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Identification of growing bacteria during litter decomposition in freshwater through $H_2^{18}O$ quantitative stable isotope probing

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Summary

Identification of microorganisms that facilitate the cycling of nutrients in freshwater is paramount to understanding how these ecosystems function. Here, we identify growing aquatic bacteria using $H_2^{18}O$ quantitative stable isotope probing. During 8 day incubations in 97 atom % $H_2^{18}O$, 54% of the taxa grew. The most abundant phyla among growing taxa were *Proteobacteria* (45%), *Bacteroidetes* (30%) and *Firmicutes* (10%). Taxa differed in isotopic enrichment, reflecting variation in DNA replication of bacterial populations. At the class level, the highest atom fraction excess was observed for OPB41 and δ-*Proteobacteria*. There was no linear relationship between ^{18}O incorporation and abundance of taxa. δ-*Proteobacteria* and OPB41 were not abundant, yet the DNA of both taxa was highly enriched in ^{18}O. *Bacteriodetes*, in contrast, were abundant but not highly enriched. Our study shows that a large proportion of the bacterial taxa found on decomposing leaf litter grew slowly, and several low abundance taxa were highly enriched. These findings indicating that rare organisms may be important for the decomposition of leaf litter in streams, and that quantitative stable isotope probing with $H_2^{18}O$ can be used to advance our understanding of microorganisms in freshwater by identifying species that are growing in complex communities.

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Introduction

Microorganisms cycle nutrients in freshwater ecosystems, degrading organic compounds and serving as a food source for heterotrophs (Newton *et al*., 2011). Identifying these organisms, and understanding which biogeochemical processes they facilitate, is paramount to understanding how freshwater ecosystems function. Advances in molecular biological techniques, including sequencing of 16S rRNA genes (Zwart *et al*., 2002; Tamames *et al*., 2010), and shotgun sequencing (Martinez-Garcia *et al*., 2012), have elucidated biofilm and planktonic community composition in rivers and lakes. However, many of these studies are unable to distinguish between microorganisms that are growing and those that are not. Comparative analyses of bacterial DNA and RNA indicate that up to 40% of organisms in lakes may be dormant (Jones and Lennon, 2010), and that high abundance does not necessarily indicate high activity in ocean surface water (Hunt *et al*., 2012). Community analyses based on total DNA differ from those based on RNA, suggesting that not all organisms in stream microbial communities are actively growing (Besemer *et al*., 2012).

DNA-based stable isotope probing (SIP) can identify growing microorganisms in environmental samples (Radajewski *et al*., 2000; Morris *et al*, 2002; Schwartz *et al*. 2014). In SIP, organisms are exposed to an isotopically labeled substrate. Organisms that assimilate the substrate incorporate the isotope into their DNA, increasing its buoyant density. The heavy DNA can be separated from non-labeled DNA through isopycnic centrifugation and analysed, using 16S rDNA amplicon sequencing for example, to identify growing organisms in an environmental sample.

SIP studies of freshwater bacteria, to date, involve spiking sediments with a labeled carbon compound (e.g., Radajewski *et al*., 2000), showing relationships between taxonomic identity and metabolic capabilities, like methanotrophy (Radajewski *et al*., 2000; Morris *et al*., 2002; Sharp *et al*., 2014), or acetate assimilation (Osaka *et al*., 2006). These studies focus on simple

substrates, such as methane or acetate, because they can identify microorganisms with certain metabolic capabilities. However, the activity of microorganisms responsible for decomposing complex substrates, like leaf litter, is less well understood. Studies using simple carbon compounds are extremely useful in answering substrate specific questions, but cannot be used to measure population growth of all active taxa during leaf litter degradation. As decaying leaves are important drivers of the freshwater C cycle (Fisher and Likens, 1973), understanding the biological controls on their decomposition will enable a better understanding of ecosystem nutrient flow. Although small plants such as wheat have been labeled sufficiently for SIP experiments (Bernard *et al*., 2007), sufficiently labeling tree leaves is presently not technically feasible (Tu *et al*., 2013). An alternative approach is to conduct SIP experiments with ^{18}O labeled water as a substrate (Schwartz, 2007; 2009).

SIP experiments with ^{18}O differentiate growing from non-dividing taxa, because all organisms incorporate oxygen atoms from water into nucleic acids. Oxygen incorporation into DNA occurs during nucleic acid synthesis as part of cell division (Richards and Boyer, 1966), thus quantification of ^{18}O incorporation from $H_2^{18}O$ into DNA can be used as a proxy for microbial growth (Blazewicz and Schwartz, 2011). By exposing a microbial community to $H_2^{18}O$, the DNA of all growing populations will become labeled and may be analysed to characterize the growing microbial community. One important advantage of using $H_2^{18}O$ is that investigations of actively growing communities are not limited to amendments with simple substrates, but that growth on any substrate, including decaying plant litter, can be studied. Recent advances in SIP have enabled quantifying the growth of individual microbial taxa based on the degree to which they are labeled with ^{18}O (Hungate *et al*., 2015). In this study, we show that quantitative stable isotope probing (qSIP; Hungate *et al*., 2015) can be applied to aquatic ecosystems and identify actively growing aquatic bacteria during leaf litter decomposition.

Results and discussion

SIP in aquatic environments with $H_2^{18}O$ was not previously explored because it may be difficult to achieve sufficient levels of nucleic acid labeling in samples containing large amounts of non-enriched water. To overcome this problem, we *replaced* the non-enriched environmental water with 97 atom % $H_2^{18}O$; an experimental approach that may also be applicable to saturated soil or sediment samples. This allowed the DNA of the bacterial community growing on decomposing litter in an aqueous sample to become sufficiently enriched with ^{18}O derived from $H_2^{18}O$ to separate it from the non-labeled DNA ($t = -4.29$, $p = 0.01$) (Fig. 1a).

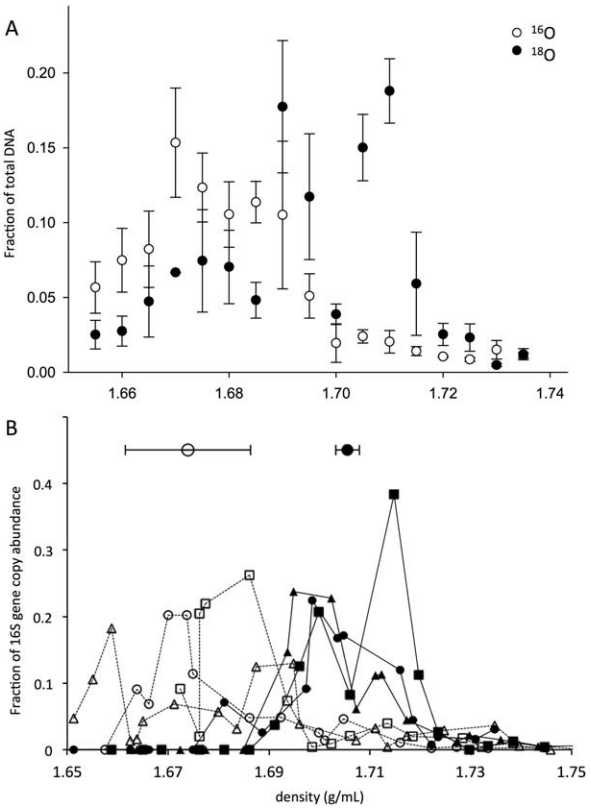

Fig. 1. A. Proportion of total DNA as a function of density in the $H_2^{16}O$ (○) and $H_2^{18}O$ (●) treatments. Each point represents the fractions within a 0.005 g density increment. Errors bars represent standard errors of the DNA concentration in the fractions. The density of DNA in the $H_2^{18}O$ treatments was higher than the density of DNA from the $H_2^{16}O$ controls ($t = -4.29$, $p = 0.01$).
B. Fraction of 16S rRNA genes as a function of density for *Desulfovibrio mexicanus* in the $H_2^{16}O$ (○) and $H_2^{18}O$ (●) treatments. The different shapes represent individual replicates from the same SIP spin. Average density and standard error of the labeled and unlabeled DNA are shown.

We used bootstrapping to determine a 90% CI for the atom fraction excess isotope composition of each bacterial taxon (*sensu* Hungate *et al*., 2015). Sequencing of 16S rRNA gene amplicons generated 6.4 million reads that clustered into 834 operational taxonomic units (OTUs) and grouped into 236 single species at the 97% sequence similarity level. Taxa were considered to be growing and included into our analysis if the confidence interval for atom fraction excess did not overlap zero. Using this criterion, out of 236 taxa, 128 grew and incorporated sufficient ^{18}O isotopic tracer into their DNA (Fig. 2a). *Proteobacteria* accounted for more than 45% of the 16S rRNA genes in the growing community, followed by *Bacteroidetes* (30%) and *Firmicutes* (10%). Other studies have shown that *Proteobacteria* were the most abundant phylum of freshwater bacteria associated with leaf

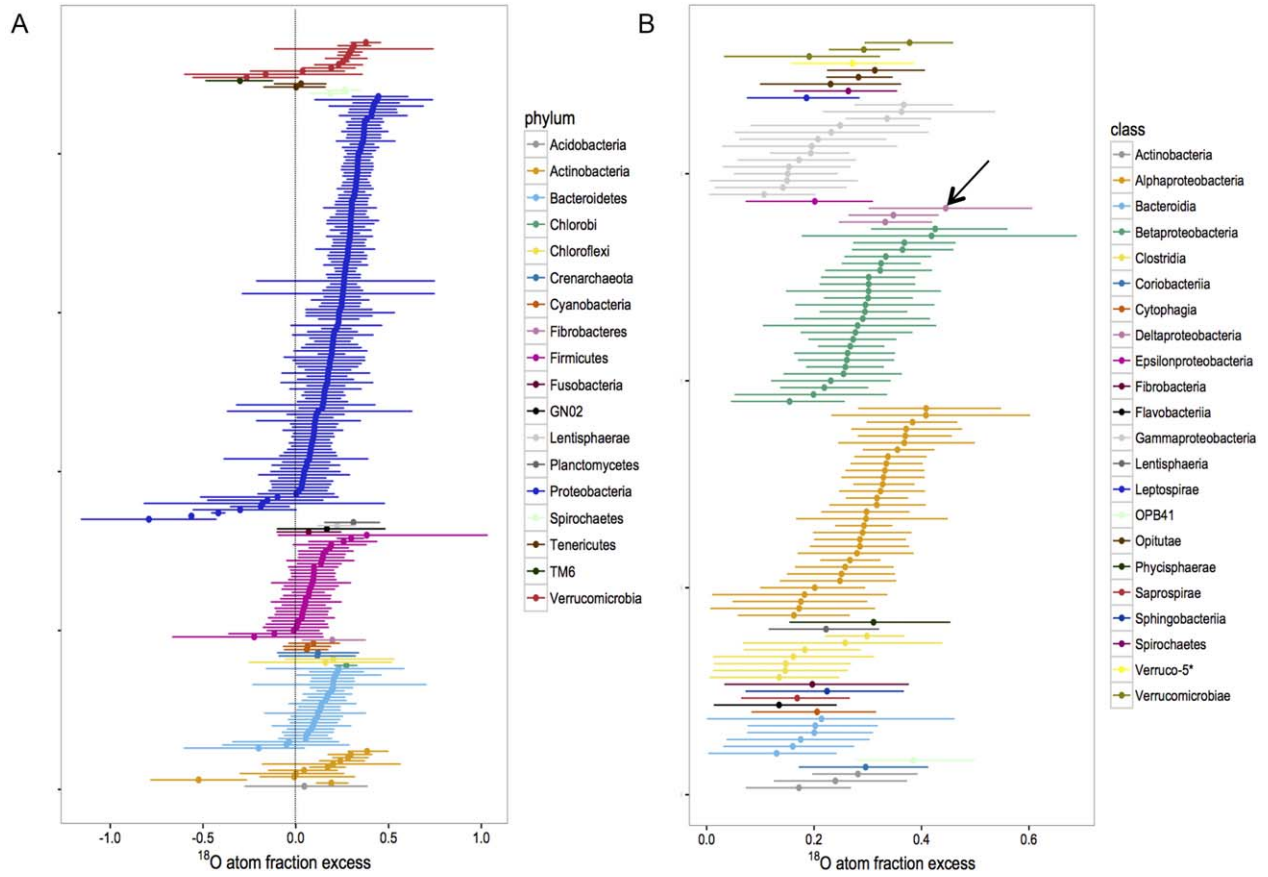

Fig. 2. Median isotopic enrichment of individual taxa with 90% confidence intervals.
A. This shows the taxa colored by phylum. Taxa with confidence intervals that did not overlap zero incorporated significant amounts of ^{18}O and were considered growing. Taxa with confidence intervals that did overlap zero were excluded from further analysis.
B. This shows the growing taxa only, coloured by class. The organisms with the highest atom fraction excess, Desulfovibrio mexicanus, is indicated by an arrow. The asterisk represents subdivision 5 of the Verrucomicrobia.

litter (McNamara and Leff, 2004; Fazi *et al*., 2005), and in planktonic and biofilm communities in streams (Besemer *et al*., 2012), where they break down recalcitrant carbon compounds found in leaf litter, or utilize degradation products and plant leachate like acetate and phenol (Hutalle-Schmelzer, 2009).

Quantitative SIP allowed identification of individual taxa with large ^{18}O enrichment, a proxy for growth (Fig. 2). Of the growing organisms, the greatest enrichment at the class level were observed for the unidentified Actinomycete OPB41 and δ-*Proteobacteria* (excess ^{18}O enrichment of 0.384 and 0.375 respectively). *Flavobacteriia*, *Saprospirae* and *Bacteroidia* had the lowest excess ^{18}O enrichment with 0.135, 0.169 and 0.180 respectively (Fig. 2b). At the species level, *Desulfovibrio mexicanus*, a sulfate-reducing bacterium in the phylum δ-*Proteobacteria* (Hernandez-Eugenio *et al*., 2000) had a large increase in weighted average density (Fig. 1a) and the highest atom fraction excess (0.445, Fig. 2b). Culture studies using *Escherichia coli* showed that 33% of the oxygen incorporated into newly synthesized DNA was derived from water (Hungate *et al*., 2015). The increased isotopic labeling we observed in this study may be possible in organisms that obtain most of their O atoms from water and not from food, for instance organisms that feed on lipids (Funke *et al*., 1997), organic pollutants (Woods *et al*., 2011) or labeled substrate.

DNA is primarily synthesized during cell division; therefore the DNA of a newly divided cell will be highly enriched in ^{18}O relative to a dormant or non-dividing cell. It is possible that some populations rely on recycling or scavenging deoxyribonucleotides (Finkel and Kolter, 2001) while others make them *de novo*, thereby skewing a direct relationship between ^{18}O enrichment and new cells formed. However, we do expect that organisms that synthesize deoxyribonucleotides incorporate more ^{18}O per base pair DNA than nucleotide recycling/scavenging taxa. To relate enrichment levels directly to changes in cell densities also requires assumptions about the DNA extraction efficiency and the number of 16S rRNA genes in a bacterial genome (Klappenbach *et al*., 2000).

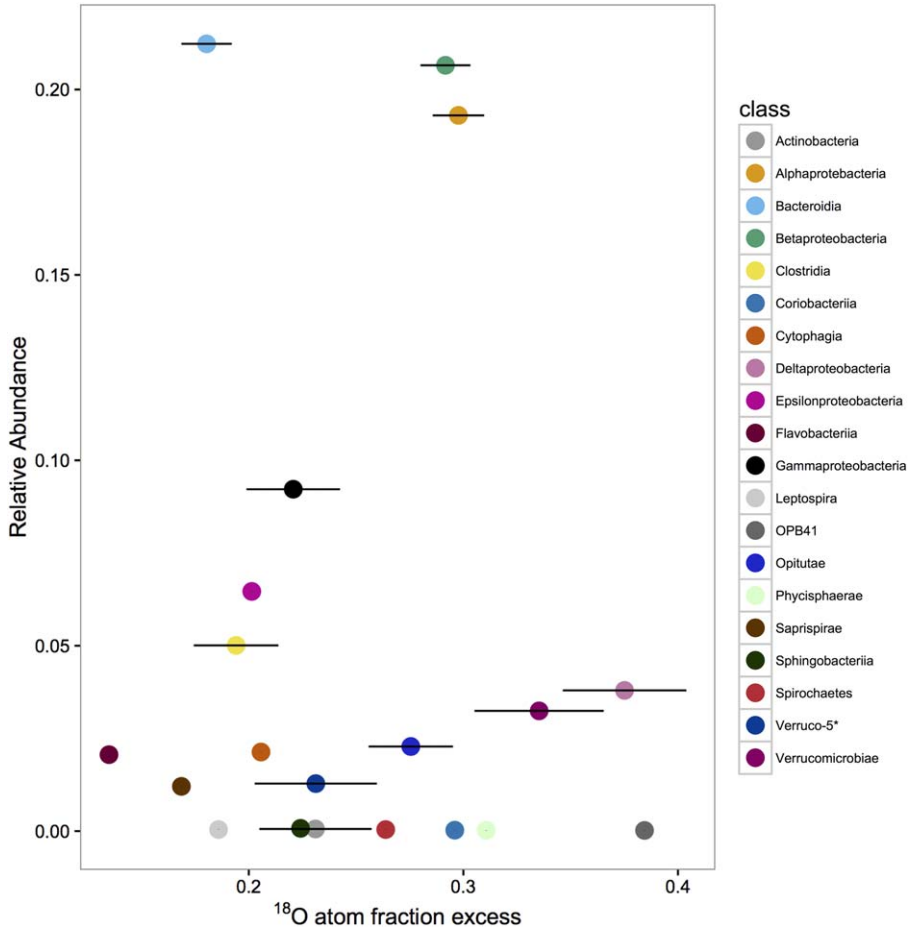

Fig. 3. Relationship between relative abundance and ^{18}O isotopic enrichment of DNA, an indicator of growth rate. Error bars represent standard errors. The asterisk represents subdivision 5 of the Verrucomicrobia.

We did not observe a linear relationship between the enrichment of a taxon and the abundance of that taxon, measured as the product of the fraction of the total sequences characterized and the number of bacterial 16S rRNA gene copies (Fig. 3). The DNA of the most abundant taxon at the class level, the *Bacteroidia* (21%), was not highly enriched, while other abundant taxa, such as the α- and β-*Proteobacteria*, had marginally above average ^{18}O enrichment values. Other taxa, for example *Cytophagia* and γ-*Proteobacteria*, were less enriched (0.206 and 0.221 excess ^{18}O enrichment) and less abundant (2.1% and 9% respectively). The δ-*Proteobacteria* were not very abundant at 3.8% of the community, and the unidentified Actinomycetes OPB41 were quite rare (0.018%), yet both taxa were highly enriched. Jones and Lennon (2010) found an inverse relationship between taxon abundance and taxon activity in lake microbial communities, and low abundant members of the 'rare biosphere' have been described as ecologically significant, possibly providing a seed bank (Fierer and Lennon, 2011; Lennon and Jones, 2011) that can influence microbial diversity and community composition in response to seasonal (Gilbert *et al.*, 2012), or environmental changes (Sogin *et al.*, 2006).

Field observations of stream bacteria on leaves show that species richness increases through time (Harrop *et al.*, 2009), and our study provides a snapshot of these bacterial population dynamics during litter decomposition. Abundant taxa with low ^{18}O incorporation into their genomes could have been active during early stages of leaf litter decomposition, when the labeled water had not been added yet. Copiotrophic organisms like the *Bacteroidetes* (Fierer *et al.*, 2007), for instance, may be able to colonize freshly added Populus fremontii leaves early and grow quickly. The *Actinobacteria*, major contributors to the decomposition of litter in freshwater environments (e.g., Newton *et al.*, 2011; Besemer *et al.*, 2012), only comprised a very small portion of the community in our study, possibly because they are involved during later stages of decomposition (Snajdr *et al.*, 2010). We sampled one point in time to demonstrate the feasibility and utility of this technique. Multiple sampling dates could

reveal if there is a shift through time in the growing bacterial assemblage.

This study demonstrates isotopic labeling of freshwater bacteria with ^{18}O from ^{18}O-water through a quantitative approach that quantifies nucleic acid enrichment of individual taxa. There are multiple applications for this technique, including: (1) using qSIP to compare activity levels of other microbial species, such as fungi, during leaf litter decomposition, (2) combining qSIP with field surveys to characterize microbial growth rates under different environmental conditions, (3) combining qSIP with manipulative experiments to determine how microbial growth of different taxonomic groups is affected (e.g., Morrissey *et al.*, 2016).

Experimental procedures

Incubations

Senescent leaves from 10 *Populus fremontii* trees were collected using bridal veil netting at Beaver Creek, AZ, and air-dried in the fall of 2013. Stream water and sediment were also collected from Beaver Creek in January of 2015. Freshwater microcosm were set up in 15mL Falcon tubes in triplicate as follows: 2 g of sediment, 9 mL of stream water and 50 *Populus fremontii* leaf discs (0.13 g – 0.15 g) were incubated at room temperature in a shaker at 160 r.p.m. for 10 days to allow bacteria to colonize the leaves (Paul *et al.*, 1977). The lids of the Falcon tubes were left slightly unscrewed to allow air exchange. After 10 days the Falcon tubes were centrifuged at 2250 $\times$ *g* for 10 min, and the supernatant was removed. The stream water was then replaced by 1mL of $H_2^{18}O$ (treatment) or sterile nanopure water (control), and the microcosms were incubated for an additional 8 days. DNA was extracted from leaf discs using a MoBio Powersoil Powerlyzer DNA extraction kit following the manufacturer's instructions with the addition of an initial 5 min incubation at 72°C after the bead solution was added.

Centrifugation and fractionation

To separate DNA by density, 1000 ng of DNA was added to 2.51 mL of cesium chloride (1.9 g mL^{-1}), 0.49 mL of gradient buffer (200mM trisaminomethane (Tris), pH 8, 200 mM potassium chloride, 2 mM Ethylenediaminetetraacetic acid (EDTA) in an OptiSeal® ultracentrifuge tube (Beckman Coulter, Brea, CA). The tubes were spun at 127,000 $\times$ g at 18°C for 72 h using a Beckman TLN-100 rotor in an Optima™ MAX ultracentrifuge (Beckman Coulter, Brea, CA).

Approximately 3 mL of the cesium chloride gradient was separated into fifteen 200 μL fractions. The density of each fraction was measured using a Reichert AR 200 handheld digital refractometer (Reichert Technologies, Buffalo, NY). The DNA present in each fraction was precipitated with isopropanol, cleaned with ethanol and resuspended in 50μL of Tris-EDTA buffer. The DNA concentrations were determined with the Qubit (Invitrogen, Carlsbad, CA) HS dsDNA assay. A density curve with the proportion of total DNA as a function of density in the $H_2^{16}O$ and $H_2^{18}O$ treatments was graphed to determine if the ^{18}O isotope was incorporated into DNA. We used a Student's *t*-test to compare the weighted average densities of the ^{16}O and ^{18}O treatments.

qPCR

Quantitative PCR was used to measure bacterial 16S rRNA gene abundance. Standard curves were generated using 10-fold serial dilutions of 16S rRNA gene amplicons which were prepared using soil DNA and primers 515F(5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) (Caporaso *et al.*, 2012) containing Illumina sequence adaptors P5 (5′- AATGATACGGCGACCACCGA) and P7 (5′-CAAGCAGAAGACGGCATACGA) at the 5′ end of each primer, respectively, to prevent decreased primer efficiency due to amplicon degradation (Dhanasekaran *et al.*, 2010). The 8 μL qPCR reactions contained 0.2 mM of each primer, 0.01 U μL^{-1} Phusion HotStart II Polymerase (Thermo Fisher Scientific, Waltham MA), 1X Phusion HF buffer (Thermo Fisher Scientific), 3.0 mM $MgCl_2$, 6% glycerol and 200 μM dNTPs. The assay was performed on an Eppendorf Master-cycler ep Realplex system (Eppendorf, Westbury NY), using a program of 95°C for 1 min followed by 44 cycles of 95°C for 30 s, 64.5°C for 30 s and 72°C for 1 min. Gel electrophoresis was performed to confirm the size of the amplified products. Bacterial gene copy numbers were calculated using a regression equation for each assay relating the cycle threshold (Ct) value to the known number of copies in the standards. All qPCR reactions were run in triplicate.

Sequencing

Two PCR steps were used to process the samples (Berry *et al.*, 2011). Each sample was first amplified using primers 515F and 806R. This was done in triplicate 8 μL PCR reactions containing 1 mM of each primer, 0.01 U μL^{-1} Phusion HotStart II Polymerase (Thermo Fisher Scientific, Waltham MA), 1X Phusion HF buffer (Thermo Fisher Scientific), 3.0 mM $MgCl_2$, 6% glycerol and 200 μM dNTPs. PCR conditions were 95°C for 2 min; 15 cycles of 95°C for 30 s, 55°C for 30 s and 60°C for 4 min. Initial PCR reaction products were checked on a 1% agarose gel, pooled, 10-fold diluted, and used as template in the subsequent tailing reaction with region-specific primers that included the Illumina

flowcell adapter sequences and a 12 nucleotide Golay barcode (15 cycles identical to initial amplification conditions). Products of the tailing reaction were purified with carboxylated SeraMag Speed Beads (Sigma-Aldrich, St. Louis, MO) at a 1:1 v/v ratio as described in Rohland and Reich (2012), and quantified by Picogreen fluorescence. Equal quantities of the reaction products were then pooled; the library was bead-purified once again (1:1 ratio), quantified by qPCR using the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA), and loaded at 11 pM (including a 30% PhiX control) onto an Illumina MiSeq instrument (San Diego, CA) using 2 x 150 paired-end read chemistry. The flowcell produced 973 $\pm$ 114 K clusters per mm^2, returning over 14 million clusters passing filter.

Data analysis

The DNA sequences were analysed with the software package Quantitative Insights into Microbial Ecology v 1.7 (QIIME) (Caporaso *et al*., 2010a). For quality filtering, the default score was changed from 25 to 30. Open reference OTU picking was performed at 97% identity using uclust (Edgar, 2010). The most abundant sequence for each OTU was aligned with PyNAST (Caporaso *et al*., 2010b) against the Greengenes v13_5 database (DeSantis *et al*., 2006). Taxonomy was assigned using Ribosomal Data Project classifier (Wang *et al*., 2007) and a phylogenetic tree was built. Any OTUs that accounted for less than 0.05% of the total sequences were discarded (Bokulich *et al*., 2013). The bacterial libraries were rarefied so that sequencing efforts did not affect diversity comparisons. The QIIME L7 species level OTU table was used for subsequent analyses. The L7 table groups together OTUs that are phylogenetically similar without accounting for intraspecific genetic diversity in the V4 region (Clayton *et al*., 1995; Acinas *et al*., 2004). OTUs that have been assigned the same species may represent different strains, but may also be sequencing errors that artificially inflate diversity (Kunin *et al*., 2010). All sequences have been deposited at MG-RAST, project ID 239736.

Isotopic composition of individual taxa after exposure to $H_2^{18}O$ was calculated as described by Hungate and colleagues (2015). Briefly, for each fraction the total number of 16S rRNA gene copies was measured using qPCR, and the proportion of 16S rRNA gene copies for each bacterial taxon within that fraction was determined by multiplying the total number of 16S rRNA gene copies by the relative abundances obtained from sequencing. The density for each bacterial taxon was computed as a weighted average, summing the densities across all fractions times the total number of 16S rRNA gene copies in that fraction expressed as a proportion of the total 16S rRNA gene copies. The increase in weighted density relative to the weighted density of the unlabeled treatments was calculated. We determined the GC content of the DNA for each taxon, based on its density, using the relationship of GC content and density based on a pure culture study by Hungate and colleagues (2015). The GC content was then used to calculate the molecular weights and the corresponding values of ^{18}O isotope composition for each taxon (see Supporting Information for calculations). Bootstrap resampling (with replacement, 1000 iterations) of replicates within each treatment was used to estimate taxon-specific 90% confidence intervals for the change in density and the corresponding value of ^{18}O atom fraction excess isotope composition. We measured the abundances of a taxon at the class level as a fraction of the total sequences characterized times the number of bacterial 16S rRNA gene copies. All calculations were performed in R (R Development Core Team, 2011).

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