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Stable isotope probing with ^{18}O-water to investigate microbial growth and death in environmental samples

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Growth and mortality of microorganisms have been characterized through DNA stable isotope probing (SIP) with ^{18}O-water in soils from a range of ecosystems. Conventional SIP has been improved by sequencing a marker gene in all fractions retrieved from an ultracentrifuge tube to produce taxon density curves, which allow estimating the atom percent isotope composition of each microbial taxon's genome. Very recent advances in SIP with ^{18}O-water include expansion of the technique to aquatic samples, investigations of microbial turnover in soil, and the first use of ^{18}O-water in RNA-SIP studies.

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Introduction

Life requires water as a solvent for biomolecules, but also as a substrate in many biological reactions, so that oxygen atoms from water are incorporated into biomolecules. For instance, water is added to fumarate to produce malate in the TCA cycle and is used to produce β-hydroxyacyl-coA during β-oxidation of fatty acids [1]. Intermediates of the TCA cycle can serve as precursors of purine or pyrimidine, the nitrogenous bases of nucleic acids. Through these and other pathways, when organisms grow in the presence of ^{18}O-labeled water, ^{18}O atoms are incorporated into their biomolecules, including their nucleic acids [2]. Sufficient ^{18}O atoms can be incorporated into newly-synthesized DNA to allow separation from non-labeled DNA along a cesium chloride density gradient, a technique known as stable isotope probing (SIP) [3–6]. Because all organisms use water as a substrate in enzymatic reactions, ^{18}O-water is considered a universal substrate in SIP experiments. DNA is primarily synthesized when cells divide, and therefore labeled DNA is only present in populations that have grown while exposed to ^{18}O-water [7]. There are important benefits of ^{18}O-SIP, such as characterization of growing populations in processes where ^{13}C-substrates are not available or are too expensive, or where catabolism is mostly co-metabolic, or when the carbon substrate assimilated by microorganisms is unknown. In this article, we review the applications of SIP with ^{18}O-water, highlighting recent efforts to quantify microbial growth, death and turnover. We conclude by identifying a set of research priorities for SIP-based studies of intact microbial assemblages (Table 1).

Labeling microbial DNA with ^{18}O-water

SIP experiments require microorganisms to be exposed to enough ^{18}O-water to label nucleic acids sufficiently for separation from nonlabeled nucleic acids by density along a cesium chloride gradient. The degree of separation is a function of the isotopic composition of the added water and of the duration of exposure. For *Escherichia coli* cells grown in culture, 23.75 atom % ^{18}O-water for 24 hours was required to separate labeled from non-labeled DNA, but superior separation was achieved with even higher atom % values of ^{18}O [4]. Given the complexity and increased variability of intact microbial communities, initial SIP experiments in soils focused on rewetting of dry soils with 97 atom% ^{18}O-water. Native soil water can be replaced by drying a soil sample and rewetting with ^{18}O-water, arguably simulating a drying–rewetting cycle. Simply allowing soil to air dry at room temperature and adding 200 μL/g soil of ^{18}O-water results in sufficiently labeled soil water to enable SIP. Conceivably, SIP with ^{18}O-water could also be applied to wet soils — or even sediments — by replacing a large fraction of the native water with 97 atom% ^{18}O-water. For example, by flushing a soil sample multiple times with ^{18}O-water, one could increase the ^{18}O content of soil water without causing a drying–rewetting cycle. With these approaches, SIP with ^{18}O-water could be applied to a wide variety of hydrologic conditions that occur in soils. It is also possible to incubate decaying organic matter in ^{18}O-water, simulating organic matter decomposition in freshwater habitats. For instance, growth of microbial populations on leaves that decompose rapidly can be compared to population dynamics on more recalcitrant leaves.

Table 1

Strengths and limitations of SIP with ^{18}O-water

Strengths	Limitations
Identifies all growing microbial populations	Does not directly link microbial growth to a specific substrate
Does not require previous knowledge of microbial substrate source.	^{18}O-water is expensive so that experiments are limited to small samples
Allows identification of microbial populations that grow on complex substrates that are difficult to label with heavier isotopes.	^{18}O-water can evaporate, limiting incubation times
Allows study of impact of environmental factors, such as pH or temperature, on microbial growth	Many environmental samples contain large amounts of non-enriched water, which needs to be replaced with labeled water through drying or flushing.
Allows study of microbial mortality	

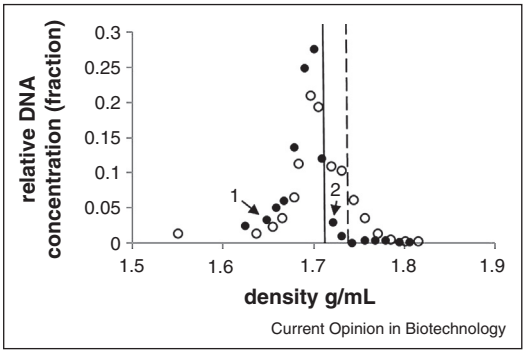
Soils are incubated with ^{18}O-water for varying lengths of time, depending on the growth characteristics of the microbial community. Labeled DNA may not be detected if a soil sample is incubated too briefly. Using conventional methods of SIP detection, Blazewicz *et al.* [8] found that labeled DNA could be detected 24 hours after ^{18}O-water was added but not 3 hours after wet up. The longer a sample is incubated with ^{18}O-water, the greater the potential for label turnover, as labeled oxygen atoms incorporated into biomolecules of growing microorganisms are consumed by other growing populations, creating a scenario where some organisms are incorporating ^{18}O from both water and organic substrates. This recycling of ^{18}O atoms may explain why, after longer incubations, three DNA bands can appear following ultracentrifugation [4]. In contrast to SIP studies in temperate soils, longer incubations may be needed to detect growth in polar ecosystems. In the McMurdo Dry Valleys, soils were incubated with ^{18}O-water in the field for 30 days because bacterial growth rates at freezing temperatures were very low [9].

After incubation, nucleic acids are extracted from soil and separated along a cesium chloride gradient through isopycnic centrifugation. DNA will distribute along the gradient according to the guanine–cytosine (GC) content of the DNA, which affects its buoyant density in cesium chloride [10,11]. The DNA will also distribute along the gradient according to the extent of labeling with ^{18}O atoms. After centrifugation, each sample is divided into density fractions, and the density and DNA concentration of each fraction are determined [12]. Earlier SIP studies used fluorescent stains to visualize DNA bands [4–6,13], but gradient fractionation is now preferred because it provides a more precise comparison of labeled and non-labeled samples. The DNA concentration is graphed versus the density in each fraction to generate a community density graph (Figure 1). The DNA in fractions from soil incubated with ^{18}O-water generally have higher densities than DNA in fractions from soil incubated with natural abundance water. This shift in density indicates that the nucleic acids have become labeled with the heavy isotope. Sequencing the fractions where the shift is most apparent then reveals the taxa that are more abundant in the denser fractions of the labeled treatments compared to the unlabeled treatments; these taxa have assimilated the label and, in the case of SIP with ^{18}O-water, have grown during the incubation.

Recent ^{18}O-water SIP studies

Unlike SIP experiments with other substrates, which focus on assimilation of specific substrates by microbial populations, SIP with ^{18}O-water examines growth of all populations in environmental samples. Recent studies have used this method to characterize population dynamics of microbial communities in a variety of soil environments. For instance, SIP with ^{18}O-water was used to detect microbial growth in soils from the McMurdo Dry Valleys in Antarctica, a polar desert with very low organic matter content [9]. Incubation of soil samples with ^{18}O-water in the field showed that bacteria were indeed growing in these soils and were not simply recent

Figure 1



Community density profiles of a control sample (●) to which non-labeled water was added and a treatment sample (○) to which ^{18}O-water was added. The solid and dashed lines represent density thresholds that could separate the labeled heavy fractions from the non-labeled light fractions. The control samples marked with '1' or '2' are used in the text to explain how these subjective thresholds will bias the results against organisms with low GC genomes or in favor of organisms with high GC genomes. DNA was quantified through fluorimetry.

immigrants, blown in by the wind from nearby, more productive, habitats.

In experiments with non-labeled toluene and ^{18}O-water, toluene degrading bacteria were identified through SIP [13]. Microorganisms obtain O atoms from both water and nutrients. Because toluene does not contain O atoms, the DNA of organisms that primarily utilize toluene as a C source will have higher ^{18}O content than DNA of growing microorganisms that utilize more oxidized C sources. The higher ^{18}O composition of toluene degraders was therefore apparent as a 3rd, more dense, band of DNA along the cesium chloride gradient [13]. While toluene degraders could also be characterized via ^{13}C-SIP, ^{18}O-SIP offers a broader view: ^{18}O-SIP detects growth agnostic of carbon sources, so enables the study of organisms that respond indirectly to the altered resource environment caused by toluene addition. The use of ^{18}O-water could be applied to any perturbation that alters growth, whether to the addition of a complex mixture of organic cocktails for which sufficient ^{13}C labeling is prohibitive, or to changes in environmental conditions independent of C sources (e.g. temperature, pH, or other nutrients). The graphical abstract in this manuscript depicts the work flow of a hypothetical ^{18}O-SIP experiment in which the effect of temperature on microbial growth is investigated. Not shown are the non-labeled control incubations which are required to calculate how much a taxon's genome is labeled during a SIP experiment. By comparing the degree to which microbial populations become labeled among the two different incubation regimes, it is feasible to identify temperature responsive taxa.

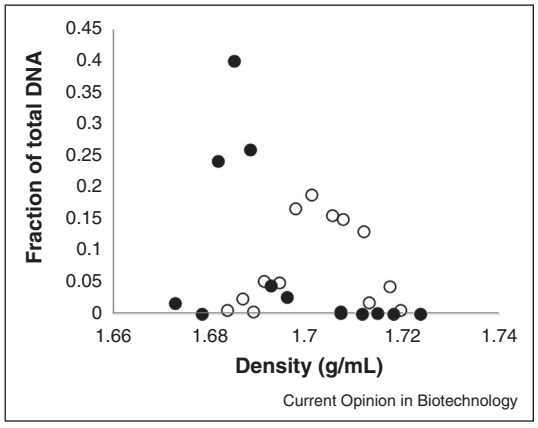
DNA SIP with ^{18}O-water, coupled with quantitative PCR (qPCR), was used to quantify growth of soil bacteria and fungi following the rewetting of a seasonally-dried California annual grassland [8]. Growth was linear between one and seven days after rewetting for both bacteria and fungi, and a dynamic assemblage of growing and dying organisms resulted in relatively stable total population size, despite dramatic microbial turnover following the sudden change in soil moisture. In a study of soil priming, where native soil carbon is respired in response to fresh carbon inputs, SIP with ^{18}O-water revealed changes in the diversity and composition of growing bacterial assemblages, elucidating the microbial dynamics underlying priming in soil [14]. This example captures the use of ^{18}O-water to characterize indirect effects, important in many ecological communities [15], because the growth responses of some organisms were not directly tied to use of the added, ^{13}C-labeled substrate. SIP with ^{18}O-water was also used to identify fast-growing soil bacteria associated with pulses of trace gases from different ecosystems following rewetting events [16]. A large fraction of bacteria that responded to rewetting were below detection limits in the dry soils, indicating that these rare taxa may play an important role in microbial communities, and hence in ecosystem function. Thus, in a variety of environments, ^{18}O-water SIP has been successfully used to detect microbial growth, revealing specific taxa associated with important ecosystem dynamics.

Quantifying microbial growth with ^{18}O-water SIP

In any SIP experiment, the mere presence of a taxon's 16S rRNA gene in a high-density fraction does not confirm that the population has incorporated the heavier isotope during the incubation. It is critical to show that the taxon's genome has a higher concentration of the heavier isotope in the labeled treatment than in the control. In other words, it is essential to isolate the incorporation of the isotope tracer into a microbial population from natural variation in genomic GC content. In the community density graph shown in Figure 1, a researcher may elect to use the density threshold indicated by the solid line. At densities above this threshold, DNA concentrations in the labeled treatment clearly are much higher than those in the control treatment. Alternatively, it would also be reasonable to select the density threshold described by the dashed line in Figure 1 after which DNA concentrations in the control samples approach the detection limit. Regardless of which threshold is used, subsequent analyses will likely include errors where either a taxon will be identified as growing when it has not, or identified as not growing when in fact it has. Taxa with genomes of low GC content, like those abundant in the fraction labeled with a '1' in Figure 1, will occur in less dense fractions in the cesium chloride gradient (i.e. toward the left of the density graph). These taxa will need to strongly assimilate the heavy isotope to become sufficiently labeled to cross the threshold into the heavy fraction. Consequently, it is likely these taxa will be characterized as non-growing even when they have produced new DNA and assimilated substantial quantities of the heavier isotope. In contrast, taxa with genomes of higher GC content, such as taxa abundant in the fraction labeled with a '2' in Figure 1, will have to assimilate far less heavy isotope to be identified as growing. Thus, there is potential bias in this classic SIP approach toward identifying high-GC organisms as labeled and low-GC organisms as non-labeled. This bias applies to any SIP experiment interpreted this way, whether it uses ^{18}O, ^{13}C, or other tracers. Past studies account for this potential bias by using analyses of all fractions through DNA fingerprinting [17,18] or pyrosequencing [19] to identify specific microbial populations that become labeled. These studies quantified label incorporation by measuring density shifts of a taxon's genome during SIP experiments.

By dividing density fractions qualitatively into only labeled or non-labeled DNA, all quantitative information about heavy isotope assimilation is lost. Soil microbial

Figure 2



A taxon density curve for the genus Arthrobacter is calculated by multiplying the proportion of the taxon's sequences in a sequencing library with the total number of 16S rRNA genes in a SIP fraction as measured with quantitative PCR. Fractions from a sample incubated with non-labeled water are represented by filled symbols while fractions from a sample incubated with ^{18}O-water are labeled with open symbols.

taxa presumably grow at different rates and therefore assimilate different quantities of ^{18}O during SIP experiments. An alternative to identifying each taxon as either growing or non-growing is to transform the typical, categorical response, to a continuous one that directly relates to growth. Quantitative SIP (qSIP) is an experimental approach that avoids the pitfalls of assigning heavy and light fractions and retains the quantitative information of the extent to which each taxon is labeled with ^{18}O [20]. From this quantitative assessment of labeling, it is feasible to estimate a growth rate for each taxon. Instead of splitting the density gradient into heavy and light fractions, in qSIP each fraction is sequenced separately for a target gene, like the 16S rRNA gene. Taxon density curves (Figure 2) are then produced by multiplying the proportion of a taxon's 16S rRNA sequence by the total number of 16S rRNA gene copies as determined through qPCR. As in standard SIP, samples without a heavy isotope are compared to those with an added heavy isotope. The shift between these curves provides a basis for quantifying the change in density for each individual taxon caused by isotope incorporation. Because the density shift is calculated relative to the taxon's density measured without the added isotope tracer, this approach quantifies the degree of labeling for all taxa, regardless of GC content [20]. This sets the stage for exploring taxonomic variation in a fundamental ecological trait: growth rate.

Using ^{18}O-water SIP to study microbial death and turnover

There are two different strategies to study microbial mortality through SIP with ^{18}O-water [8,21]. First, one can estimate the abundance of a taxon in non-labeled DNA at the beginning and end of an incubation. Populations that have declined in abundance during the incubation include individuals that have died, and their DNA will have been degraded. This approach measures mortality in non-growing microbial populations. It is important to confirm that the decline in abundance is real, and that the genome of the taxon has not simply increased in density because it was labeled and shifted to another part of the density profile, causing an apparent decline in the lower density region. This is another advantage of the qSIP approach, because the abundance of a given taxon across all density regions is assessed. This approach also assumes that all non-labeled DNA is part of viable non-growing cells, while it is likely that a fraction of non-labeled DNA is extracellular or present in non-viable but intact cells. A second approach is to first label DNA of growing cells with ^{18}O by incubating a soil sample in ^{18}O-water, after which the labeled water can be repeatedly flushed out of the soil with unlabeled water. Subsequently, the decline in ^{18}O content of the DNA, which represents mortality of newly grown cells, can be measured through isotope ratio mass spectrometry (IRMS) analysis, or through qSIP on a taxon-specific basis. This approach would miss mortality of intact cells that have died but with DNA that has not yet been degraded. Interestingly, the few SIP studies that have considered mortality show large turnover rates of the microbial community, with as much as half of microorganisms dying within a week [8].

RNA-SIP with ^{18}O-water to characterize RNA dynamics in microbial populations

Studies of RNA-SIP with ^{18}O-water have been conducted recently. Angel and Conrad [22] studied the activation cascade in soil crusts by characterizing organisms that produced new ribosomal RNA after soil crusts were rewetted with ^{18}O-water. SIP analysis of an incubation time series showed that not all populations produce new rRNA instantaneously, indicating that there is an ordered progression of reactivation of microbial populations as soil crusts are rewetted. Rettedal and Brozel [23] compared growing bacterial populations to bacterial populations that made new ribosomes as revealed by DNA SIP or rRNA SIP with ^{18}O-water. They incubated soil samples for 38 days and found that both DNA and rRNA SIP identified similar communities, indicating that most dominant OTUs in the total nucleic acid extracts contained active members. Thirty-eight days is a long time to expose a microbial community to a labeled tracer, and it is likely that the label was recycled as microbial biomass turned over during the incubation. RNA SIP can offer a different perspective of microbial activity than DNA SIP because it only requires organisms to utilize the substrate in assimilatory processes, and because RNA labeling occurs more rapidly, allowing for shorter incubation periods. The use of non-labeled rRNA analysis as an activity measure has been challenged by other researchers [24]. It

is likely that RNA SIP with ^{18}O-water will change our perspectives of RNA dynamics and microbial activity in soil.

Directions for future SIP research

Over the last decade, ^{18}O-water SIP has enabled a deeper understanding of the roles individual taxa play in the ecological dynamics of intact microbial assemblages. Recent, novel applications are laying a quantitative foundation for measuring the growth, death, turnover, and activity of microbial populations, and linking those rates to ecosystem processes. These developments present a variety of fruitful avenues for advancing ^{18}O-water SIP research, including, firstly, developing new techniques to improve the resolution for detecting labeling by heavy isotopes, secondly, investigating the relationships between quantitative isotopic labeling and growth or mortality rates for individual taxa, and thirdly, exploring the ecological implications of congruent and contradictory findings for RNA-SIP versus DNA-SIP.

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