Validation of Heavy-Water Stable Isotope Probing for the Characterization of Rapidly Responding Soil Bacteria

Zachary T. Aanderud and Jay T. Lennon

W. K. Kellogg Biological Station and Department of Microbiology & Molecular Genetics.

Michigan State University, Hickory Corners, Michigan

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Rapid responses of bacteria to sudden changes in their environment can have important implications for the structure and function of microbial communities. In this study, we used heavy-water stable isotope probing (H$_2^{18}$O-SIP) to identify bacteria that respond to soil rewetting. First, we conducted experiments to address uncertainties regarding the H$_2^{18}$O-SIP method. Using liquid chromatography-mass spectrometry (LC-MS), we determined that oxygen from H$_2^{18}$O was incorporated into all structural components of DNA. Although this incorporation was uneven, we could effectively separate 18O-labeled and unlabeled DNAs derived from laboratory cultures and environmental samples that were incubated with H$_2^{18}$O. We found no evidence for ex vivo exchange of oxygen atoms between DNA and extracellular H$_2$O, suggesting that 18O incorporation into DNA is relatively stable. Furthermore, the rate of 18O incorporation into bacterial DNA was high (within 48 to 72 h), coinciding with pulses of CO$_2$ generated from soil rewetting. Second, we examined shifts in the bacterial composition of grassland soils following rewetting, using H$_2^{18}$O-SIP and bar-coded pyrosequencing of 16S rRNA genes. For some groups of soil bacteria, we observed coherent responses at a relatively coarse taxonomic resolution. Following rewetting, the relative recovery of Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria increased, while the relative recovery of Chloroflexi and Deltaproteobacteria decreased. Together, our results suggest that H$_2^{18}$O-SIP is effective at identifying metabolically active bacteria that influence soil carbon dynamics. Our results contribute to the ecological classification of soil bacteria while providing insight into some of the functional traits that influence the structure and function of microbial communities under dynamic soil moisture regimes.

One of the overarching goals of microbial ecology is to understand the contributions of different taxa to ecosystem processes. This seemingly straightforward task is often hindered by the tremendous complexity of microbial communities. For example, environmental samples typically contain thousands of taxa (44), but a large fraction of this diversity is comprised of dormant or inactive individuals (10, 24, 30). Furthermore, microbial communities are dominated by rare taxa (49), and it is unclear whether the activity of these microorganisms is important for understanding the biogeochemical fluxes of material and energy in different ecosystems.

One approach that has been used successfully for assigning function to microbial taxa is stable isotope probing (SIP). This technique involves the addition of an isotopically labeled substrate (e.g., $^{13}$CH$_4$) to an environmental sample (39). Following a sufficient incubation period, isotopically enriched macromolecules (e.g., lipids or nucleic acids) can be extracted and linked to a targeted microbial process, such as methane oxidation (31), contaminant degradation (27), or nitrogen fixation (3).

Recently, heavy water (H$_2^{18}$O) was introduced as a novel substrate to be used with SIP (48). Two features make H$_2^{18}$O-SIP attractive for linking microbial structure and function. First, it has the potential to be more sensitive than some nucleic acid-based SIP techniques that employ other labeled substrates. For example, most SIP studies use substrates that have only one additional neutron compared to the most abundant naturally occurring isotope (e.g., $^2$H versus $^1$H, $^{13}$C versus $^{12}$C, or $^{15}$N versus $^{14}$N). In contrast, $^{18}$O has two more neutrons than the most abundant naturally occurring oxygen isotope ($^{16}$O). Therefore, the incorporation of a single $^{18}$O atom compared to a single $^{13}$C or $^{15}$N atom should increase the degree of physical separation between labeled and unlabeled fractions during the isopycnic centrifugation step of SIP. Second, most SIP studies to date have used isotopically labeled substrates that target somewhat phylogenetically constrained metabolic processes (e.g., methane oxidation). In contrast, H$_2^{18}$O-SIP has the potential to label all metabolically active (RNA-SIP) or growing (DNA-SIP) microorganisms due to the universal requirements of water for cellular maintenance and biosynthesis (48).

Although H$_2^{18}$O-SIP holds promise for linking the identity and activity of microorganisms, there are some important assumptions and unknowns that remain to be addressed. The location and evenness of $^{18}$O incorporation into nucleic acids are not well established. For example, $^{18}$O has the potential to be located in the phosphate group, in deoxyribose/ribose sugars, and in nucleobases (i.e., cytosine, guanine, and thymine), since all of these molecular components contain oxygen. There are three specific pathways by which $^{18}$O can be incorporated into the phosphate group of nucleic acids. First, $^{18}$O can be exchanged between H$_2$O and inorganic phosphate species of...
ATP, which is facilitated by pyrophosphatases (6). Second, the F1 component of ATPase can promote isotopic exchange so that the γ-phosphate group of ATP contains 18O (25). The 18O-labeled ATP may then catalyze the formation of glucose-6-phosphate, which serves as a precursor of ribose-5-phosphate and, eventually, nucleic acid biosynthesis. Third, H318O can be used as a substrate to convert nucleoside diphosphates to deoxyribonucleoside triphosphates (33). Furthermore, 18O may be incorporated into pyrimidine nucleobases during hydrolysis reactions with citric acid cycle intermediates (e.g., aspartate and carbamoyl phosphate), into deoxyribose via glucogenesis, and into pentose phosphate pathways that use small carbon and oxygen precursors (34). Variation in the evenness of 18O incorporation in all of these pathways may lead to differences in the degree of separation between labeled and unlabeled DNAs during SIP, which can have important implications for drawing conclusions about the metabolic activity of a microbial population (3). Another concern is the potential exchange between 18O and 16O in nucleic acids following DNA replication or RNA transcription. For example, it is fairly well documented that oxygen isotopic exchange can occur between water and a variety of substances, including metal complexes (36, 53), organic matter (55), and monosaccharides such as ribose and deoxyribose (7), but the extent of this phenomenon for nucleic acids remains unclear. If a significant amount of isotopic exchange occurs between nucleic acids and cytoplasmic or extracellular water, then it could be problematic to associate microbial taxonomic shifts with metabolic processes by using H318O-SIP.

Despite some methodological uncertainties, H218O-SIP is a promising technique for linking the activity of microorganisms to changes in their environment. In particular, H318O-SIP may help to assign ecological significance to groups of bacteria that respond to sudden changes in soil moisture. In nature, rainfall patterns can create rewetting events that elicit dramatic changes in soil microbial dynamics and ecosystem activity (18, 20, 58). For example, dry soils generate large pulses of CO2. In this study, we conducted a series of experiments to address some of the methodological questions surrounding the H318O-SIP technique. First, we used liquid chromatography-mass spectrometry (LC-MS) to determine the location and evenness of 18O incorporation within bacterial DNA. Second, we conducted H318O-SIP experiments with DNA from Escherichia coli to test for the exchange of 18O-18O between DNA and extracellular H2O. Third, we tracked the incorporation of 18O into bacterial DNA to assess whether H318O-SIP can be used to capture the rapid metabolic responses generated from soil rewetting. We then used bar-coded pyrosequencing of 16S rRNA genes to identify bacterial taxa that responded to a rewetting event in grassland soils and thus contributed to measured pulses of CO2.

**Materials and Methods**

**Location of 18O in DNA.** To evaluate the location and evenness of 18O incorporation into bacterial DNA, we measured the incorporation of oxygen atoms from H218O into the nucleotide dTMP in a population of Escherichia coli cells using LC-MS. Because the mass of dTMP does not overlap with those of any of the other nucleoside monophosphates (i.e., guanosine, adenosine, and cytidine), this nucleotide offered the best mass spectrum for identifying 18O incorporation. In triplicate, we obtained labeled (18O) and unlabeled (16O) DNAs by inoculating 10 μl of log-phase E. coli (K-12 strain MG1655) into 140 ml of 10% Luria-Bertani (LB) liquid medium containing 1.5 ml of H218O (97 atom% 18O; Isotech, Sigma-Aldrich, St. Louis, MO) and unlabeled nucleosulfate-free water (H318O), respectively. Cells were incubated for 5 h at 37°C on a shaker (150 rpm). Genomic DNA was extracted from E. coli by using a UltraClean microbial DNA isolation kit (MoBio, Carlsbad, CA) and was eluted in 150 ml of nucleosulfate-free H2O. Genomic DNA (~3 μg) was then digested with DNease 1 (Invitrogen Corporation, Carlsbad, CA) for 48 h at 37°C to create dTMP. Each 300-μl digestion mixture contained 150 ml of DNA, 30 μl of 10× DNease buffer, 5 μl of 10 mg/ml DNease 1, and an enzyme/substrate ratio of 1:80. We terminated the reaction by placing the digestion mixtures at ~20°C. The resulting dTMP was purified with a Novagen SpinPrep PCR cleanup kit (EMD Chemicals, Gibbstown, NJ) to remove polymerases and then stored at −20°C until being run on the LC-MS. We used an LC-MS system consisting of a Thermo Fisher Scientific Xevo G2 TOF Ultima API LC-MS, a Waters 2795 separation module, and an Acquity UPLC BEH C18 column (2.1 mm by 100 mm by 1.7 μm; Waters Corporation, Milford, MA) with eluent ions of 1000–2000 m/z. The resulting data were analyzed with MassLynx 4.0 software (Waters Corporation, Milford, MA).

**Ex vivo exchange of 18O in DNA.** We tested for the ex vivo exchange of 18O in bacterial DNA by using H318O-SIP. Specifically, we tracked changes in the location of 18O-labeled DNA in cesum trifluoroacetate (CtFA; GE Healthcare, Salt Lake City, UT) gradients following isopycnic centrifugation before and after the incubation. DNA was incubated in H318O. First, 18O-labeled and unlabeled DNA (10 μg) separately in H218O (500 μl) for 7 days at 4°C. Next, H318O-SIP was performed in triplicate, using the procedures described above. Finally, we quantified the amounts of DNA in the different density fractions by using quantitative PCR (qPCR) with primers targeting a fragment of the 16S rRNA gene (see “qPCR detection of 18O-labeled DNA”). The resulting quantities of 16S rRNA gene copies were standardized by expressing the number copies in each fraction to the number of gene copies in the buoyant density fraction that contained the maximum number of 16S rRNA gene copies. This standardization of copy numbers within the gradient for each replicate helped to determine the locations of 18O-labeled and unlabeled DNAs. For a given replicate, the entire gradient was analyzed in the same qPCR assay to minimize the potential for qPCR bias between runs. We tested for the effects of isotopic exchange on 18O-labeled DNA by comparing the ratios of maximum quantities of 16S rRNA gene copies in the different density fractions (i.e., 11 fractions, with densities ranging from 1.351 to 1.649 g ml−1) before and after the incubation, using two-way analysis of variance (ANOVA). Assumptions of ANOVA were tested using the Shapiro-Wilk test for normality and Levene’s test for homogeneity of variance (46).

**H318O-SIP procedure.** We followed the DNA-SIP procedure outlined by Neufeld et al. (35), with a few modifications to improve the recovery of the 18O-labeled DNA from unlabeled DNA. Approximately 1 μg of genomic DNA (ranging from 0.85 to 1.3 μg) was loaded into 4.7-ml OptiSeal polyallomer tubes (Beckman Coulter Inc., Brea, CA) containing CtFA with a buoyant density of 1.61 g ml−1. The CtFA mixture was prepared in bulk before centrifugation, with each 4.7-ml tube receiving approximately 2.9 ml of CtFA and 1.75 ml of nuclease-free H2O. The DNA was added to each tube in 45 μl of nuclease-free H2O to help maintain similar starting densities between tubes. Heavy and light DNAs were separated in each tube through isopycnic ultracentrifugation at a speed of 65,135 rpm (177,000 × g), for 72 h. Tubes were balanced within ±0.005 g, loaded into a TLA 100 rotor (Beckman Coulter Inc., Brea, CA), and centrifuged on an Optima Max-XP benchtop ultracentrifuge (Beckman Coulter Inc., Brea, CA) at 20°C. We retrieved DNA in 20 fractions (235 μl each) from the resulting gradient, using a fraction recovery system (Beckman Coulter Inc., Brea, CA) connected to a single-syringe infusion pump (Cole-Farmer, Vernon Hills, IL) set at a rate of 0.47 ml min−1. DNA was then replaced with nuclease-free water and centrifuged for approximately 5 min. We incorporated two blanks without DNA in each ultracentrifugation step to calculate the densities of the 20 fractions and create a standard CtFA buoyant density gradient (n = 12) for our specific SIP conditions. Buoyant densities of all fractions were weighed on a microbalance (Sartorius, Goettingen, Germany).
Germany). DNA was precipitated from all fractions through two isopropanol (molecular biology grade) washes. Each fraction received 600 µl of ice-cold isopropanol and was incubated overnight at −20°C. These fractions were then centrifuged at 14,000 × g, for 40 min, followed by a 10-min centrifugation in 200 µl ice-cold isopropanol. DNAs in all fractions were resuspended in 30 µl of nuclease-free H2O.

qPCR detection of 18O-labeled DNA. We performed qPCR assays to determine the distribution of DNA within the CsTFA gradient. Following centrifugation and fractionation, qPCR was used on all 20 fractions from each SIP run, using a Mastercycler EP Realplex qPCR machine (Eppendorf, Hamburg, Germany) and SYBR green to quantify bacterial DNA obtained with primers that targeted regions of the 16S rRNA gene. Each 30-µl reaction mixture contained the following: 13.5 µl of 2.5× SYBR Rox Real master mix (5 Prime, Inc., Gaithersburg, MD); 0.5 µl of forward primer Eub338 and 0.5 µl of reverse primer Eub518, which make up a universal bacterial primer set (17); 1 µl of DNA template; and 14.5 µl of nuclease-free H2O. The qPCR standard was generated from a soil bacterium (Micromonas sp.) by use of a Topo TA cloning kit (Invitrogen). We extracted plasmids from transformed cells (45) and used M13 forward and reverse primers from the cloning kit to generate PCR products that we used for our standard curve (102 to 107 copies/µl). The coefficients of determination (r2) for our assays ranged from 0.96 to 0.99, while amplification efficiencies (E) varied between 0.98 and 0.99. Based on melting curve analyses, we found no evidence for primer dimers. The resulting quantities of rRNA gene copies were normalized by expressing gene copies as ratios to the maximum quantity of copies per grassland replicate, similar to the normalized by expressing gene copies as ratios to the maximum quantity of copies fell between 0.93 and 0.99. Based on melting curve analyses, we found no ex vivo

Grassland soil sampling. We collected soils from the successional grassland treatment of the 177th Term Ecological Research (LTER) site in southwestern Michigan. This replicated land-use treatment consists of 1-ha plots that comprise a mixture of perennial grasses and forbs that became established after the field was abandoned from agricultural activity in 1989. The dominant plant species in the T7 treatment include Solidago canadensis (L.), Apocynum cannabinum (L.), and Phleum pratense (L.) (43). Our sampling was conducted on 2 September 2008 following summer dry-down of the soils. We collected and pooled 10 random soil cores (10 cm by 2 cm) from three randomly selected grassland treatments. On average, annual precipitation at the KBS LTER site is 890 mm, with half falling as snow, and the mean annual temperature is 9.7°C (http://www.lternet.edu/sites/kbs). All soils are fine-loamy, mixed, mesic Typic Hapludalfs with a total soil C content of 1.3% and N content of 0.13%, pH of 5.5, and cation exchange capacity of 5.5 cmol kg−1. Soil samples were collected from the Ap horizon, sieved to 2 mm, and used in 18O-SIP microcosms within 5 h of removal.

Soil rewetting and H2O-SIP. We performed H18O-SIP experiments to estimate the rate of 18O incorporation into the DNA of soil bacteria. This information was then used to link bacterial taxa to the metabolic activity associated with soil rewetting (see “Soil CO2 production”). For both of these objectives, we simulated rewetting events with H18O in microcosms containing grassland soil. Microcosms consisted of 40-ml borosilicate glass vials with polypropylene, sili-coneximex screw caps with microcosm diameters of 3 g of grassland soil. Soils were incubated with H18O for different durations (0, 48, and 72 h). The experiment was initiated by evenly applying 600 µl of H18O (97 atom% 18O; Isotech, Sigma-Aldrich, St. Louis, MO) to the surface of each grassland soil microcosm (3 grassland replicates × 3 sampling times = 9 microcosms). The rewetting increased gravimetric soil moisture 5-fold, from −0.05 to 0.25 g H2O g soil−1. Prior to rewetting, gravimetric water content was estimated as the difference in the mass of soil before and after drying at 105°C for 48 h. The microcosms were incubated at 25°C in a temperature-controlled incubator. Total genomic DNA was extracted from the microcosm soil by using a PowerSoil DNA isolation kit (MoBio, Carlsbad, CA). We performed H18O-SIP on the resulting DNA by using the methods described above.

Soil CO2 production. We quantified the effects of rewetting on soil microbial metabolism by measuring the evolution of CO2 in the headspace of the microcosms over the duration of the experiment. To accurately measure transient CO2 pulse dynamics and the rate of CO2 production, microcosms were destructively harvested at 2 h increments over a 9-h time span, resulting in a total of 27 experimental units (3 grasslands × 9 sampling times from 0 to 96 h = 27 microcosms). For the 0-, 48-, and 72-h sampling times, we measured soil respiration after rewetting with H18O. For the remaining time points (12, 24, 36, 48, and 96 h), rewetting was performed with nuclease-free unlabeled water. We evaluated the rate of soil respiration by removing 1 ml of gas from the headspace of each microcosm with a 1-ml syringe, measuring the CO2 concentration (ppm) in the gas on a CO2 gas analyzer (Li-820; Li-Cor Biosciences, Lincoln, NE), and regressing the total amount of CO2 respired (µg C–CO2 g soil−1) by the harvest time of each microcosm. We stored microcosms at −20°C until further processing.

Taxonomic responses to rewetting. To link bacterial taxa to moisture-induced pulses of CO2, we pyrosequenced the 16S rRNA genes from bacteria in the 0- and 72-h H18O soil incubations. Specifically, we selected, pooled, and sequenced two fractions within the 0-h gradients (fractions 12 and 13, with buoyant densities in CsTFA of 1.531 to 1.546 g/ml) to identify bacterial taxa immediately following the rewetting event. We then selected, pooled, and sequenced two fractions within the 72-h gradients containing 18O-labeled DNA (fractions 9 and 10, with buoyant densities in CsTFA of 1.574 to 1.585 g/ml). This combined SIP procedure was performed on soil from three replicate grassland plots. We used bar-coded pyrosequencing of the 16S rRNA gene to characterize shifts of bacterial taxa following rewetting. The pyrosequencing procedures are described in greater detail elsewhere (16, 23). Briefly, 16S rRNA genes were amplified using a 27F and 338R primer set with unique 12-nucleotide (nt) error-correcting Golay bar codes under the following thermal cycle conditions: an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 30 s, and extension at 72°C for 90 s. PCR amplicons from each sample were pooled at approximately equimolar concentrations prior to pyrosequencing. Samples were sequenced at the Environmental Genomics Core Facility at the University of South Carolina in a 454 Life Sciences FLX genome sequence (Roche, Branford, CT) instrument. Sequences were analyzed according to the methods outlined by Fierer et al. (16). To ensure the accuracy and quality of pyrosequencing, only sequences of ≥200 bp were included in the analysis (22). Phylotypes were identified using Megablast at a minimum coverage of 99% and a minimum pairwise identity of 97%. The phylogenetic identities of sequences were determined with BLAST searches against the Greengenes database (http://greengenes.biology.gatech.edu) (12), using an E value cutoff of 1e−10 and the Hugenholtz taxonomy.

To assess compositional responses to rewetting, we first calculated the relative recoveries of taxonomic groups as the total number of phylotypes in a given group divided by the total number of phylotypes present in each sample. We restricted our analyses to taxa that comprised more than 1% of all phylotypes and demonstrated ≥0.5% differences in relative recovery. Next, shifts in bacterial composition in response to rewetting were assessed for each grassland replicate (n = 3) by quantifying the change in relative recovery of taxa between 0 and 72 h of H18O application. We tested for the significance of these shifts in bacterial composition following rewetting by constructing 95% confidence intervals (sample mean ± [1.96 × standard error]) around the mean change for each taxonomic group. If the confidence intervals overlapped with zero, we concluded that there was no significant change in the relative recovery of a taxonomic group following rewetting. Furthermore, we quantified changes in bacterial taxon richness over the rewetting periods by using Chao1 diversity estimation (e) for each of the H18O-SIP runs.

RESULTS

Location of 18O in DNA. Results from LC-MS revealed that oxygen from H18O was incorporated into all structural components of dTMP of E. coli, including the phosphate group, deoxyribose, and thymine. Naturally occurring dTMP has a molar mass of 322 and contains a total of eight oxygen atoms; four of these are in the phosphate group, two in the pentose sugar, and two in the thymidine nucleobase. If any of the 16O atoms were replaced by 18O, we would expect the molar mass of dTMP to increase by 2, for a total possible mass shift of 16 in a fully 18O-labeled dTMP molecule. After incubating unlabeled E. coli DNA with H218O, the resulting dTMP peaked predominantly at a mass-to-charge ratio (m/z) of 321 in the LC-MS spectrum (Fig. 1A). After incubating E. coli with H18O, however, the resulting dTMP exhibited substantial peaks at 321, 325, 327, 331, 333, and 335 m/z (Fig. 1A). These multiple peaks suggest that the number of 18O atoms incorporated into dTMP is variable, ranging from approximately 4 to 14 extra neutrons (or 2 to 7 18O atoms). The largest of these peaks occurred at 333 and 335 m/z, corresponding to the addition of six and seven 18O atoms, respectively. Because dTMP contains only eight O atoms and no structural component of
DNA contains only one O atom, we can infer that $^{18}$O incorporation from H$_2^{18}$O occurred in all three components of DNA (Fig 1B). The relatively smaller peaks throughout the spectrum, at 322 m/z in the unlabeled DNA and at 324, 326, and 328 m/z in the $^{18}$O-labeled DNA, most likely occurred from the addition of an extra neutron into DNA from $^{13}$C. This phenomenon is common and can most likely be attributed to the natural abundance of $^{13}$C/$^{12}$C (≈1%) in the E. coli inoculum and the medium used in the H$_2^{18}$O incubations.

**Ex vivo exchange of $^{18}$O in DNA.** Our results suggest that it is unlikely that H$_2^{18}$O-SIP is strongly affected by ex vivo isotopic exchange. $^{18}$O in the $^{18}$O-labeled DNA from E. coli did not undergo ex vivo exchange with oxygen from H$_2$O. If significant isotopic exchange had occurred, we would have expected there to be a decrease in the buoyant density of $^{18}$O-labeled DNA following incubation. In contrast, there was no difference in the buoyant density for the fraction with the maximum quantity of $^{18}$O-rRNA genes within 11 fractions of the gradient (with densities ranging from 1.531 to 1.649 g ml$^{-1}$) before and after the 7-day incubation in H$_2^{18}$O. Therefore, $^{18}$O-labeled DNA possessed the same buoyant density and retained its extra neutrons from $^{18}$O following incubation in unlabeled H$_2$O. $^{18}$O-labeled DNA resided in fractions with densities of 1.561 to 1.585 g/ml, while unlabeled DNA resided in fractions with densities of 1.531 to 1.548 g/ml.

**Soil rewetting and H$_2^{18}$O-SIP.** As expected, bacteria in the dry grassland soil rapidly responded to rewetting. CO$_2$ accumulated in microcosms at a rate of 1.34 µg C-CO$_2$ g$^{-1}$ soil h$^{-1}$ (linear regression $r^2 = 0.76; P < 0.0001$). By 72 h, CO$_2$ concentrations had increased from 3.04 ± 0.35 (mean ± standard error of the mean [SEM]) to 113 ± 9.11 µg C-CO$_2$ g$^{-1}$ soil. At the same time, $^{18}$O from H$_2^{18}$O was rapidly incorporated into the DNA of metabolically active soil bacteria. Based on H$_2^{18}$O-SIP time series, the $^{18}$O incorporation into DNA...
caused the buoyant density of DNA to increase by 0.013 to 0.054 g/ml within the first 48 h (Fig. 3). We observed an increase in the denser CsTFA fractions (i.e., fractions 8 to 11, with buoyant densities of 1.561 to 1.598 g/ml) in the 72-h incubation, suggesting that bacteria continued to produce 18O-labeled DNA over the duration of the experiment. However, at both 48 and 72 h, there was always a substantial quantity of unlabeled rRNA genes present in the less dense CsTFA fractions (i.e., fractions 12 to 14, with buoyant densities of 1.531 to 1.548 g/ml).

**Taxonomic responses to rewetting.** Based on pyrosequencing of the 16S rRNA genes from H218O-SIP, we found that soil bacteria exhibited different responses to rewetting (Fig. 4A). Bacterial taxa were considered positive responders if they produced new cells labeled with 18O and increased in relative recovery following rewetting. For example, the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria all increased in relative recovery following rewetting. The most dra-

![Graph](image-url)

**FIG. 4.** Taxonomic responses to soil rewetting. Values are mean changes (n = 3) in relative recoveries of taxa after rewetting (72 h) and before rewetting (0 h) with H218O for phyla or classes (A) and families (B). Asterisks denote taxonomic groups that responded significantly to rewetting based on the overlap of 95% confidence intervals with zero change in relative recovery.

### CsTFA buoyant density (g mL⁻¹)

**FIG. 3.** Rapid incorporation of 18O from H218O into bacterial DNA following rewetting of dried soils. Panels show the resulting distributions of 16S rRNA genes from replicate (n = 3) grassland soils in CsTFA gradients. Soils were incubated with H218O for 0 h (A), 48 h (B), and 72 h (C). Values are mean ratios of maximum quantities of rRNA genes (n = 3) ± SEM from qPCRs.
matic change occurred for the Alphaproteobacteria, which increased in their relative recovery by 10%. Together, these positively responding taxa accounted for 30% ± 1.7% of all phylotypes prior to the rewetting event. In contrast, the relative recovery of the Chloroflexi and Deltaproteobacteria, which accounted for 3.2% ± 0.4% of the initial phylotypes, decreased after the rewetting event. The response of some bacterial taxa to rewetting was variable. For example, the mean changes in relative recovery of Acidobacteria, Actinobacteria, Bacteroidetes, Gemmatimonadetes, TM7, and Verrucomicrobia ranged from −3.7 to 0.9%; the 95% confidence intervals around the average shifts in relative recoveries overlapped with zero, indicating that these taxonomic groups did not respond significantly to rewetting. This variability could be explained in part by examining the bacterial responses at a finer taxonomic resolution. At the family level, we found that nine families increased in relative recovery by at least 0.5% following the rewetting event, and three of these nine came from phyla with variable responses (Fig. 4B). For example, we found that the Rubrobacteraceae (Actinobacteria phylum), Acidobacteriaceae (Acidobacteria phylum), and Gemmatimonadaceae (Gemmatimonadetes phylum) all responded positively and significantly to rewetting. The remaining six families belonged to phyla that demonstrated coherent positive responses to rewetting. Within the Alphaproteobacteria, we observed significant positive responses by the Caulobacteraceae, Hyphomicrobiaceae, and Sphingomonadaceae. In particular, the relative recovery of the Sphingomonadaceae increased by 10% following rewetting. In addition, we saw positive responses by the Comamonadaceae (Betaproteobacteria) and the Xanthomonadaceae (Gammaproteobacteria). Based on Chao1 estimates, there was no difference between the numbers of phylotypes in the initial (0 h) and final (72 h) SIP samples (5,963 ± 587.0 versus 6,679 ± 204.1 [mean ± SEM], respectively; paired t test \( t_2 = 1.15 \) \( [P = 0.42] \)).

**DISCUSSION**

Over the past decade, SIP has emerged as an effective tool for linking the structure and function of microbial communities. In this study, we evaluated some of the mechanisms and underlying assumptions of \( ^{18} \text{O}-\text{SIP} \) (48). In laboratory experiments with *E. coli*, we found that \(^{18} \text{O}\) was incorporated into all of the structural components of DNA and that these labeled materials did not exchange with extracellular water. With environmental samples, we measured rapid (48 to 72 h) incorporation of \(^{18} \text{O}\) into the DNA of soil bacteria, which coincided with the production of soil CO\(_2\). Consistent with previous studies, we found that many taxa belonging to the Proteobacteria were able to respond rapidly to changes in soil environmental conditions. We discuss some of the functional traits that may be involved in the tolerance of fluctuating soil moisture and the contribution of these organisms to ecosystem activity.

**Validation of \(^{18} \text{O}-\text{SIP}\).** LC-MS data retrieved from labeled dTMP indicated that \(^{18} \text{O}\) from \(^{18} \text{O}\) was incorporated into all structural components of DNA. However, this incorporation was variable, consisting of as few as two \(^{18} \text{O}\) atoms to as many as seven \(^{18} \text{O}\) atoms per nucleotide. There is additional evidence that \(^{18} \text{O}\) incorporation into microbial DNA can be variable. For example, after 2-week incubations of soils with 95 atom% \(^{18} \text{O}\), three bands of DNA (instead of two) were observed in cesium chloride gradients stained with SYBR green 1 (48). It was hypothesized that the additional band of DNA belonged to “cross-feeding” taxa, which over time preyed upon or decomposed \(^{18} \text{O}\)-labeled organisms and their cellular constituents. Alternatively, an additional band could reflect semiconservative DNA replication (48). Although our incubations with *E. coli* were much shorter than those in previous work with environmental samples (48), the preferential labeling of different DNA components may also give rise to variability in the buoyant densities in \(^{18} \text{O}-\text{SIP}\). Specific pathways for DNA synthesis directly incorporate \(^{18} \text{O}\) from H\(_2\)\(^{18} \text{O}\) (i.e., formation of phosphate), while others derive \(^{18} \text{O}\) from both water and oxygen-containing organic compounds. For example, it was shown that oxygen associated with C-3 and C-5 of deoxyribose was derived predominantly from glucose, not from the surrounding cytoplasmic water (41). Thus, any unlabeled C substrate from growth media or the surrounding environment (e.g., soil) has the potential to be incorporated into deoxyribose over \(^{18} \text{O}\) from H\(_2\)\(^{18} \text{O}\), but this interaction may change as more carbon becomes labeled with \(^{18} \text{O}\). Regardless of the uneven labeling during \(^{18} \text{O}-\text{SIP}\), \(^{18} \text{O}\) incorporation was sufficient to clearly separate fractions of \(^{18} \text{O}\)-labeled and unlabeled DNAs from both cultures and environmental samples (Fig. 2 and 3). In addition, we found that the \(^{18} \text{O}\) in \(^{18} \text{O}\)-labeled DNA from *E. coli* did not undergo any detectable *ex vivo* exchange with \(^{18} \text{O}\) from unlabeled H\(_2\)\(^{18} \text{O}\). These results suggest that \(^{18} \text{O}\) incorporation into DNA is relatively stable and that H\(_2\)\(^{18} \text{O}\)-SIP is appropriate for making inferences about the metabolic activity of microbial taxa. Future studies, however, should evaluate whether *in vivo* isotopic exchange associated with processes such as DNA repair (54) facilitates \(^{18} \text{O}\)-\(^{18} \text{O}\) substitutions.

**Linking \(^{18} \text{O}-\text{SIP}\) to soil \text{CO}_2\) production.** In a previous study, separation of \(^{18} \text{O}\)-labeled and unlabeled DNAs was achieved after incubating soil for 6 to 21 days (48). In this study, we measured consistent shifts in the buoyant density of 16S rRNA genes from soil bacteria after only 48 h of incubation with H\(_2\)\(^{18} \text{O}\) (Fig. 2 and 3). This shorter incubation time may reduce the occurrence of artifacts associated with exposure of environmental samples to laboratory conditions for prolonged periods. In addition, a 3-day H\(_2\)\(^{18} \text{O}\) incubation is a time frame that captures rapid pulses of ecosystem activity generated from rewetting of dry soils (19). Therefore, it is likely that many of the \(^{18} \text{O}\)-labeled bacterial taxa contributed, at least in part, to the observed pulses of CO\(_2\) that followed rewetting. There are a number of important caveats, however, that must be considered in using H\(_2\)\(^{18} \text{O}\)-SIP to make inferences about the contributions of soil microorganisms to CO\(_2\) pulses. First, not all bacteria generate CO\(_2\). For example, ammonia-oxidizing bacteria use NH\(_4^+\) as an electron donor and O\(_2\) as an electron acceptor; CO\(_2\) is not generated as a byproduct of their metabolism. Second, it is possible that some heterotrophic bacteria contributed to soil CO\(_2\) production but did not grow and become labeled with \(^{18} \text{O}\). For example, soil bacteria may have responded to rewetting but used H\(_2\)\(^{18} \text{O}\) predominantly for catabolism to meet maintenance energy requirements (e.g., motility, energized cell membrane, DNA repair, etc.) in the absence of new cell growth (52). These active but nonreplicating bacteria would presumably be detected if...
Coherent taxonomic responses to rewetting. Given the tre- mendous diversity of microorganisms, recent attention has fo-cused on whether the ecological responses of bacteria can be predicted at relatively high levels of taxonomic resolution (38). In other words, can we make generalizations about how phyla respond to environmental changes, or must we understand bacterial behaviors on a strain-by-strain basis? In general, our results provide evidence that bacterial responses to rewetting are coherent at fairly coarse taxonomic levels of resolution. Specifically, the Betaproteobacteria and Gammaproteobacteria exhibited moderate increases in relative recovery, and Alphaproteobacteria increased by 10% following soil rewetting. This positive response represents the rapid growth of ~500 individ- ual phylotypes within the Alphaproteobacteria alone. In particu- lar, we found that the Sphingomonadaceae—a group of Gram-negative, aerobic heterotrophs that are common in soils (26, 40)—were chiefly responsible for the increase in relative recovery of the Alphaproteobacteria following rewetting. Con- versely, the relative recovery of Chlorflexi and Delphaceo- bacteria declined after rewetting. This reduction in relative recov- ery may reflect the fact that some bacteria experience stress by sudden changes in osmotic pressure and are unable to rapidly grow following rewetting (47). Alternatively, a decrease in rel- ative recovery could represent a dilution effect whereby neu- trally responding taxa were less likely to be detected in our pyrosequencing efforts owing to the increased probability of recovering positively responding taxa. Furthermore, it is pos- sible that we missed the responses of other bacterial taxa since we evaluated only two 18O-labeled fractions within the H2,18O- SIP study; other taxa may have been recovered in fractions immediately adjacent to the fractions evaluated in our study.

The response of bacterial taxa to rewetting is likely to be influenced by the distribution of functional traits. For example, fast-growing (r-selected), copiotrophic microorganisms have high rates of resource uptake (i.e., V_{max}) that would allow them to take advantage of sudden increases in substrate availability that accompany soil rewetting events (47). In contrast, slow- growing (k-selected), oligotrophic microorganisms benefit from having a higher affinity (i.e., lower half-saturation constant [K_{s}]) for growth-limiting resources, which may allow them to persist under less productive conditions (e.g., pro- longed periods of drought). Using this framework, our data are consistent with the view that the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria exhibit copiotrophic behavior, while some of the Chloroflexi, Delphaceobacteria, and other nonresponsive taxa may include oligotrophic bacteria. Specifically, the sphingomonads are recognized as a copio- trophic group of soil bacteria. These microorganisms exhibit extreme metabolic versatility, as demonstrated by their ability to consume a wide range of organic carbon substrates, includ- ing glucose, polysaccharides, and aromatic hydrocarbons (1, 28, 29). The taxonomic responses from our rewetting experi- ment are consistent with other studies that have attempted to classify the ecology of soil bacteria. For example, the relative recovery of Betaproteobacteria increased along a carbon min- eralization gradient (15), while the relative recoveries of Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria from different land-use types responded positively to the addition of simple carbon substrates such as glucose, gly- cine, and citric acid (13).

It seems likely, however, that functional traits other than those associated with resource acquisition may also contribute to bacterial rewetting responses. For example, many Proteobacteria, including the Sphingomonadaceae, secrete a diverse compilation of exopolymeric substances (11, 14). These sub- stances can retain moisture in dry soils (37, 42) and allow bacteria to capitalize on resources when they become available. Also, the presence of aquaporins, channel proteins that enhance the permeability of cell membranes, should allow bacteria to rapidly expel osmolytes following rewetting and thus maintain equilibrium with their environment (8, 47). Of the sequenced Gram-negative bacteria with aquaporins, approxi- mately 80% belong to phyla that responded positively to rewetting (i.e., Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria) (50). Other traits that may be import- ant for soil microbes that experience drying-rewetting cycles are motility (9), tolerance to low oxygen (32), and the ability to enter and exit a dormant state (24).

Summary and future applications. Our findings demon- strate that H2,18O-SIP is a viable method for identifying bac- teria that rapidly respond to soil rewetting and potentially contribute to pulses of ecosystem activity (e.g., CO2 flux). A number of studies have documented the effects of rewetting stress on individual strains of soil bacteria (2, 21, 42, 51), but this information comes largely from a phylogenetically re- stricted group of cultured bacteria. In examining an intact soil bacterial community, we found that there were coherent pos- itive responses of the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria to rewetting. Additional studies are needed to elucidate the mechanisms behind these patterns, especially given the predicted changes in precipitation regi- mens under future climate change scenarios (56, 59). Besides understanding bacterial responses to fluctuations in moisture, H2,18O-SIP has many other potential applications in microbial ecology. For example, this form of SIP may help to identify temperature-sensitive microorganisms involved in decomposi- tion, characterize the activity of functional genes, or serve as an isotopic backdrop to identify bacteria that are capable of re- mediating contaminated ecosystems. Last, H2,18O-SIP could also be used in combination with metagenomic approaches (5) to characterize genes that are regulated under conditions caus- ing fluctuations in microbial growth and activity.

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RNA-SIP were used instead of DNA-SIP (57). Lastly, there were nonbacterial taxa (e.g., archaea, fungi, and nematodes) in our grassland soil samples that were not targeted by our PCR primers but almost certainly produced CO2 in response to rewetting. Therefore, it is important to acknowledge that our H2,18O-SIP assay captured some microorganisms that did not contribute to CO2 pulses but also missed some microorganisms that did contribute to CO2 pulses.

H2,18O-SIP AND RAPID MICROBIAL RESPONSES

18O-SIP is a viable method for identifying bacteria that rapidly respond to soil rewetting and potentially contribute to pulses of ecosystem activity (e.g., CO2 flux). A number of studies have documented the effects of rewetting stress on individual strains of soil bacteria (2, 21, 42, 51), but this information comes largely from a phylogenetically re- stricted group of cultured bacteria. In examining an intact soil bacterial community, we found that there were coherent pos- itive responses of the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria to rewetting. Additional studies are needed to elucidate the mechanisms behind these patterns, especially given the predicted changes in precipitation regi- mens under future climate change scenarios (56, 59). Besides understanding bacterial responses to fluctuations in moisture, H2,18O-SIP has many other potential applications in microbial ecology. For example, this form of SIP may help to identify temperature-sensitive microorganisms involved in decomposi- tion, characterize the activity of functional genes, or serve as an isotopic backdrop to identify bacteria that are capable of re- mediating contaminated ecosystems. Last, H2,18O-SIP could also be used in combination with metagenomic approaches (5) to characterize genes that are regulated under conditions caus- ing fluctuations in microbial growth and activity.

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