm^{-3}). Addition of ^{18}O-water (Figure 3B) caused a similar increase in density, 0.0041 g cm^{-3}, but the 90% confidence interval for this increase also overlapped zero, spanning -0.0011 to 0.0090 g cm^{-3}. The incubations receiving ^{18}O-water and supplemental glucose (natural abundance isotope composition) exhibited the largest increase in average DNA density, 0.0090 g cm^{-3}, and in this case the 90% confidence limit did not overlap zero (0.0065 to 0.0125 g cm^{-3}). These comparisons estimate the change in density of DNA fragments encoding the 16S rRNA gene across all

Applied and Environmental Microbiology

taxa considered together. Figure 3 also illustrates the density distributions often used in SIP experiments to visualize the qualitative cutoff between labeled and unlabeled regions suitable for sequencing.

Sequencing all fractions allowed visualizing analogous density distributions for individual taxa. Figure 4 shows three taxa to illustrate the concept, showing graphically the manner in which the density of labeled (W_{LABi}) and unlabeled (W_{LIGHTi}) DNA is calculated for each taxon (equation 3). For example, the density of an unidentified genus in the family *Micrococcaceae* did not change with ^{18}O-water addition in the absence of supplemental glucose. For this taxon, the shift in density (Z) due to ^{18}O incorporation was -0.0002 g cm^{-3}, with the 90% confidence interval spanning -0.0046 to 0.0049 g cm^{-3} (Figure 4A). The shift in density due to ^{18}O-incorporation increased when unlabeled glucose was also added (Figure 4B, Z = 0.0169 g cm^{-3}, 90% CI, 0.0146 to 0.0194 g cm^{-3}). This bacterial taxon was therefore not incorporating the ^{18}O tracer in unamended soil, but synthesized new DNA using ^{18}O derived from H_2O in response to glucose addition. The DNA of an unidentified genus in the family *Pseudonocardiaceae* similarly exhibited no change in density in the absence of supplemental glucose (Z = 0.0005 g cm^{-3}, -0.0033 to 0.0045 g cm^{-3}), and exhibited only a slight increase in response to glucose addition (Z = 0.0040 g cm^{-3}, 0.0015 to 0.0070 g cm^{-3}, Figure 4C & D). By contrast, the density of DNA in a member of the genus *Herpetosiphonales* increased in soil without any supplemental glucose (Z = 0.0124 g cm^{-3}, 90% CI, 0.0105 to 0.0143 g cm^{-3}, Figure 4E), but the density did not further increase in response to the addition of glucose (Z = 0.0110 g cm^{-3}, 90% CI, 0.0088 to 0.0133 g cm^{-3}, Figure 4F). These results show that, by dividing the density

Applied and Environmental Microbiology

gradient into multiple fractions and sequencing each separately, one can determine changes in the density of DNA for individual taxa caused by the assimilation of stable isotope tracers.

The taxon-specific shifts in average density associated with incorporation of the heavy isotope (Figure 5) translate directly to quantitative variation in isotope composition, expressed here as atom fraction excess ^{18}O (A_{OXYGEN}, Figure 5A & B) and ^{13}C (A_{CARBON}, Figure 5C). The detection limit for a shift in density is the median change in density required to shift the lower bound of the bootstrapped 90% confidence limit above zero. As constrained by our sampling design, these values were 0.0037 g cm^{-3} for ^{18}O, and 0.0044 g cm^{-3} for ^{13}C, changes that correspond to 0.056 atom fraction excess ^{18}O and 0.081 atom fraction excess ^{13}C. No taxon exhibited a detectable decline in density in response to isotope addition (i.e., a negative mean density shift with confidence interval that did not include zero).

More than half of the bacterial genera (209) did not exhibit any detectable excess ^{18}O enrichment under control conditions without added glucose; in other words, the lower bound of the confidence intervals for these genera overlapped zero (Figure 5A). Of the 170 taxa that did exhibit detectable ^{18}O enrichment without added glucose, the corresponding values of atom fraction excess ^{18}O ranged from 0.047 (90% confidence interval, 0.001 to 0.100) in a member of the genus, *Lentzea*, to 0.354 (CI, 0.248 to 0.449) in an unidentified representative of the candidate bacterial phylum, *OD1*. With added glucose, 351 of the 379 taxa exhibited positive atom fraction excess ^{18}O (90% CIs did not

Applied and Environmental Microbiology

overlap zero), averaging 0.147 (Figure 5B), with a minimum of 0.036 (CI, 0.004 to 0.064) in an unidentified genus of the family *Ktedonobacteracea*, and a maximum of 0.365 (CI, 0.282 to 0.449) in an unidentified genus within the class *AT12OctB3* of the phylum, *Bacteroidetes*. Bacterial taxa in this soil varied in atom fraction excess ^{18}O under control conditions and in response to added glucose (Figure 5A & B). Atom fraction excess ^{13}C reflects direct assimilation of C from the added glucose (Figure 5C), and ranged from no detectable enrichment among 215 of the 379 genera, to over half of the carbon atoms comprising ^{13}C in the DNA of a member of the *Micrococcaceae* (0.525, CI 0.458 to 0.592).

GC Bias

There was no evidence of GC bias in qSIP. There were negligible differences in densities between organisms exhibiting tracer assimilation and those not exhibiting tracer assimilation (Table 2). Inferred GC contents averaged 52.3% (CI 44.6% to 57.3%) for organisms exhibiting tracer assimilation, very close to the average of 52.8% inferred GC content for taxa for which assimilation was not detected (CI 45.1% to 58.2%).

Soil Incubations: multi-element quantitative stable isotope probing

There was a strong positive relationship between increased atom fraction excess ^{18}O in response to glucose addition and the direct utilization of glucose-derived C (atom fraction excess ^{13}C; Figure 6; r^2=0.51, P<0.001). The expected relationship (solid line in Figure 6) reflects the case where glucose is the sole C source, and thus there should be an 0.33 atom fraction excess increase in ^{18}O for each 1 atom fraction excess increase in ^{13}C,

Applied and Environmental Microbiology

based on our finding that 33% of the oxygen molecules in DNA are derived from water (Figure 2). For many taxa, the increase in atom fraction excess ^{18}O in response to added glucose exceeded the expected amount (solid line in Figure 6).

Applied and Environmental Microbiology

520 **Discussion**

521 We demonstrate that stable isotope probing of bacterial assemblages in natural
522 environments can yield quantitative information about the assimilation of isotope tracers
523 into bacterial DNA with fine taxonomic resolution. This work establishes a framework
524 for coupling quantitative interpretation of stable isotope tracer experiments with
525 microbial diversity, a coupling essential for understanding how to represent microbial
526 diversity in biogeochemical models.

527

528 The shifts in density we could detect using qSIP (0.0034 to 0.0042 g cm^{-3}, Figure 5) are
529 nearly an order of magnitude smaller than those typically used to resolve the assimilation
530 of stable isotopes into newly synthesized DNA using conventional SIP, in which "light"
531 and "heavy" density fractions often differ by 0.03 g cm^{-3} (14, 42) or more (13, 24, 43).
532 For ^{13}C, the minimum required change in density for SIP has been estimated to be 0.01 g
533 cm^{-3}, corresponding to 0.2 atom fraction excess (7). The approach presented here
534 achieves higher resolution by accounting for taxonomic differences in the density of
535 DNA caused by natural variation in GC content. It may be possible to improve the
536 resolution we achieved. We collected fractions in discrete density increments of 0.0036 g
537 cm^{-3} (average difference in density between adjacent fractions), setting a limit on the
538 changes in density we could detect. This difference in density between adjacent fractions
539 we collected is comparable to the density shifts of bacterial taxa that we could resolve:
540 the mean density shift required for the lower confidence limit to exceed zero was, on
541 average, 0.0034 g cm^{-3} for ^{18}O and 0.0042 g cm^{-3} for ^{13}C. Thus, it is possible that
542 separation of the nucleic acids into finer density fractions will afford higher precision in

543 the estimates of stable isotope composition. Furthermore, our sample size was quite low;
544 higher replication would achieve finer resolution. Nevertheless, the finding that no taxon
545 exhibited a detectable decline in density in response to isotope addition is encouraging.
546 Such a result would be illogical because isotope tracer uptake cannot be negative, but
547 could arise from large random variation (natural and measurement error) and low sample
548 size. The absence here of such negative confidence intervals indicates that our
549 bootstrapping application to qSIP is not particularly subject to false positive inference.
550
551 The resolution achieved by sequencing individual density fractions, though an
552 improvement over traditional SIP, is still very coarse compared to the resolution achieved
553 with isotope ratio mass spectrometry. Detecting differences between taxa with
554 quantitative stable isotope probing (~0.05 atom fraction excess) is four orders of
555 magnitude less precise than that achieved with gas isotope ratio analysis of bulk organic
556 matter in continuous flow, where differences of 0.000005 atom fraction excess or better
557 (<0.5‰) can be resolved (44). Isopycnic centrifugation to quantify isotope composition is
558 also less precise than compound specific analysis of biomarkers, for example, of ^{13}C in
559 fatty acids, where resolution of 0.00002 atom fraction excess (or 2‰) is typical (45-47).
560 Coupling stable isotope tracing with Nano-scale secondary ion mass spectrometry (Nano-
561 SIMS) and microarrays, a coupling called Chip-SIP (48), can resolve 0.005 atom fraction
562 excess for ^{15}N and 0.001 for ^{13}C (49), considerably more precise than qSIP.
563
564 qSIP has advantages in taxonomic resolution over these other techniques. For compound
565 specific biomarkers, specific fatty acids serve as biomarkers for up to a dozen groups of

566 microorganisms, taxonomic resolution much coarser than that afforded by qSIP. Chip-
567 SIP requires nucleic acid probes, necessitating deciding *a priori* what sequences to
568 collect for isotopic analysis, and preparing microarrays implanted with those sequences
569 prior to the isotope addition. For this reason, in Chip-SIP the taxonomic resolution in the
570 isotope fluxes is influenced by information gathered without knowledge of which taxa are
571 biogeochemically important. One advantage of qSIP is that sequencing occurs after
572 isotope enrichment, enabling quantitative exploration of the biodiversity involved in
573 biogeochemistry, without having to decide where to focus *a priori*. Furthermore, the
574 taxonomic resolution possible with a microarray is limited by probe specificity and
575 fidelity, whereas the resolution afforded by qSIP is very high, equivalent to the resolution
576 of sequencing technology applied to the density fractions. Chip-SIP also requires access
577 to a Nano-SIMS, expensive and technically challenging, limiting its wide adoption in the
578 field.
579
580 Other approaches used to link element fluxes to microbial taxa are limited to target
581 organisms, such as fluorescent *in situ* hybridization (FISH) coupled with SIMS (50), or
582 halogen in situ-hybridization-SIMS (51). Bromodeoxyuridine (BrdU) uptake has been
583 proposed as a universal technique for identifying growing organisms (52) and their
584 responses to environmental perturbations (53). However, there is up to 10-fold variation
585 among taxa in the conversion between BrdU uptake and growth, unrelated to taxonomic
586 affiliation, a bias calling into question the quantitative universality of this technique (54).
587 Compared to these other techniques, qSIP can assess quantitatively the entire microbial
588 assemblage at fine taxonomic resolution, a solid foundation for exploring quantitatively

Applied and Environmental Microbiology

589 the relationships between microbial biodiversity and the biogeochemistry known to be
590 microbial.
591
592 Our finding that many bacterial taxa did not exhibit any increase in ^{18}O content under
593 control conditions (Figure 5A) is consistent with the notion that a portion of the soil
594 microbial biomass is not growing and may be metabolically inactive (55). The increase in
595 atom fraction ^{18}O and ^{13}C with added glucose indicates that glucose addition stimulates
596 bacterial growth, not just respiration. The breadth of taxa that exhibited a positive
597 response to glucose addition is consistent with glucose being a widely utilized substrate
598 (56), though there are two other possible mechanisms. First, over the 7-day duration of
599 the incubation period, glucose will be assimilated by cells that then died, releasing
600 labeled cellular constituents available to the rest of the microbial community (57). We
601 cannot distinguish between direct utilization of the added glucose and utilization of
602 labeled cellular constituents produced by another organism. This applies equally to the
603 ^{18}O-labeled and ^{13}C-labeled assemblages. Second, ^{18}O-water is a universal tracer for
604 DNA synthesis, not necessarily tied to any particular carbon source (58, 59). The
605 observed increase in atom fraction excess ^{18}O includes growth stimulation caused by the
606 carbon contained in the added glucose, along with the growth stimulation caused by
607 increased rates of utilization of other carbon sources. In contrast, atom fraction excess ^{13}C
608 in response to ^{13}C-glucose addition traces the incorporation of carbon atoms from glucose
609 (or derived from glucose via other metabolites as discussed above) into newly
610 synthesized DNA (Figure 5C). This is expected, because glucose addition stimulates
611 growth and DNA synthesis (60, 61). In summary, the effect of added glucose was

Applied and Environmental Microbiology

612 apparent as: (1) an overall stimulation of growth, independent of the specific carbon
613 substrate, and (2) as a stimulation of growth that relied directly on glucose-derived
614 carbon.
615
616 The combination of ^{18}O and ^{13}C tracers enabled quantitative partitioning of these direct
617 and indirect effects, based on the deviation in the data from the expected relationship
618 between ^{18}O and ^{13}C enrichment for organisms utilizing glucose as a sole carbon source
619 (solid line, Figure 6). One explanation for this deviation is that most taxa derive more
620 than 33% of the oxygen in DNA from environmental water. Quantitative variation in the
621 contribution of water to oxygen in DNA could occur, for example, due to the variation in
622 the oxidation state of the carbon substrate (e.g., lipids versus carbohydrates), though to
623 our knowledge this variation is not known. Given the universality of the mechanism of
624 DNA replication, it is unlikely that taxa vary widely in the contribution of water to
625 oxygen, at least when grown on a common substrate.
626
627 A more parsimonious explanation of the deviation we observed is that it represents
628 utilization of C sources other than glucose for growth. In other words, the added glucose
629 stimulated the utilization of native soil C as a growth substrate. This points to the
630 potential for quantitative stable isotope probing to test hypotheses regarding microbial
631 diversity in the commonly observed phenomenon where the addition of simple C
632 substrates to soil alters the mineralization of native soil C (16). This so-called "priming
633 effect" is common and quantitatively significant (16), but remains mechanistically
634 inscrutable. In past priming studies employing ^{13}C-SIP, some components of the

635 microbial community were found to utilize as growth substrates the ^{13}C-labeled
636 compounds added to initiate priming, though inferences about the organisms responsible
637 for priming – i.e., degrading native soil organic matter – were weak, because no
638 independent marker could validate their activity (62-64). Combining isotope tracers
639 (using both ^{13}C and ^{18}O) can help by distinguishing microorganisms that respond to the
640 original substrate pulse from those that respond indirectly by degrading soil organic
641 matter (11), an approach useful for testing hypotheses about which groups of
642 microorganisms contribute to priming. qSIP advances this one step further, by enabling
643 quantitative comparisons of microorganisms' utilization of the added substrate and of soil
644 organic matter for growth. Future analyses combining qSIP with system-level C fluxes
645 would support stronger inferences about the role of specific microorganisms in the
646 priming effect. The analysis presented here suggests that some microorganisms respond
647 to glucose addition by enhancing their rates of utilization of native soil carbon, enabling
648 additional biosynthesis (Figure 6). More generally, the taxonomic diversity of responses
649 we observed highlights the potential for this technique to provide insight into the
650 population and community ecology behind biogeochemical phenomena involving such
651 indirect effects (e.g., 16, 17).
652
653 Quantifying isotope composition is the first step in determining the rate of substrate
654 utilization in isotope tracer experiments, and the foundation for comparing rates of
655 substrate utilization and element fluxes among different taxa in intact microbial
656 communities. This work advances a quantitative approach to stable isotope probing in
657 order to elucidate taxon-specific processes that drive element cycling in intact

Applied and Environmental Microbiology

658 communities, bringing to microbial ecology the power of stable isotopes to quantify rates
659 of element fluxes into and through organisms (65, 66). Like Chip-SIP (48, 67), qSIP
660 provides a means to quantify the ecology of organisms about which we know little more
661 than the genetic fragment used to identify their unique place on the tree of life. These
662 approaches lay the groundwork for a quantitative understanding of microbial ecosystems,
663 including the types of ecological interactions previously described among macro-
664 organisms that influence ecosystem processes. Quantitative stable isotope probing adds to
665 the suite of tools that facilitate interpretation of stable isotope tracer experiments in
666 microbial communities, probing the quantitative significance of microbial taxa for
667 biogeochemical cycles in nature.
668
669 Acknowledgements. This research was supported by grants from the National Science
670 Foundation (EAR-1124078 and DEB-1321792) and the Department of Energy's
671 Biological Systems Science Division, Program in Genomic Science.
672

References

1. **Jenny H.** 1941. Factors of Soil Formation: A System of Quantitative Pedology. Dover Publications, New York.

2. **Vitousek PM.** 1990. Biological Invasions and Ecosystem Processes - Towards an Integration of Population and Ecosystem Studies. Oikos **57:**7-13.

3. **Pace NR.** 1997. A molecular view of microbial diversity and the biosphere. Science **276:**734-740.

4. **Amann RI, Ludwig W, Schleifer KH.** 1995. Phylogenetic Identification and in situ Detection of Individual Microbial Cells without Cultivation. Microbiological Reviews **59:**143-169.

5. **Muyzer G, Dewaal EC, Uitterlinden AG.** 1993. Profiling of Complex microbial-Populations by Denaturing Gradient Gel-Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S Ribosomal-RNA. Applied and Environmental Microbiology **59:**695-700.

6. **Roesch LF, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, Kent AD, Daroub SH, Camargo FAO, Farmerie WG, Triplett EW.** 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. Isme Journal **1:**283-290.

7. **Radajewski S, Ineson P, Parekh NR, Murrell JC.** 2000. Stable-isotope probing as a tool in microbial ecology. Nature **403:**646-649.

8. **Hutchens E, Radajewski S, Dumont MG, McDonald IR, Murrell JC.** 2004. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. Environmental Microbiology **6:**111-120.

9. **Sharp CE, Martinez-Lorenzo A, Brady AL, Grasby SE, Dunfield PF.** 2014. Methanotrophic bacteria in warm geothermal spring sediments identified using stable-isotope probing. FEMS microbiology ecology **90:**92-102.

10. **Schwartz E, Van Horn DJ, Buelow HN, Okie JG, Gooseff MN, Barrett JE, Takacs-Vesbach CD.** 2014. Characterization of growing bacterial populations in McMurdo Dry Valley soils through stable isotope probing with O-18-water. Fems Microbiology Ecology **89:**415-425.

11. **Mau RL, Liu CM, Aziz M, Schwartz E, Dijkstra P, Marks JC, Price LB, Keim P, Hungate BA.** 2014. Linking soil bacterial diversity and soil carbon stability. The ISME Journal **9:**1477-1480.

12. **Schildkraut CL.** 1962. Determination of Base Composition of Deoxyribonucleic Acid from its Buoyant Denstiy in CsCl. Journal of Molecular Biology **4:**430-443.

13. **Schmidt O, Horn MA, Kolb S, Drake HL.** 2015. Temperature impacts differentially on the methanogenic food web of cellulose-supplemented peatland soil. Environmental microbiology **17:** 720-734.

14. **Aanderud ZT, Jones SE, Fierer N, Lennon JT.** 2015. Resuscitation of the rare boisphere contributes to pulses of ecosystem activity. Frontiers in Microbiology **6**: 24.

15. **Jayamani I, Cupples AM.** 2015. Stable Isotope Probing and High-Throughput Sequencing Implicate Xanthomonadaceae and Rhodocyclaceae in Ethylbenzene Degradation. Environmental Engineering Science **32:**240-249.

16. **Kuzyakov Y, Friedel JK, Stahr K.** 2000. Review of mechanisms and quantification of priming effects. Soil Biology & Biochemistry **32:**1485-1498.

17. **Bingeman CW, Varner JE, Martin WP.** 1953. The effect of the addition of organic materials on the decomposition of an organic soil. Soil Science Society of America Proceedings **17:**34-38.

18. **Lohnis.** 1926. Nitrogen availability of green manures. Soil Science Society of America Journal **22:**171-177.

19. **Broadbent FE.** 1948. Nitrogen release and carbon loss from soil organic matter during decomposition of added plant residues. Proceedings Soil Science Society of America, 1947 **12:**246-249.

20. **Broadbent FE, Bartholomew WV.** 1949. The effect of quantity of plant material added to soil on its rate of decomposition. Proceedings Soil Science Society of America, 1948 **13:**271-274.

21. **Kuzyakov Y.** 2002. Review: Factors affecting rhizosphere priming effects. Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde **165:**382-396.

22. **Blagodatskaya E, Kuzyakov Y.** 2008. Mechanisms of real and apparent priming effects and their dependence on soil microbial biomass and community structure: critical review. Biology and Fertility of Soils **45:**115-131.

23. **Mesbah NM, Whitman WB, Mesbah M.** 2011. Determination of the G plus C Content of Prokaryotes, p 299-324. *In* Rainey F, Oren A (ed), Methods in Microbiology, Vol 38: Taxonomy of Prokaryotes, vol 38.

24. **Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, Friedrich MW, Murrell JC.** 2007. DNA stable-isotope probing. Nature Protocols **2:**860-866.

741 25. **Liu CM, Aziz M, Kachur S, Hsueh P-R, Huang Y-T, Keim P, Price LB.** 2012.
742 BactQuant: An enhanced broad-coverage bacterial quantitative real-time PCR
743 assay. Bmc Microbiology **12:**56.
744 26. **Fadrosh DW, Ma B, Gajer P, Sengamalay N, Ott S, Brotman RM, Ravel J.**
745 2014. An improved dual-indexing approach for multiplexed 16S rRNA gene
746 sequencing on the Illumina MiSeq platform. Microbiome **2:**6.
747 27. **Magoc T, Salzberg SL.** 2011. FLASH: fast length adjustment of short reads to
748 improve genome assemblies. Bioinformatics **27:**2957-2963.
749 28. **Edgar RC.** 2010. Search and clustering orders of magnitude faster than BLAST.
750 Bioinformatics **26:**2460-2461.
751 29. **Rideout JR, He Y, Navas-Molina JA, Walters WA, Ursell LK, Gibbons SM,**
752 **Chase J, McDonald D, Gonzalez A, Robbins-Pianka A, Clemente JC, Gilbert**
753 **JA, Huse SM, Zhou HW, Knight R, Caporaso JG.** 2014. Subsampled open-
754 reference clustering creates consistent, comprehensive OTU definitions and scales
755 to billions of sequences. PeerJ **2:**e545.
756 30. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello**
757 **EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST,**
758 **Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD,**
759 **Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann**
760 **J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-
761 throughput community sequencing data. Nat Methods **7:**335-336.
762 31. **McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A,**
763 **Andersen GL, Knight R, Hugenholtz P.** 2012. An improved Greengenes

taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J **6:**610-618.

32. **Bokulich NA, Rideout JR, Kopylova E, Bolyen E, Patnode J, Ellett Z, McDonald D, Wolfe B, Maurice CF, Dutton RJ, Turnbaugh PJ, Knight R, Caporaso JG.** 2015. A standardized, extensible framework for optimizing classification improves marker-gene taxonomic assignments. PeerJ PrePrints.

33. **McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P.** 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J **6:**610-618.

34. **Claesson MJ, O'Sullivan O, Wang Q, Nikkila J, Marchesi JR, Smidt H, de Vos WM, Ross RP, O'Toole PW.** 2009. Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One **4:**e6669.

35. **Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A, Wilkening J, Edwards RA.** 2008. The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics **9:**386.

36. **Coplen TB.** 1996. Atomic weights of the elements 1995. Pure and Applied Chemistry **68:**2339–2359.

37. **(CODATA) CoDfSaT.** 2015. The NIST Reference on Constants, Units, and Uncertainty, *on* National Institute of Standards and Technology. Accessed July 2, 2015.

38. **R Development Core Team.** 2014. R: A language and environment for statistical computing, *on* R Foundation for Statistical Computing. http://www.R-project.org/. Accessed 15 Aug 2015

39. **Rangel-Castro JI, Killham K, Ostle N, Nicol GW, Anderson IC, Scrimgeour CM, Ineson P, Meharg A, Prosser JI.** 2005. Stable isotope probing analysis of the influence of liming on root exudate utilization by soil microorganisms. Environmental Microbiology **7:**828-838.

40. **Kovatcheva-Datchary P, Egert M, Maathuis A, Rajilic-Stojanovic M, de Graaf AA, Smidt H, de Vos WM, Venema K.** 2009. Linking phylogenetic identities of bacteria to starch fermentation in an in vitro model of the large intestine by RNA-based stable isotope probing. Environmental Microbiology **11:**914-926.

41. **Darjany LE, Whitcraft CR, Dillon JG.** 2014. Lignocellulose-responsive bacteria in a southern California salt marsh identified by stable isotope probing. Frontiers in Microbiology **5**: 263

42. **Hu HW, Macdonald CA, Trivedi P, Holmes B, Bodrossy L, He JZ, Singh BK.** 2015. Water addition regulates the metabolic activity of ammonia oxidizers responding to environmental perturbations in dry subhumid ecosystems. Environmental Microbiology **17:**444-461.

43. **Jiang XJ, Hou XY, Zhou X, Xin XP, Wright A, Ji ZJ.** 2015. pH regulates key players of nitrification in paddy soils. Soil Biology & Biochemistry **81:**9-16.

44. **Brenna JT, Corso TN, Tobias HJ, Caimi RJ.** 1997. High-precision continuous-flow isotope ratio mass spectrometry. Mass Spectrometry Reviews **16:**227-258.

45. **Amelung W, Brodowski S, Sandhage-Hofmann A, Bol R.** 2008. Ccombining biomarker with stable isotope analysis for assessing the transformation and turnover of soil organic matter , p 155-250. *In* Sparks DL (ed), Advances in Agronomy, Vol 100, vol 100.

46. **Kramer C, Gleixner G.** 2008. Soil organic matter in soil depth profiles: Distinct carbon preferences of microbial groups during carbon transformation. Soil Biology & Biochemistry **40:**425-433.

47. **Treonis AM, Ostle NJ, Stott AW, Primrose R, Grayston SJ, Ineson P.** 2004. Identification of groups of metabolically-active rhizosphere microorganisms by stable isotope probing of PLFAs. Soil Biology & Biochemistry **36:**533-537.

48. **Pett-Ridge J, Weber PK.** 2012. NanoSIP: NanoSIMS applications for microbial biology. Methods in molecular biology (Clifton, NJ) **881:**375-408.

49. **Mayali X, Weber PK, Brodie EL, Mabery S, Hoeprich PD, Pett-Ridge J.** 2012. High-throughput isotopic analysis of RNA microarrays to quantify microbial resource use. Isme Journal **6:**1210-1221.

50. **Orphan VJ, House CH, Hinrichs KU, McKeegan KD, DeLong EF.** 2001. Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. Science **293:**484-487.

51. **Behrens S, Losekann T, Pett-Ridge J, Weber PK, Ng WO, Stevenson BS, Hutcheon ID, Relman DA, Spormann AM.** 2008. Linking microbial phylogeny to metabolic activity at the single-cell level by using enhanced element labeling-catalyzed reporter deposition fluorescence in situ hybridization (EL-FISH) and NanoSIMS. Applied and Environmental Microbiology **74:**3143-3150.

833 52. **Urbach E, Vergin KL, Giovannoni SJ.** 1999. Immunochemical detection and
834 isolation of DNA from metabolically active bacteria. Applied and Environmental
835 Microbiology **65:**1207-1213.
836 53. **Goldfarb KC, Karaoz U, Hanson CA, Santee CA, Bradford MA, Treseder**
837 **KK, Wallenstein MD, Brodie EL.** 2011. Differential growth responses of soil
838 bacterial taxa to carbon substrates of varying chemical recalcitrance. Frontiers in
839 Microbiology **2**.
840 54. **Hellman M, Berg J, Brandt KK, Hallin S.** 2011. Survey of bromodeoxyuridine
841 uptake among environmental bacteria and variation in uptake rates in a
842 taxonomically diverse set of bacterial isolates. Journal of Microbiological
843 Methods **86:**376-378.
844 55. **Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella**
845 **G.** 2003. Microbial diversity and soil functions. European Journal of Soil Science
846 **54:**655-670.
847 56. **Stotzky G, Norman AG.** 1961. Factors limiting microbial activities in soil.
848 Archiv fur Mikrobiologie **40:**341-369.
849 57. **Manefield M, Griffiths RI, Bailey MJ, Whiteley AS.** 2006. Stable isotope
850 probing: A critique of its we in linking phylogeny and function. Nucleic Acids
851 and Proteins in Soil **8:**205-216.
852 58. **Blazewicz SJ, Schwartz E.** 2011. Dynamics of O-18 Incorporation from H (2)
853 (18) O into Soil Microbial DNA. Microbial Ecology **61:**911-916.
854 59. **Schwartz E.** 2007. Characterization of growing microorganisms in soil by stable
855 isotope probing with H218O. Appl Environ Microbiol **73:**2541-2546.

60. **Nannipieri P, Johnson RL, Paul EA.** 1978. Criteria for measurement of microbial-growth and activity in soil. Soil Biology & Biochemistry **10:**223-229.

61. **McMahon SK, Wallenstein MD, Schimel JP.** 2009. Microbial growth in Arctic tundra soil at-2 degrees C. Environmental Microbiology Reports **1:**162-166.

62. **Bernard L, Mougel C, Maron PA, Nowak V, Leveque J, Henault C, Haichar FZ, Berge O, Marol C, Balesdent J, Gibiat F, Lemanceau P, Ranjard L.** 2007. Dynamics and identification of soil microbial populations actively assimilating carbon from 13C-labelled wheat residue as estimated by DNA- and RNA-SIP techniques. Environ Microbiol **9:**752-764.

63. **Haichar FZ, Achouak W, Christen R, Heulin T, Marol C, Marais MF, Mougel C, Ranjard L, Balesdent J, Berge O.** 2007. Identification of cellulolytic bacteria in soil by stable isotope probing. Environ Microbiol **9:**625-634.

64. **Nottingham AT, Griffiths H, Chamberlain PM, Stott AW, Tanner EVJ.** 2009. Soil priming by sugar and leaf-litter substrates: A link to microbial groups. Applied Soil Ecology **42:**183-190.

65. **Peterson BJ, Fry B.** 1987. Stable Isotopes in Ecosystem Studies. Annual Review of Ecology and Systematics **18:**293-320.

66. **Schimel DS.** 1993. Isotopic Techniques in Plant, Soil and Aquatic Biology: Theory and application of tracers.

67. **Mayali X, Weber PK, Pett-Ridge J.** 2013. Taxon-specific C/N relative use efficiency for amino acids in an estuarine community. FEMS Microbiology Ecology **83:**402-412.

879 Figure Legends

880 Figure 1. Conceptual model of the quantitative stable isotope probing technique, from
881 sample collection to determining the density of 16S rRNA gene fractions for individual
882 taxa and their corresponding values of atom % stable isotope composition. Note: except
883 for the addition of the stable isotope tracer at the beginning of the incubation, all steps are
884 applied identically to both labeled and unlabeled samples. Artwork by Victor Leshyk.

885

886 Figure 2 (A) The ^{18}O composition of *E. coli* DNA as a function of the ^{18}O composition of
887 water in the growth medium. Solid line is the regression ($^{18}O_{DNA}$ = 0.3339 x $^{18}O_{H2O}$ +
888 0.0004, n=15, $P<0.001$, $R^2=0.976$). (B) The average density of *E. coli* DNA as a function
889 of the ^{18}O composition of the DNA (density = 0.0644 x atom fraction ^{18}O + 1.6946
890 $R^2 = 0.852$, n=15)

891

892 Figure 3. The relative abundance of bacterial 16S rRNA genes, measured through
893 quantitative PCR, as a function of density of DNA. Isotope treatments are shown with
894 filled symbols while natural abundance controls are shown with open symbols.
895 Comparison of soil samples incubated with (A) ^{12}C-glucose and ^{13}C-glucose, (B) ^{16}O-
896 H_2O and ^{18}O-H_2O, and (C) ^{16}O-H_2O plus ^{12}C-glucose and ^{18}O-H_2O plus ^{12}C-glucose. The
897 dotted lines represent the density that separates labeled from non-labeled DNA in
898 traditional SIP. The distribution of densities in each replicate tube yielded an estimate of
899 the average density for that tube, indicated by the horizontal position of the large symbols
900 and error bars at the top of each panel (bars show 90% CIs, with n=3; the vertical position
901 of these symbols does not convey meaning).

Applied and Environmental Microbiology

902

903

904 Figure 4. Frequency distribution of the 16S rRNA gene as a function of density of DNA
905 for three bacterial taxa without added glucose (left panels) and with added (natural
906 abundance $\delta^{13}C$) glucose (right side panels) for three different taxa: unidentified genera
907 in the families Micrococacceae (A & B) and Pseudonocardiaceae (C & D), and genus
908 *Herpetosiphonales* (E & F). Open symbols and dashed lines show the density distribution
909 for the incubation where all substrates had natural abundance isotope composition, and
910 filled symbols and solid lines show the distribution with ^{18}O-water. Different shapes
911 represent individual replicates within a treatment combination. For each replicate, the
912 area under the curve sums to 1. The distribution of densities for each taxon in each
913 replicate yielded an estimate of the average density for that taxon, indicated by the
914 horizontal position of the large symbols and error bars at the top of each panel (bars show
915 90% CIs, with n=3; note, the vertical position of the large symbols does not convey
916 meaning).

917

918 Figure 5. The taxon-specific shift in average density of DNA (g cm^{-3}, lower horizontal
919 axis) and the corresponding atom fraction excess of ^{18}O or ^{13}C (upper horizontal axis)
920 between incubations with enriched and natural abundance substrates. Changes in DNA
921 density were caused by ^{18}O incorporation from water (A) without or (B) with added
922 natural abundance glucose, or by (C) ^{13}C incorporation from added ^{13}C-labeled glucose.
923 Bars show bootstrapped medians and 90% CIs.

924

Applied and Environmental Microbiology

925 Figure 6. Atom fraction ^{13}C with added ^{13}C-glucose and the shift in atom fraction ^{18}O
926 caused by added ^{12}C-glucose across groups of bacteria. The solid black line represents the
927 expected relationship if organisms derived 100% of their carbon from the added glucose
928 and 33% of their oxygen from ^{18}O water. The difference between the solid line and points
929 falling above it is the indirect effect of added glucose on the utilization of other carbon
930 substrates, reflecting the difference between the total growth stimulation caused by
931 glucose addition and the stimulation based on direct reliance on the added glucose. Points
932 show means with standard errors of the mean (n=3).
933

Table 1. Definitions of indices, variables, and calculated quantities used in modeling excess atom fraction ^{18}O for each bacterial taxon.

Indices:

i	taxon
j	replicate (or tube) within a treatment
k	fraction (within a replicate)
I	number of taxa
J	number of replicates (within a treatment)
K	number of fractions (within a replicate)

Variables:

f_{jk}	total number of 16S rRNA gene copies per µL (all taxa combined) in fraction k of replicate j (copies μL^{-1})
p_{ijk}	proportion of the total number of 16S rRNA gene copies per µL that are taxon i in fraction k of replicate j (unitless)
x_{jk}	density of fraction k of replicate j (g cm^{-3})

Calculated quantities:

y_{ijk}	number of 16S rRNA gene copies per µL of taxon i in fraction k of replicate j (copies μL^{-1})

Applied and Environmental Microbiology

950	y_{ij}	total number of 16S rRNA gene copies per µL of taxon *i* in replicate *j* (copies μL^{-1})
951	W_{ij}	observed weighted average density for taxon *i* in replicate *j* (g cm^{-3})
952	W_{LABi}	mean observed weighted average density for taxon *i* in the labeled treatment (mean across all replicates of the treatment
953		with the heavy isotope) (g cm^{-3})
954	W_{LIGHTi}	mean observed weighted average density for taxon *i* in the unlabeled (i.e., natural abundance) treatment (mean across
955		all replicates in all treatments without heavy isotopes) (g cm^{-3})
956	G_i	guanine + cytosine content of taxon *i* (unitless)
957	$H_{CARBONi}$	average number of carbon atoms per DNA nucleotide for taxon *i*
958	M_{LIGHTi}	observed molecular weight of the DNA fragment containing the 16S RNA gene for taxon *i* in the unlabeled (i.e.,
959		natural abundance) treatment (g mol^{-1})
960	$M_{HEAVYMAXi}$	theoretical molecular weight of the DNA fragment containing the 16S RNA gene for taxon *i* assuming maximum
961		labeling by the heavy isotope (g mol^{-1})
962	M_{LABi}	observed molecular weight of the DNA fragment containing the 16S RNA gene for taxon *i* in the labeled treatment (g
963		mol^{-1})
964	Z_i	difference in observed weighted average densities of taxon *i* for the labeled and unlabeled treatments (g cm^{-3})
965	$A_{OXYGENi}$	excess atom fraction of ^{18}O in the labeled versus unlabeled treatment for taxon *i* (unitless)

966 $A_{CARBONi}$ excess atom fraction of ^{13}C in the labeled versus unlabeled treatment for taxon *i* (unitless)

967

Table 2. Density (g cm^{-3}) of DNA for taxa exhibiting or not exhibiting tracer assimilation in the three tracer experiments. Values are means and standard deviations.

	Density (g cm^{-3})	
Tracer	assimilating	not assimilating
^{18}O-H_2O	1.6905 ± 0.0031	1.6912 ± 0.0033
^{18}O-H_2O with glucose	1.6896 ± 0.0033	1.6894 ± 0.0045
^{13}C-glucose	1.6890 ± 0.0030	1.6900 ± 0.0036

968

Applied and Environmental Microbiology

969

970

Applied and Environmental Microbiology