Quantitative Stable Isotope Probing with $H_2{}^{18}O$ reveals that most bacterial taxa in soil synthesize new ribosomal RNA.

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Abstract

Most soil taxa are thought to be dormant, or inactive, yet the extent to which they synthetize new rRNA is poorly understood. We analyzed ^{18}O-composition of RNA extracted from soil incubated with $H_2{}^{18}O$ and used quantitative stable isotope probing to characterize rRNA synthesis among microbial taxa. RNA was not fully labeled with ^{18}O, peaking at a mean of 23.6 ± 6.8 atom percent excess (*APE*) ^{18}O after 8 days of incubation, suggesting some ribonucleotides in soil were more than 8 days old. Microbial taxa varied in the degree to which they incorporated ^{18}O into their rRNA over time. Additionally, there was no correlation between the *APE* ^{18}O of bacterial rRNA and their rRNA to DNA ratios, suggesting that the ratios were not appropriate to measure ribonucleotide synthesis. Our study indicates that, on average, 94% of soil taxa produced new rRNA and therefore were metabolically active.

Keywords: RNA-quantitative Stable Isotope Probing, *APE* ^{18}O of rRNA, rRNA density shift, rRNA: DNA ratio, microbial activity, soil prokaryotes/microbes

Most bacteria in soil are thought to be dormant (Babiuk and Paul, 1970; Mayfield, 1977; Lundgren, 1981; Alvarez *et al.*, 1998; Sherr *et al.*, 1999; Luna *et al.*, 2002; Khomutova *et al.*, 2004; Wang *et al.*, 2014), while only a small active fraction controls ecosystem processes (Mengoni *et al.*, 2005; Aanderud *et al.*, 2015). Active bacterial cells have higher metabolic rates than dormant cells leading to higher protein and rRNA synthesis. Growth is not required for metabolic activity (Blazewicz *et al.,* 2013). In contrast, during dormancy bacteria transition into a state of very low metabolic activity (Jones and Lennon, 2010; Bär *et al.,* 2016). RNA concentrations are expected to decrease as most metabolic processes are halted, while DNA concentrations may remain relatively stable because dormant cells do not die. Accordingly, the relative abundances of ribosomal RNA (rRNA) and DNA extracted from environmental samples are commonly used as indicators of microbial metabolic activity (DeLong *et al.,* 1989; Poulsen *et al.*, 1993; Muttray and Mohn, 1999; Kamke *et al*., 2010). (Baldrian *et al*., 2012; Brettar *et al*., 2012; Foesel *et al*., 2014). However, rRNA to DNA ratios among taxa in microbial communities vary substantially, often unrelated to metabolic activity, suggesting RNA alone may not be a reliable indicator of active populations (Blazewicz *et al*., 2013).

Stable isotope probing (SIP) can assess microbial activity independent of rRNA to DNA ratios. SIP with ^{18}O labeled water is especially powerful for assessing growth and activity of microbial communities because water is a universal substrate for nucleic acid synthesis (Schwartz, 2007). In this study, we incubated 2 grams of soil with 400 µl of sterile 95 atom % $H_2{}^{18}O$ or with 400 µl of sterile, natural abundance ^{18}O-water, for 1, 4 and 8 days (N=18), and extracted total RNA following each incubation. Newly synthetized ^{18}O-containing RNA has higher buoyant density than old RNA, and can be separated through isopycnic ultracentrifugation on a cesium trifluoroacetate (CsTFA) density gradient. We fractionated the ultracentrifuged

RNA, purified the fractions and sequenced a fragment of the 16S rRNA gene from complementary DNA (cDNA) as described in Document S1. Sequencing data were analyzed using a QIIME 1.7 based (Caporaso *et al.* 2010a) chained workflow (Krohn, 2016) https://github.com/alk224/akutils-v1.2. To assess rRNA synthesis of individual taxa, we measured the incorporation of ^{18}O into rRNA by calculating the taxon specific shift in rRNA density and by converting it to atom percent excess (*APE*) ^{18}O using a freely available R code https://bitbucket.org/QuantitativeSIP/qsip_repo. *APE* ^{18}O indicated the excess of ^{18}O atoms in microbial rRNA relative to natural abundance of the isotope, and was used to estimate rRNA synthesis rate. We were interested in assessing temporal patterns and variation in rRNA synthesis rates among soil microbial populations using qSIP, and in comparing our results to RNA to DNA ratios.

All taxa contained ^{18}O-labeled rRNA after 4 days of incubation with $H_2{}^{18}O$. Densities of their non-labeled rRNA varied slightly around the mean (1.7808 ± 0.0011 g/ml), whereas densities of their labeled rRNA substantially differed on each day (Figure 1). This pattern likely reflects taxonomic variation in the rate of metabolic activity (Campbell and Kirchman, 2012; Männistö *et al.*, 2016) or differential reliance among taxa on *de novo* ribonucleotide synthesis (Ebbole and Zalkin, 1987; Berg *et al*., 2002) versus ribonucleotide salvaging (Koch, 1970; Callaghan *et al*., 2005; Deutscher, 2006). If ribonucleotides are synthesized *de novo*, ^{18}O will be assimilated throughout the ribonucleotide, in addition to its assimilation into phosphodiester bonds (Richards and Boyer, 1966; Chaney *et al.*, 1972), which will increase ^{18}O composition of rRNA more than recycling alone.

Entirely dormant soil taxa were absent in our study, which challenges the widely accepted idea that dormancy is widespread among microbial taxa in the environment (Stevenson, 1978; Cole, 1999; Luna *et al*., 2002; Jones and Lennon, 2010; Lennon and Jones, 2011). We would observe many populations with non-labeled rRNA (i.e. containing ^{18}O only at the natural abundance level), if dormancy was a common survival strategy of soil bacteria. However, our observations do not preclude that members of a microbial population were not synthesizing new rRNA. Our observation of a weak correlation between rRNA to DNA ratio and *APE* ^{18}O of rRNA of taxa (Spearman's rank-order correlation, $\rho(574) = -0.082$, $p = 0.051$, Figure 2), suggests that the ratio may be a poor proxy for metabolic activity despite its positive correlation with microbial growth rate in pure cultures (Kjeldgaard and Kurland, 1963; Rosset *et al.*, 1966; Kerkhof and Ward, 1993; Muttray and Mohn, 1999; Muttray *et al.*, 2001; Worden and Binder, 2003). We expected that taxa with high rRNA to DNA ratios would have highly labeled rRNA (Rozsak and Colwell 1987) but this was not observed.

We observed a significant temporal increase in ^{18}O content for total RNA ($F_{2,4}$ = 15.404, p = 0.013, Figure S1 and S2) and for RNA of phyla (Figure S3) because RNA is thought to turn over rapidly (Wellington *et al*., 2003; Lillis *et al*., 2009), with rates ranging from 20% per day (Ostle *et al*., 2003) to 25% per hour (Yuan and Shen 1975). We expected that most RNA would be labeled with ^{18}O shortly after $H_2{}^{18}O$ addition, but modeled rRNA turnover varied between 9 to 18% per day, which was slower than previously reported. The labeled RNA had approximately 23% of its oxygen atoms replaced with ^{18}O, indicating that either some of the rRNA that was formed prior to $H_2{}^{18}O$ addition remained intact, that the rRNA was newly synthesized but partly made with ribonucleotides that were more than 8 days old, or that newly synthesized ribonucleotides obtain part of their oxygen from organic substrates. Assuming that 50% of oxygen atoms came from $H_2{}^{18}O$ and 50% come from organic substrates, (Chaney *et al.*, 1972), the isotopic composition of rRNA would be 50% at the fast turnover rate and ~42% at the slower turnover rate and should have increased only minimally over time. The increase in ^{18}O composition of RNA over time suggested that increasingly more ribonucleotides were synthesized and that the turnover rate of ribonucleotides in soil is on the order of ~23% per week

Our knowledge of ribosome biosynthesis and degradation derives mostly from pure culture experiments but it appears that rRNA dynamics are different among bacteria in soil. $H_2{}^{18}O$-RNA qSIP provides a different perspective of microbial activity than rRNA to DNA ratios because qSIP characterizes and quantifies taxa that synthetize new nucleic acids and is therefore not subject to biases introduced by nucleic acids from dead or inactive populations.

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Conflict of Interest

The authors declare no conflict of interest.

Supplemental Information

Supplementary information is available at *The ISME Journal's* website.

Accession numbers. All sequences have been deposited in NCBI SRA (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.

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313 **Figure Legends**

314 Figure 1: Shifts in total RNA density after soils were incubated with $H_2{}^{18}O$ for 1, 4 or 8 days.
315 Bars show means ± standard deviation. The RNA density shifts significantly increased over time
316 as shown ($p = 0.013$).

317

318 Figure 2: Densities of rRNA extracted from soil incubated with $H_2{}^{18}O$ (♦) or $H_2{}^{16}O$ (♦) at three
319 time points. Panel A: rRNA densities of taxa detected on day 1, panels B and C: rRNA densities
320 of taxa detected on day 4 and 8 respectively. Taxa are ranked by the same alphabetical order in
321 each panel. Symbols represent means ± standard deviations.

322

323 Figure 3: Atom percent excess (*APE*) ^{18}O of rRNA of major soil phyla on three time points (open
324 bars: day 1, black bars: day 4, gray bars: day 8). Significant temporal increase in APE ^{18}O of
325 rRNA is indicated by *. Bars show means ± standard deviation.

326

327 Figure 4: Relationship between rRNA to rDNA ratios and atom percent excess (APE) ^{18}O of
328 rRNA among soil taxa on three time points: open symbols – day 1, black symbols – day 4 and
329 gray symbols – day 8.

330

331 Figure S1: Density curves of total RNA extracted from soil incubated with $H_2{}^{18}O$ (●) (n = 3) or
332 $H_2{}^{16}O$ (○) (n = 3) at three time points (panel A: day 1, panel B: day 4, panel C: day 8) expressed
333 as a percentage of the whole RNA sample.

334