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Microbial rRNA Synthesis and Growth Compared through Quantitative Stable Isotope Probing with $H_2{}^{18}O$

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ABSTRACT Growing bacteria have a high concentration of ribosomes to ensure sufficient protein synthesis, which is necessary for genome replication and cellular division. To elucidate whether metabolic activity of soil microorganisms is coupled with growth, we investigated the relationship between rRNA and DNA synthesis in a soil bacterial community using quantitative stable isotope probing (qSIP) with $H_2{}^{18}O$. Most soil bacterial taxa were metabolically active and grew, and there was no significant difference between the isotopic composition of DNA and RNA extracted from soil incubated with $H_2{}^{18}O$. The positive correlation between ^{18}O content of DNA and rRNA of taxa, with a slope statistically indistinguishable from 1 (slope = 0.96; 95% confidence interval [CI], 0.90 to 1.02), indicated that few taxa made new rRNA without synthesizing new DNA. There was no correlation between rRNA-to-DNA ratios obtained from sequencing libraries and the atom percent excess (APE) ^{18}O values of DNA or rRNA, suggesting that the ratio of rRNA to DNA is a poor indicator of microbial growth or rRNA synthesis. Our results support the notion that metabolic activity is strongly coupled to cellular division and suggest that nondividing taxa do not dominate soil metabolic activity.

IMPORTANCE Using quantitative stable isotope probing of microbial RNA and DNA with $H_2{}^{18}O$, we show that most soil taxa are metabolically active and grow because their nucleic acids are significantly labeled with ^{18}O. A majority of the populations that make new rRNA also grow, which argues against the common paradigm that most soil taxa are dormant. Additionally, our results indicate that relative sequence abundance-based RNA-to-DNA ratios, which are frequently used for identifying active microbial populations in the environment, underestimate the number of metabolically active taxa within soil microbial communities.

KEYWORDS rRNA, DNA, quantitative stable isotope probing, qSIP, atom percent excess ^{18}O values of nucleic acids, APE ^{18}O, density shift, rRNA-to-DNA ratio, relative sequence abundance, microbial activity, microbial growth, soil, atom percent excess, environmental microbiology, soil microbiology

Growing bacteria require many proteins to replicate their DNA and divide and therefore must also have a large pool of ribosomes to synthesize these proteins. Ribosome concentrations in pure cultures correlated positively with microbial growth rates: fast-growing bacteria contained relatively more ribosomes, measured as rRNA-to-DNA ratios, than slowly growing bacteria (1–5). rRNA-to-DNA ratios have been extended to environmental studies of multispecies bacterial communities, in which the ratios are used as indicators of bacterial metabolic activity (6–8). By comparing relative abundances in sequencing libraries from RNA and DNA directly extracted from the environment, ostensibly active and growing microbial populations, with high rRNA-to-DNA ratios, and inactive microbial populations, with low rRNA-to-DNA ratios, can be identified (9).

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Growth of microbial taxa can be characterized by DNA stable isotope probing (DNA-SIP) with $H_2{}^{18}O$ (10). This technique identifies organisms that assimilate and grow on isotopically labeled substrates (11), and detection of DNA with high ^{18}O content provides direct evidence for microbial growth because oxygen from water is incorporated into bacterial genomes during replication (10). $H_2{}^{18}O$-DNA-SIP has been applied to study microbial growth in a wide range of ecosystems, including terrestrial (12–15) and aquatic (16) habitats.

Whereas DNA-SIP investigates DNA replication, a measure of cell division, RNA-SIP characterizes RNA synthesis, an indicator of microbial metabolic activity (17–20). Thus, stable isotope probing of rRNA may detect metabolically active populations that are not growing, and comparison of RNA- and DNA-SIP results can characterize the relationship between metabolic activity and microbial population growth.

Few studies have simultaneously compared DNA- and RNA-SIP results of microbial communities. Most used ^{13}C-containing substrates (18, 20, 21) and generally found that incorporation of the isotope into microbial RNA (mRNA or rRNA) was faster than incorporation into DNA. There has been only one study that investigated microbial growth and activity in soil using DNA- and RNA-SIP with $H_2{}^{18}O$ (22). This study found that, after incubation of the soil with $H_2{}^{18}O$, the bacterial communities recovered in heavy RNA-SIP fractions were similar to communities characterized through bulk RNA analysis. The community represented in the heavy fractions of DNA was also similar to the community in nonfractionated DNA, leading the investigators to propose that most soil microorganisms were active and grew during the incubation with $H_2{}^{18}O$, but their study did not quantitatively assess the isotopic enrichment of individual taxa.

In the present study, we used quantitative stable isotope probing (qSIP) (23) with $H_2{}^{18}O$ to investigate rRNA synthesis and growth of microbial taxa in soil. Following incubation of the soil with $H_2{}^{18}O$, we calculated shifts in rRNA and DNA densities of bacterial taxa using all SIP fractions and used the density shift to calculate the ^{18}O composition of nucleic acids. This approach not only captures a greater number of taxa, including taxa present in fractions of intermediate densities, but also quantifies the atom percent excess ^{18}O values (APE ^{18}O) of rRNA and DNA for all members of the community and is unaffected by taxon-specific GC content. In addition to qSIP analysis, we compared ratios of rRNA to DNA in sequencing libraries to the isotopic composition of rRNA or DNA for all taxa. Our objective was to elucidate whether metabolic activity, assessed by measuring rRNA synthesis, and growth, assessed by measuring DNA synthesis, were coupled for all soil taxa. We hypothesized that growing populations will also be metabolically active. Additionally, we were interested to find if nongrowing taxa synthetized new rRNA of if microorganisms were able to grow with minimal rRNA synthesis.

RESULTS

Labeling of microbial RNA and DNA. Incubation of soil with $H_2{}^{18}O$ resulted in incorporation of ^{18}O into microbial RNA and DNA (Fig. 1). The presence of ^{18}O within nucleic acids increased their densities so that substantial weighted average density (WAD) shifts were observed after isopycnic centrifugation (Fig. 1, $P < 0.05$, on all days). There was no significant interaction between the density shift of RNA or DNA and time ($F_{1.012,\ 4.048} = 0.383$, $P = 0.572$, Greenhouse-Geisser epsilon $= 0.506$), showing that incorporation of ^{18}O into RNA was similar to incorporation of ^{18}O into DNA on each day. However, there was a significant main effect for time ($F_{1.012,\ 4.048} = 15.620$, $P = 0.016$, Greenhouse-Geisser epsilon $= 0.506$), with an increase in nucleic acid density over time. Specifically, RNA density shifts significantly increased from 0.0067 ± 0.0018 g/ml on day 1 to 0.0165 ± 0.0056 g/ml on day 8 ($F_{2,\ 4} = 15.404$, $P = 0.013$); mean changes in DNA density shifts were similar to those observed for RNA but were not statistically significant ($F_{1,\ 2} = 5.501$, $P = 0.144$). ^{18}O isotope ratio mass spectrometry (IRMS) analysis of total RNA and DNA samples showed similar trends over time. The isotopic enrichment, expressed as delta (δ) ^{18}O values, is provided in Table S1 in the supplemental material.

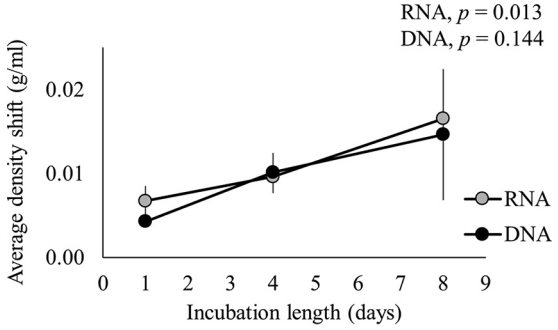

FIG 1 Density shifts of RNA or DNA extracted from soil incubated with $H_2{}^{18}O$ for 1, 4, or 8 days. Bars show the means ± standard deviations ($n = 3$). *P* values indicate statistical significance for a main effect of time.

Correlation between labeled RNA and DNA for taxa. There was a strong positive correlation between the overall atom percent excess (APE) ^{18}O of rRNA and DNA [ρ(345 simultaneously detected taxa) = 0.859, $P < 0.005$] (Fig. 2). Positive values of APE ^{18}O indicate isotopic enrichment of nucleic acids above natural abundance levels of ^{18}O (approximately 0.2 atom%), which resulted from assimilation of $H_2{}^{18}O$ followed by incorporation of the isotope during nucleic acid synthesis. For all time points, the incorporation of ^{18}O into rRNA and DNA was described by the following equation: APE ^{18}O of DNA = 0.96 × APE ^{18}O of rRNA − 0.001, with the 95% confidence interval (CI) around the slope from 0.90 to 1.02 (model II regression, major axis [MA] analysis), indicating that, for a given taxon, the amount of ^{18}O assimilated into rRNA was not different from the amount of ^{18}O assimilated into DNA. The number of taxa simultaneously detected in DNA and RNA qSIP analyses was 83 on day 1, 126 on day 4, and 136 in day 8 (Table 1). Taxa unique to each library type could not be included in the correlations. There were 106, 52, and 73 unique taxa in the rRNA libraries on days 1, 4, and 8, respectively, and 28, 74, and 51 unique taxa in the DNA libraries on days 1, 4, and 8, respectively (Table 1).

Correlation between labeled RNA and DNA for phyla. APE ^{18}O of rRNA and DNA were also correlated for individual soil phyla (Fig. 3). All correlations were positive, and the APE ^{18}O of the nucleic acids increased over time similarly to the overall correlation shown in Fig. 2. The confidence intervals around the regression slopes overlapped 1 for all phyla except *Acidobacteria*, which had a slope significantly less than 1 (slope = 0.72; 95% CI around the slope, 0.61 to 0.86). Some groups, for instance, *Thaumarchaeota*, *Chloroflexi*, or *Verrucomicrobia*, had lower APE ^{18}O of their nucleic acids than other groups, including *Actinobacteria* or *Proteobacteria* (Fig. 3). The regressions also demonstrated that, within each phylum, taxa varied in ^{18}O compositions of their DNA and rRNA. For example, among the *Actinobacteria*, *Streptomycetaceae* contained highly

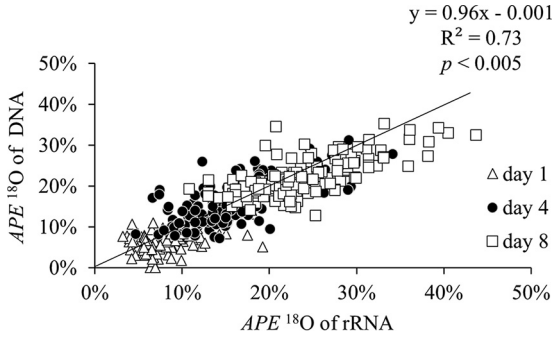

FIG 2 Correlation between atom percent excess (APE) ^{18}O values of DNA and rRNA among soil taxa on days 1, 4, and 8. The equation shown is for the overall regression, which includes all time points. The black line represents a 1:1 ratio, where the APE ^{18}O of DNA and rRNA are equal.

TABLE 1 Number of taxa detected in rRNA or DNA libraries at each time point

Day	No. of taxa present in RNA libraries	No. of taxa present in DNA libraries	No. of taxa unique to RNA libraries	No. of taxa unique to DNA libraries	No. of taxa shared between RNA and DNA libraries	No. of taxa present in RNA or DNA libraries
1	189	111	106	28	83	217
4	178	200	52	74	126	252
8	209	187	73	51	136	260

^{18}O-labeled rRNA (43.72% ± 7.41%) and DNA (32.51% ± 9.44%), whereas *Rubrobacter* had much lower ^{18}O content of rRNA (16.61% ± 4.78%) and DNA (16.34% ± 6.76%) even after 8 days of incubation with $H_2{}^{18}O$. Similar differences were observed among taxa from other phyla (Fig. 3).

Correlation between RNA-to-DNA ratio and nucleic acid labeling. Across taxa, ratios of rRNA to DNA in sequencing libraries were not correlated with their APE ^{18}O of DNA or rRNA [$\rho(335) = 0.025$, $P = 0.649$ for DNA, and $\rho(574) = -0.082$, $P = 0.051$ for rRNA] (Fig. 4A and B), indicating that the ratios were not a sound indicator of metabolic activity. Ratios of relative abundances of RNA to DNA are commonly used to assess microbial activity in nature. Active taxa are characterized by high ratios (or at least ratios of >1), and inactive taxa are characterized low ratios (ratios of <1). We therefore expected that taxa with high ratios would also be highly labeled with ^{18}O due to substantial rRNA synthesis or growth, but this was not observed. The ratios were greater than 1 for only 35 (44%) taxa on day 1, 70 (56%) taxa on day 4, and 57 (44%) taxa on day 8. These proportions, which supposedly indicate active taxa, were considerably lower than proportions of active taxa identified through qSIP.

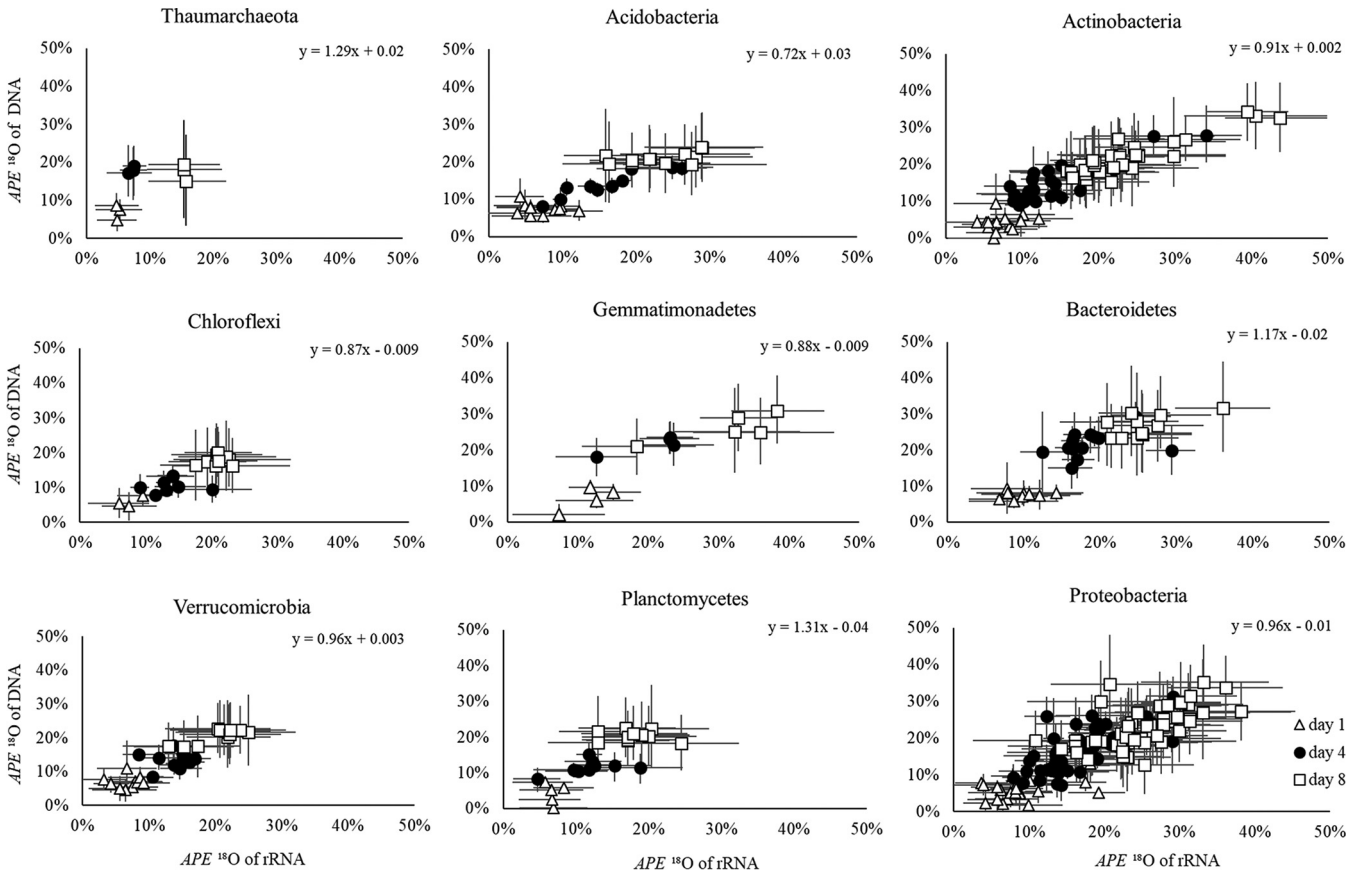

FIG 3 Correlations between APE ^{18}O of DNA and rRNA of major soil phyla at incubation times of 1, 4, and 8 days. For each phylum, the equation represents the overall relationship, comprising all time points, between the two variables. Symbols represent taxa within the phylum, and error bars represent 95% confidence intervals of the APE ^{18}O values.

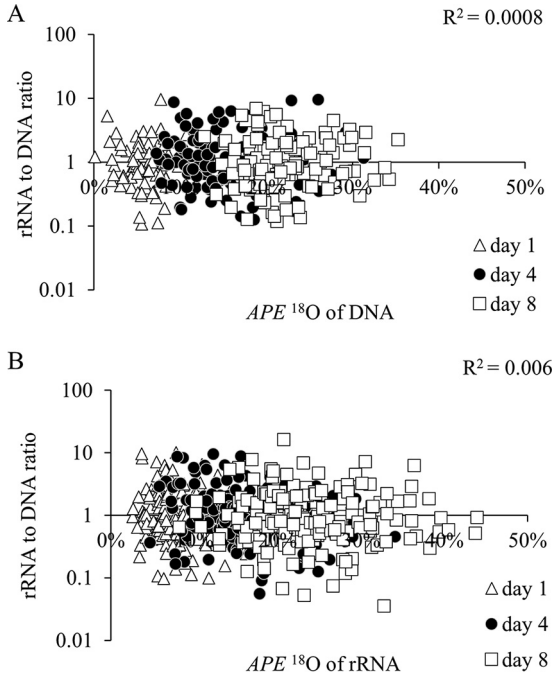

FIG 4 Log scale relationship between taxon-specific ratios of rRNA to DNA in sequencing libraries and the APE ^{18}O of DNA (A) or rRNA (B) at days 1, 4, and 8.

DISCUSSION

We expected bacterial RNA to become significantly more labeled with ^{18}O than DNA because RNA turns over faster than DNA (24–27) and because bacteria can be metabolically active without growing (28, 29). We hypothesized that, compared to RNA, a small fraction of the DNA extracted from soil incubated with $H_2{}^{18}O$ would be new (i.e., labeled with ^{18}O) because DNA is present in dormant or dead cells (30), whereas high RNA concentrations are associated with cellular activity (31–33). Additionally, we hypothesized that all RNA extracted from soil incubated with $H_2{}^{18}O$ would be new and labeled with ^{18}O after 8 days of incubation. Assuming that half of the oxygen atoms in RNA come from water (34), the APE ^{18}O of new RNA should be at least 50%. However, only ~23% of O atoms in RNA were ^{18}O, indicating that approximately half of the ribonucleotides must have been made prior to $H_2{}^{18}O$ addition and persisted throughout the incubation.

Although we found evidence for old rRNA in soil, we also found that most soil taxa were synthesizing new rRNA and were growing. Overall, rates of rRNA and DNA synthesis were statistically indistinguishable from each other in this soil bacterial community, a result that contrasts sharply with the expectation that RNA synthesis could occur without DNA replication or that metabolism in soil is largely independent of microbial growth. A number of labeled taxa were detected only in the rRNA or DNA libraries. This suggested that extremely rare taxa can also be metabolically active and can grow.

The degree to which taxa incorporated ^{18}O from water into their nucleic acids varied, indicating differences in growth and metabolic rates among bacterial populations in soil. Taxa with low APE ^{18}O of rRNA and DNA likely simply had lower growth or metabolic rates than taxa with high APE ^{18}O of nucleic acids. For instance, *Chloroflexi* or *Planctomycetes* express oligotrophic characteristics (35, 36), thus possibly explaining the low APE ^{18}O of their nucleic acids. In contrast, *Bacteroidetes* are known copiotrophs, and *Proteobacteria* comprise many copiotrophic genera (36, 37), which can explain the higher growth and metabolic activity of these groups in a rewetted soil. Alternatively, variation in the APE ^{18}O of nucleic acids among populations may reflect the presence of old rRNA and DNA synthetized prior to $H_2{}^{18}O$ addition. The larger the amount of

older nucleic acids that persist through the incubation, the lower is the measured ^{18}O composition of the nucleic acid extracts.

Unlike ^{13}C substrates, which require *de novo* nucleotide synthesis to become incorporated into nucleic acids (20), ^{18}O from $H_2{}^{18}O$ can become incorporated into new rRNA and new DNA during polymerization of salvaged nucleotides (38, 39). Salvaged nucleotides were previously present in nucleic acids while *de novo* nucleotides were newly synthesized. In an environment with high concentrations of $H_2{}^{18}O$, *de novo* nucleotides will contain more ^{18}O than salvaged nucleotides because ^{18}O may be incorporated into the ribose sugar or the base during nucleotide synthesis. In contrast, nucleic acids generated with salvaged ribonucleotides incorporate ^{18}O isotopes only as branch oxygen atoms in phosphodiester bonds that link nucleotides together (40). Consequently, the isotopic composition of RNA or DNA in soil microorganisms incubated with $H_2{}^{18}O$ is impacted by the source of nucleotides used in their synthesis. Microorganisms that heavily salvage nucleotides will have lower ^{18}O compositions of nucleic acids even during high rRNA or DNA synthesis than microorganisms that newly synthesize nucleotides (12). It is possible that ^{13}C-RNA-SIP studies could fail to capture organisms that salvage ribonucleotides in early stages of an experiment, while $H_2{}^{18}O$-based SIP studies would find these populations to contain RNA with only moderate ^{18}O content.

Within a cell, newly synthetized RNA and DNA molecules should have similar ^{18}O isotopic compositions because both nucleic acids are made from rapidly interchanging nucleotide pools. Ribonucleotide reductase catalyzes the conversion of ribonucleotides to deoxyribonucleotides (41, 42), so that it is likely that both ribonucleotides and deoxyribonucleotides, which can be salvaged or synthetized *de novo*, have the same ^{18}O composition. It is only when a cell synthesizes RNA but not DNA that we would expect to find differences in ^{18}O composition between the nucleic acids.

The isotopic composition of rRNA and DNA can be related quantitatively to microbial activity (rRNA synthesis) and growth (DNA synthesis) if degradation rates of old and new, ^{18}O-enriched, nucleic acids are similar. The degree of ^{18}O incorporation then reflects the level of activity or growth for each taxon. Currently, ratios of relative abundance of rRNA to DNA in sequencing libraries are often used to assess microbial activity in soil (see, for example, references 7 and 8), and in aquatic environments (see, for example, references 43, 44, and 45). Taxa with ratios below 1 are considered inactive (9, 46) because they are represented by fewer rRNA than DNA sequences (47). This may occur when other populations have very high rRNA-to-DNA ratios or when a substantial fraction of DNA, originally derived from taxa with low ratios, is released extracellularly and becomes adsorbed to the soil matrix (30). The extant population could be highly active but misclassified as inactive because of the extracellular (old) DNA. Sequencing DNA does not distinguish between old and new nucleic acids, but it is possible to do so using stable isotope probing.

SIP analysis of bacterial nucleic acids showed that 99.5% of taxa were synthesizing new rRNA while 100% of taxa had grown at the end of the incubation. In contrast, rRNA-to-DNA ratios indicated that only 44% of taxa were active. Many studies documented the presence of inactive taxa in the environment (27, 33, 48) through measuring the relative abundance ratios in sequencing libraries. This study finds that taxa with low rRNA-to-DNA ratios contain newly synthesized nucleic acids, suggesting that relative abundance ratios may not be appropriate for identifying active populations in soil. We find that rRNA-to-DNA ratios underestimated the number of active taxa. Recently, Steven and colleagues (49) have also found that rRNA-to-DNA ratios tend to misclassify active populations as dormant, with a rate of false detection as high as 47% when the simulated communities comprised populations that differed in the rRNA amplification (amount of ribosomes per cell) associated with a specific growth rate (49).

qSIP reflects the uptake of an isotope by entire populations, not by single microorganisms. It is therefore impossible to determine what fractions (if any) of a microbial population are more or less active relative to each other. It is likely that individual cells have different metabolic rates and thus assimilate different amounts of ^{18}O, but our

approach detects only the isotopic enrichment of the whole group. Identifying regions of DNA and RNA capable of differentiating ecotypes and using these as targets for qSIP analysis could provide finer resolution. Still, despite this shortcoming, we were able to draw interesting conclusions about microbial taxa, certainly applicable to important questions in microbial ecology. Adding water to dry soils does not provide an energy or a carbon source, but it still likely stimulates the growth and activity of some populations, some of which otherwise may have remained inactive and nongrowing. The qSIP approach using ^{18}O-labeled water could therefore potentially overestimate growth and activity of some populations. Nevertheless, addition of 200 mg of water certainly had less impact on microbial growth and activity than, for example, addition of an equivalent amount of carbon. Furthermore, we were able to detect significant differences in the APE ^{18}O of nucleic acids among taxa as some populations did not assimilate significant amounts of ^{18}O, indicating that the technique is able to distinguish differential rates of growth and activity among taxa in complex communities. Last, this study focused on only one ecosystem; other ecosystems may show different patterns of microbial activity and growth when explored through qSIP. Further research is therefore needed to explore microbial community dynamics in different ecosystems and to assess whether qSIP consistently provides evidence for high metabolic activity and growth of microbial taxa in the environment.

Conclusion. Contrary to our expectation, we found a strong correlation between the ^{18}O composition of a microbial population's rRNA and DNA with a slope approximating 1. This indicates that it is rare for a cell to synthesize new rRNA without synthesizing DNA. We observed some taxa that had less enrichment in rRNA than DNA, possibly indicating that they contained rRNA prior to $H_2{}^{18}O$ addition. We also found that the nucleotides of most dominant soil taxa were enriched in ^{18}O, suggesting that a large fraction of the soil microbial community appears active rather than dormant. Last, our study suggests that rRNA-to-DNA ratios may underestimate the number of active taxa in natural ecosystems.

MATERIALS AND METHODS

Sample collection. Soil samples were obtained from the A-horizon (~20 cm deep) at three locations ($n = 3$) (34°57′21.879″N, 111°45′14.859″W; 34°57′21.289″N, 111°45′14.189″W; and 34°57′21.591″N, 111°45′14.683″W) near Sedona, AZ, USA, in April 2014. This soil was characterized as sandy loam with an average pH of 6.95 ± 0.42, with a soil moisture content of 4.11% ± 0.24% at the time of sampling. The organic matter content was 13.7% ± 1.7%, and concentrations of NO_3-N and P were 13.5 ± 7.0 and 14.3 ± 0.6 ppm, respectively. The samples were immediately transported at room temperature to Northern Arizona University. Following sieving through a 2-mm-pore-size sieve, the soils were air dried overnight on clean metal trays.

Sample processing. Two grams of soil was incubated with 400 μl of 95 atom% $H_2{}^{18}O$ or with 400 μl of sterile, natural-abundance [^{18}O]water in 15-ml Falcon tubes for 1, 4, or 8 days in the dark. Total RNA and DNA were extracted from 1 gram of soil using an RNA PowerSoil Total RNA isolation kit and DNA elution accessory kit (Mo Bio Laboratories, Carlsbad, CA) immediately at the end of each incubation. All extracts were visualized on 1% agarose E-gels (Life Technologies, Grand Island, NY) and quantified with a Qubit 2.0 Fluorimeter (Life Technologies) and Qubit RNA assay kit or double-stranded DNA (dsDNA) assay kit (Life Technologies). Total RNA was digested with DNA-free DNase treatment removal reagents (Ambion, Life Technologies, Grand Island, NY) and visualized on E-gels, and the concentration of the treated RNA was determined with the Qubit.

Isopycnic ultracentrifugation. Nucleic acids were ultracentrifuged in 3.3 ml OptiSeal polyallomer tubes (Beckman Coulter) using an Optima MAX benchtop ultracentrifuge (Beckman Coulter, Fullerton, CA). For RNA samples, 2.585 ml of 1.99 g/ml cesium trifluoroacetate (CsTFA) solution (GE Healthcare, Piscataway, NJ), 110 μl of deionized formamide (Sigma-Aldrich, St. Louis, MO), and 505 μl of RNase-free water (Life Technologies, Grand Island, NY) were combined. For DNA samples, a cesium chloride (CsCl) solution consisting of 2.7 ml of saturated CsCl and 400 μl of gradient buffer (200 mM Tris, pH 8.0, 200 mM KCl, and 2 mM EDTA) was prepared. For ultracentrifugation of RNA, 3.2 ml of the CsTFA solution and 1 μg of total RNA were added to each tube and centrifuged in a TLN-100 rotor at 60,000 rpm (127,000 × *g*) at 18°C for 72 h. For centrifugation of DNA, 3.1 ml of the CsCl solution and 1 μg of DNA were added to each tube. DNA was centrifuged identically to RNA. CsTFA and CsCl gradients were separated into 100-μl or 150-μl fractions using a manual fraction recovery system (Beckman Coulter) for RNA samples or an automated fractionation system (Brandel, Gaithersburg, MD) for DNA samples. All parts of the manual system were cleaned with ethanol (EtOH), and all parts of the automated Brandel system were thoroughly cleaned with ethanol and air between each sample. Additionally, samples incubated with natural-abundance [^{18}O]water were processed separately from samples incubated with $H_2{}^{18}O$ to minimize potential carryover of the label. The density of each fraction was measured with

a digital refractometer (Reichert Ophthalmic Instruments, Depew, NJ). RNA fractions were purified by adding 200 μl of ice-cold isopropanol and 100 ng of ultrapure glycogen (Affymetrix, Santa Clara, CA) to each 1.5-ml tube. Tubes were vortexed and incubated at −20°C overnight. Purification was done as described by Whiteley et al. (50). DNA fractions were purified by combining two volumes of water, one volume of isopropanol, and 100 ng of ultrapure glycogen (Affymetrix, Santa Clara, CA). Tubes were also vortexed and stored at room temperature overnight. DNA was further purified by centrifuging the fractions for 15 to 30 min at 13,400 × *g*, discarding the supernatant, adding 500 μl of 70% EtOH, centrifuging for 5 min at the same speed, discarding the supernatant again, centrifuging the tubes one more time at 13,400 × *g* for 30 s, and finally removing traces of the supernatant. Pellets were air dried for 10 min and resuspended in 20 μl of 1× Tris-EDTA (TE) buffer. Final concentrations of nucleic acids in each fraction were measured using a Qubit fluorimeter (Life Technologies).

cDNA was obtained from RNA samples, including fractionated and nonfractionated samples, using a Maxima H-Minus First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA) and random pentadecamer primers (Eurofins MWG Operon, Huntsville, AL). Each 20-μl reaction mixture contained 5 μl of RNA template, 5.0 μM primer, 0.5 mM concentrations of the deoxynucleotide triphosphates (dNTPs), 1× reverse transcriptase (RT) buffer, and sterile water.

Sequencing and data processing. Libraries were prepared from fractionated and nonfractionated cDNA or DNA by preamplifying the 16S rRNA gene with the PCR primers 515F (5′-GTGCCAGCMGCCGC GGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) (Eurofins MWG Operon, Huntsville, AL). PCR was carried out in 8-μl reaction mixtures containing 1 μl of template (DNA or cDNA), 4 μl (2×) Phusion Mastermix (water, 10× RedJuice [40% 1 M Tris, pH 8.5, phenol red, 60% glycerol]) with 5× HF buffer (10 mM dNTPs, Phusion HSII polymerase), 2.4 μl nuclease-free water, 0.25 μl (50 M) $MgCl_2$, and 0.06 μl (25.0 mM) of each primer. Preamplification was done in triplicate using a Tetrad PCR thermal cycler (Bio-Rad, Hercules, CA). Cycling conditions started with a 2-min denaturation step at 95°C, followed by 15 cycles of 30 s at 95°C, 30 s at 55°C, 4 min at 60°C, and a final hold at 10°C. The three PCR replicates were pooled, visualized (1% agarose gel), and diluted 10-fold with Tris-Cl (pH 8.0). One microliter of the diluted PCR products was amplified with 515F and 806R indexed primers (51). Carboxyl-modified (0.1%) Sera-Mag Magnetic Speed-beads (Thermo Fisher Scientific, Freemont, CA) in 18% polyethylene glycol (PEG) were used to purify the indexed amplicons, which were then quantified with a Quant-it PicoGreen double-stranded DNA assay kit (Life Technologies). A PerkinElmer automatic liquid handler (Waltham, MA) was used to pool the amplicons, with final concentrations between 2 and 3.5 nM. Pools were subsequently bead purified with the Sera-Mag beads and quantified with a Kapa SYBR Fast qPCR kit (KAPA Biosystems, Inc., Wilmington, MA). DNA and cDNA libraries were sequenced in separate runs on an Illumina MiSeq platform. All runs used the 2-by-150 paired-end read chemistry and followed identical protocols. Sequencing was done at the Environmental Genetics and Genomics Facility (EnGGen) at Northern Arizona University.

An in-house chained workflow (52), which is based on QIIME, version 1.7 (51), was used to process the sequencing data. Information about the workflow can be found at GitHub (https://github.com/alk224/akutils-v1.2). First, sequences were screened and filtered for PhiX contamination (5 to 6%). Next, reads and index files were joined and demultiplexed. The chained workflow was used for chimera filtering, operational taxonomic unit (OTU) picking (swarm algorithm, open reference, 97% similarity), sequence alignment (Greengenes, version 13_5 database) (53), and taxonomy assignment (Ribosomal Data Project classifier) (54). Singletons were removed. OTU tables were filtered and summarized. FastTree (55) was used to generate a phylogenetic tree, and PyNAST (56) was used to produce a filtered sequence alignment file. Normalized and nonnormalized core diversity analyses were run using a rarefaction depth of 5,000 sequences per sample for cDNA and 2,500 sequences per sample for DNA libraries to maximize the number of retained samples in each library type. The resulting L7 (species-level) nonnormalized OTU tables, which comprised 591 cDNA libraries and 521 DNA libraries and contained 665 and 930 taxa, respectively, were used in subsequent analyses.

IRMS analysis. Nonfractionated RNA and DNA samples were quantified with a Quant-iT RiboGreen RNA assay kit (Invitrogen, Carlsbad, CA) or a Quant-iT PicoGreen dsDNA assay kit (Invitrogen) and diluted with UltraPure salmon sperm DNA solution (Invitrogen, Carlsbad, CA) to 200 μg of oxygen. The diluted samples were pipetted into silver capsules (Costech Analytical Technologies, Inc., Valencia, CA), and the capsules were placed in a 96-well tray, set on a heat block at 50°C until water evaporated, closed, weighted, and sent to the Stable Isotope Facility at the University of California, Davis, for isotopic analysis on an Elementar PyroCube (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a Isoprime VisION (Isoprime Ltd., Stockport, United Kingdom, a unit of Elementar Analysensysteme GmbH, Hanau, Germany).

Data preparation for qSIP analysis. For qSIP analysis, we retained sequences of taxa that were present in at least 40% of fractions from one sample or present in at least two of the three replicate field samples. This filtering removed ~470 rare taxa from the cDNA libraries (here referred to as rRNA libraries) and ~750 taxa from the DNA libraries. These taxa were rare and comprised less than 5.5% of the rRNA and less than 10.7% of the DNA libraries. We conserved as many taxa as possible for the analysis for which we had reliable and robust data. A taxon was therefore omitted only if we could not reliably calculate the weighted average density (WAD) for its DNA or RNA. Because reliably calculated WADs could indicate no isotope labeling, there was no inherent bias toward growing organisms in this analysis.

Weighted average densities were calculated for both types of nucleic acids. The sample-specific WAD_s was obtained as follows: the concentration of nucleic acids (RNA or DNA) in each fraction was multiplied by the density of the fraction. The products were summed across density fractions (the

numerator in the equation below) and divided by the sum of the concentrations of nucleic acids across all fractions (the denominator in the equation below):

$$\mathrm{WAD}_s = \frac{\sum_{k=1}^{K} \left(\left[\text{nucleic acid}\right] \times \text{density}\right)}{\sum_{k=1}^{K} \left[\text{nucleic acid}\right]} \quad (1)$$

where WAD_s is the weighted average density of a nucleic acid sample (*s*) (i.e., RNA or DNA), *k* is the fraction of a nucleic acid sample, *K* is the total number of fractions from a nucleic acid sample, [nucleic acid] is the concentration of a nucleic acid in each fraction (either RNA or DNA), and density is the density of a nucleic acid in each fraction.

The WADs of nucleic acids (rRNA or DNA) were also obtained for individual taxa (*t*) (WAD_t). The calculations were identical to those for whole nucleic acid samples described above, except that the concentration of rRNA was multiplied by taxon-specific relative abundance in the rRNA libraries, and the concentration of DNA was multiplied by taxon-specific relative abundance in the DNA libraries.

$$\mathrm{WAD}_t = \frac{\sum_{k=1}^{K} \left(\mathrm{RelAb}_t \times \left[\text{nucleic acid}\right] \times \text{density}\right)}{\sum_{k=1}^{K} \left(\mathrm{RelAb}_t \times \left[\text{nucleic acid}\right]\right)} \quad (2)$$

where WAD_t is the weighted average density of a nucleic acid of a taxon (*t*), *k* is the fraction of a nucleic acid sample, *K* is the total number of fractions from a nucleic acid sample, RelAb_t is the relative abundance of a taxon (*t*) in the sequencing library (i.e., rRNA or DNA library), [nucleic acid] is the concentration of a nucleic acid in each fraction, and density is the density of a nucleic acid in each fraction.

A correction, which was based on the ^{18}O isotopic composition of each nucleic acid type, was applied to WAD_s and WAD_t. The isotopic composition of RNA and DNA was obtained by IRMS analysis (see Table S1 in the supplemental material) and was expressed as atom percent excess ^{18}O (APE ^{18}O) of RNA or DNA using the fractional abundance (*F*) and isotope mixing models. First, the δ^{18}O values were converted to atom percent values as follows: $F = [(\delta/1{,}000 + 1)R_{st}]/[(\delta/1{,}000 + 1)R_{st} + 1]$, where *F* is fractional abundance (atom percent), δ is the sample-specific delta ^{18}O value obtained by IRMS analysis, R_{st} is the ratio of molar abundance of heavy to light isotopes in the Vienna Standard Mean Ocean Water (VSMOW). The ^{18}O/^{16}O ratio is 2005.20 ppm.

Next, the atom percent ^{18}O of each sample was determined using the isotope mixing model:

$$pA + pB = 1 \quad (3)$$

$$\mathrm{At\%}A \times pA + \mathrm{At\%}B \times pB = \mathrm{At\%\ mix} \quad (4)$$

where *pA* is the proportion of RNA or DNA sample, *pB* is the proportion of salmon sperm DNA required for dilution of RNA or DNA samples for IRMS analyses, At%*A* is the atom percent ^{18}O of the RNA or DNA sample, At%*B* is the atom percent ^{18}O of salmon sperm DNA, and At% mix is the atom percent ^{18}O of the mixture of the RNA or DNA sample diluted with salmon sperm DNA.

To obtain the isotopic enrichment of each labeled RNA or DNA sample relative to the natural abundance of the isotope, we calculated its atom percent excess ^{18}O (APE ^{18}O). This was done by subtracting the APE ^{18}O of a nonlabeled sample from the APE ^{18}O of a corresponding labeled sample. The APE ^{18}O of each RNA or DNA sample was then converted to an expected WAD_s using the equations below:

$$\text{Expected WAD}_s = 0.0744 \times \text{APE } ^{18}\text{O of RNA} + 1.7803 \quad (5)$$

$$\text{Expected WAD}_s = 0.0644 \times \text{APE } ^{18}\text{O of DNA} + 1.6946 \quad (6)$$

The first equation was developed using the known molecular weight of the rRNA molecule at different ^{18}O isotopic compositions (natural abundance and 100% ^{18}O content), verified by empirical measurements of the density of RNA at natural abundance of ^{18}O. Details about the second equation, for DNA, were provided by Hungate et al. (23). All calculated WAD_s values, here referred to as measured WAD_s, were adjusted using the expected WAD_s:

$$\text{Correction factor} = \text{expected WAD}_s - \text{measured WAD}_s \quad (7)$$

$$\text{Corrected WAD}_s = \text{measured WAD}_s + \text{correction factor} \quad (8)$$

The WADs were adjusted individually for each sample and corresponding fractions. The statistical software package R (57) was used to determine all taxon-specific shifts in nucleic acid density as well as all taxon-specific atom percent excess ^{18}O (APE ^{18}O) values of rRNA and DNA as described by Hungate et al. (23). The code was adapted for RNA analysis, which included setting the GC content to 50%, molecular weight to 339.49, and number of oxygen atoms to 7. All computer code can be found at https://bitbucket.org/QuantitativeSIP/qsip_repo.

Ratio calculations. Relative rRNA-to-DNA ratios were obtained by dividing a taxon-specific relative abundance in nonfractionated 16S rRNA libraries by its relative abundance in the nonfractionated DNA (i.e., 16S rRNA gene-based) libraries.

Statistical analyses. Analysis of APE ^{18}O of rRNA and DNA was performed in R (57). Other statistical analyses were performed in SPSS (58), with statistical significance set to an alpha (α) of 0.05. The Shapiro-Wilk test, Levene's test, and Mauchly's test were used to test for assumption of normality, homogeneity of variances, and sphericity, respectively. Additionally, we used a *t* test to determine if shifts in nucleic acid densities significantly differed from zero. A two-way mixed analysis of variance (ANOVA)

was used to test for a significant interaction between density shift or APE ^{18}O of nucleic acids of phyla and time. Last, we used a Spearman's rank order correlation test and a model II regression analysis in R to assess the strength and significance of the correlation between taxon-specific APE ^{18}O of rRNA and DNA and between the ratio of relative abundance in rRNA to DNA libraries and the APE ^{18}O of DNA or rRNA of taxa. Correlations and regressions were generated only with taxa that were present in both the DNA and RNA libraries. These taxa were referred to as the shared taxa. All other analyses included taxa that were present in only one library type, either DNA or RNA. These taxa were referred to as the unique taxa.

Accession number(s). All sequences have been deposited in the NCBI Sequence Read Archive (SRA) under BioSample accession numbers SAMN07960499 to SAMN07960874, SAMN07965143 to SAMN07965605, and SAMN07968111 to SAMN07968486. Data can directly be accessed at https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP123236.

Data availability. Workflow used for sequencing data processing is available at https://github.com/alk224/akutils-v1.2. R code used for calculating taxon-specific APE ^{18}O of nucleic acids is available at https://bitbucket.org/QuantitativeSIP/qsip_repo.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02441-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We declare that we have no conflicts of interest.

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