Research protocol on stable isotope probing to elucidate the role of soil microorganisms in nutrient cycling and soil quality

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Introduction

Stable isotope probing is a technique that is used to identify the microorganisms in environmental samples that use a particular growth substrate. The method relies on the incorporation of a substrate that is highly enriched in a stable isotope, such as $^{13}$C, $^{15}$N or $^{18}$O and allows identification of active microorganisms by the selective recovery and analysis of isotope-enriched cellular components. DNA and rRNA are the most informative taxonomic biomarkers and $^{13}$C-labelled molecules can be purified from non-labeled nucleic acid by density-gradient centrifugation. The future holds great promise for SIP, particularly when combined with other emerging technologies such as metagenomics.

The report is divided into five separate sections: 1. An introduction to centrifugation and stable isotope probing, 2. Experimental design of stable isotope probing experiments, 3. A detailed experimental protocol for stable isotope probing experiments 4. A discussion of possible applications of stable isotope probing to nutrient cycling and soil quality is given, and 5. A list of published SIP studies is given in the Reference section. Stable Isotope Probing sometimes refers to the study of incorporation of stable isotopes into any biomolecule including RNA, DNA, phospholipid fatty acids, and proteins. This report is limited to the application of stable isotope probing to the study of DNA and RNA.
VI. **Introduction to centrifugation and stable isotope probing.**

Centrifugation has played an extremely important role in biological research. It has been used to separate cell types, organelles within a cell and a variety of biological macromolecules. Among the most famous researchers was the Swedish chemist Theodore Svedberg who received the Nobel Prize in 1926. The Svedberg unit, for sedimentation rate is named after him, and he was the first to show that pure proteins could be separated from each other through ultra-centrifugation.

There are several types of centrifugation including differential centrifugation, rate zonal centrifugation and isopycnic centrifugation. In differential centrifugation particles of various densities are sedimented at different rates thereby separating them. In rate zonal centrifugation the sample is not homogenously distributed throughout the centrifuge tube but rather is layered on top of the density gradient. Differences in sedimentation rates are still exploited to separate particles from each other. It is less likely that particles will cross contaminate in Rate zonal centrifugation than in differential centrifugation. In isopycnic centrifugation the particles do not sediment. Instead the density gradient is designed in such a way that the particles will migrate within the density gradient to the location where the particles density is equal to the density of the gradient. Isopycnic centrifugation is used in SIP so that both labeled and non-labeled DNA hang in the middle of the tube.

In isopycnic centrifugation a variety of gradient media are used to separate biomolecules. In SIP cesium chloride (CsCl) is most commonly used to separate labeled DNA from non-labeled DNA while cesium trifluoroacetate (CsTFA) is used to separate labeled RNA from non-labeled RNA. However in the 1960’s and 1970’s other media, such as sodium iodide, were also employed to separate labeled DNA molecules.
In SIP a highly labeled substrate, as much as 99 atom% but rarely less than 50 atom%, is added to an environmental sample. Microorganisms in the sample will assimilate the substrate thereby incorporating heavy isotopes from the substrate into their nucleic acids. These nucleic acids will have higher buoyant densities than those of organisms that did not assimilate the labeled substrate, either because they were not active or because they assimilated other non-labeled substrates present in the environmental sample. The difference in buoyant density is subsequently exploited to separate the labeled nucleic acids from non-labeled nucleic acids along a density gradient of CsCl or CsTFA, generated in an ultracentrifuge. The labeled nucleic acids are subsequently recovered from the tube and can be analyzed in a variety of ways including quantitative PCR or next generation sequencing such as pyrosequencing or sequencing on the illumina platform.
Stable Isotope Probing has its origins in the 1950’s and 60’s when a large number of researchers focused on how DNA in cells replicated. The classic studies of Meselson and Stahl (1958) showed a new strand DNA formed by using an old strand as a template also known as semi-conservative replication. In these experiments DNA was labeled with $^{15}$N, the heavier stable isotope of nitrogen. The researchers were able to analyze the DNA in their experiments after one generation through isopycnic centrifugation; 2 distinct DNA bands formed in their ultra-centrifuge tubes, one containing $^{14}$N atoms while the other had incorporated $^{15}$N atoms. When labeled DNA from subsequent generations where included in the comparisons a third DNA band appeared. The only reasonable interpretation of these results was that, after one generation, DNA with one $^{14}$N strand and one $^{15}$N strand formed and DNA in which both strands contained $^{15}$N appeared in subsequent generations thereby establishing the principle of semiconservative DNA replication. During this important period of innovation in the field of centrifugation, and biology in general, studies were limited to pure cultures and labeled substrates were not administered to environmental samples. These were experiments in the fields if cell and molecular biology and not microbial ecology.

The first report of a Stable Isotope Probing study was published in the journal Nature in 2000 and was authored by Radajewski, Ineson, Parkeh and J.C. Murrell. The report was entitled “stable-isotope probing as a tool in microbial ecology” and involved the use of $^{13}$C-methane to label DNA of methane oxidizers in environmental samples. Subsequently numerous SIP studies appeared not only using $^{13}$C labeled substrates but also compounds labeled with $^{15}$N or $^{18}$O. Theoretically any atom present in nucleic acids including, Carbon, Nitrogen, Oxygen, Hydrogen and Phosphorus, can be used in SIP to label nucleic acids. Phosphorus has only radioactive isotopes and to date no SIP
Overview of Stable Isotope Probing. (Taken from Marc G. Dumont & J. Colin Murrell *Nature Reviews Microbiology* 3, 499-504 (June 2005) doi:10.1038/nrmicro1162)
studies have employed this element. Furthermore the element is relatively heavy compared to Carbon, Nitrogen and Oxygen and a one or two neutron difference between the isotopes would only increase buoyant density by a small fraction. Deuterium can be used in SIP. DNA from E.coli grown in D2O based media can be separated along a CsCl gradient from DNA extracted from E.coli grown in media with H2O, but to date no environmental studies employing deuterium have been published. Hydrogen atoms on molecules may exchange more readily with water molecules, which may complicate interpretation of SIP experiments that use deuterium. Most of the SIP studies to date are in the field of bioremediation and focus on the degradation of pollutants in environmental samples.

II Stable Isotope Probing experimental design

Before designing a SIP experiment, it is important that the researcher is aware of several limitations imposed on SIP experiments and she must adjust her experimental design to accommodate these limitations.

Cost of isotopes limits size of experiments

SIP experiments require that nucleic acids are heavily labeled, in excess of 50 atom%, and therefore the substrate added to an environmental sample must also be heavily labeled. Highly labeled substrates are expensive. 95 atom % H218O for instance can cost upwards of $200 a mL. Also it is important that the label of the added substrate is not diluted with non-labeled substrates already present in the soil. If for instance, the object is to study degradation of labeled litter in soil the carbon already present in soil may dilute the added labeled litter. A small amount of labeled litter in a large amount of non-labeled soil will not produce nucleic acids sufficiently labeled for
SIP experiments. Consequently SIP experiments are often conducted on small quantities of soil (1-5 grams) and often relatively large quantities of substrate are added to produce enough labeled nucleic acids. As discussed below these experimental limitations may lead to fertilizer artifacts.

**Most SIP experiments to date involve a single labeled substrate**

To perform SIP a highly labeled substrate must be added to an environmental sample. Usually this substrate is purchased from a chemical company and is delivered to the environmental sample in pure form. For some substrates, including $^{13}$CO$_2$, $^{13}$CH$_4$ and H$_2^{18}$O, that is representative of environmental conditions. But in other studies, especially those focusing on pollution degradation or carbon metabolisms in soil, the addition of a single substrate does not conform to realistic circumstances. For instance, it is highly unlikely that pure benzene or glucose is ever added to an environmental sample. More likely, benzene pollutes soil in combination with other compounds including toluene, ethylbenzene and xylene. Thus SIP experiments in which multiple labeled substrates are added to a soil remain rare. Some scientists would argue that adding multiple substrates at once defeats the main purpose of SIP experiments which is to identify microbial populations that assimilate specific compounds. However, in studies of soil quality or nutrient cycling we are especially keen to understand the role of microorganisms in degrading complex cocktails of substrates such as litter or organic nitrogen.

**Type of stable isotope impacts separation of labeled DNA from non-labeled DNA**

Stable isotopes are more dense because the nuclei of the atom incorporates extra neutrons. An extra neutron can shift the density of a small element substantially but
may have less impact, on a relative basis, on larger elements. Furthermore the concentrations of different elements in nucleic acids are not equivalent. Thus while $^{15}\text{N}$ is on a percentage basis, relative to $^{14}\text{N}$, only slightly less heavy than $^{13}\text{C}$ is to $^{12}\text{C}$, there is substantially less N in nucleic acids than C, so that it is more difficult to label nucleic acids sufficiently for SIP with $^{15}\text{N}$ than $^{13}\text{C}$.  

$^{13}\text{Carbon}$

There is a long and rich history of the use of carbon isotopes in biology dating back to the classical experiments on photosynthesis of Kammen, Benson and Calvin employing $^{14}\text{C}$. The first experiments that demonstrated stable isotope probing was feasible in environmental samples used $^{13}\text{C}$ labeled methane. The methane was the sole carbon source for methane oxidizers and as the communities were incubated with $^{13}\text{C}$ methane substantial quantities of $^{13}\text{C}$ (DNA) formed. Methane was a particularly good choice for the first SIP experiments because it is relatively easy to remove methane from soil samples so that in an incubation with newly added $^{13}\text{C}$-methane almost all the added methane is labeled with $^{13}\text{C}$. In studies of litter decomposition this is not feasible because soil organic matter cannot be removed from soil without severely disturbing the sample. Therefore the added labeled litter will be diluted by the $^{12}\text{C}$ in the soil organic matter. Microorganisms in soil are often limited by the availability of labile carbon. Therefore SIP studies that employ labile carbon are particularly susceptible to fertilization artifacts. Most SIP experiments to date involved $^{13}\text{C}$ substrates, partly because there is great interest in metabolism of organic compounds in our environment, but also because $^{13}\text{C}$ labeled substrates are readily available. Specifically, most studies to date have focused on degradation of pollutants in the environment and assimilation of methane or simple compounds such as acetate.
**¹⁵Nitrogen**

As previously mentioned ¹⁵N was used by Messelson and Stahl to study DNA replication in E. Coli. ¹⁵N has also been used in SIP to study nitrogen fixation and to identify pollutant degraders in environmental samples. The table below lists the limited number of studies that have used ¹⁵N SIP. It is the most difficult of the elements to use in SIP because ¹⁵N labeled nucleic acids are only slightly more dense than ¹⁴N labeled nucleic acids. The maximum shift in buoyant density that can be achieved in CsCl gradients for ¹⁵N labeled nucleic acids is approximately 0.016 g ml⁻¹ DNA, relative to 0.036 g ml⁻¹ for ¹³C labeled isotopes. GC content of microbial genomes can result in DNA samples that vary in buoyant density by as much as 0.05 g ml⁻¹. Some researchers employ a DNA binding dye, bis-benzimide and further discussed later in the report, to help separate ¹⁵N labeled DNA from non-labeled DNA.

**¹⁸Oxygen**

Paul Boyer and coworkers studied DNA replication in *E. coli* using H₂¹⁸O to label DNA showing that branch oxygen atoms of *E. coli* DNA are almost entirely derived from water and that ¹⁸O labeling of DNA is not due to formation of hydration shells around DNA. When analyzing environmental samples of microorganisms by stable isotope probing (SIP), labeling the DNA with H₂¹⁸O, instead of organic or nitrogenous compounds, offers important advantages because water cannot be used as an energy, carbon, or nitrogen source. As a result, addition of the label is unlikely to influence microbial growth rates in soil directly and microbial communities can be exposed to the label for long periods of time because they are not exposed to abnormally high substrate
concentrations. Because all organisms incorporate water into their DNA, performing SIP with H$_2^{18}$O is a method for identifying microorganisms that have grown during incubation with H$_2^{18}$O, as well as, microorganisms that have not grown (i.e., did not incorporate the label) but survived the incubation. Though there are fewer oxygen atoms in DNA than carbon (Radajewski et al., 2003), more neutrons can be added to DNA through $^{18}$O than any other isotope. There are, on average, 11 oxygen atoms per nucleotide unit in DNA whereas on average 3.75 nitrogen and 14.75 carbon atoms are present per unit DNA. The carbon and nitrogen isotopes used in SIP contain one extra neutron relative to $^{12}$C or $^{14}$N while $^{18}$O has 2 more neutrons than the more prevalent oxygen isotope $^{16}$O. Thus by labeling DNA with $^{18}$O it is possible to introduce 22 extra neutrons per unit DNA while only 3.75 or 14.75 extra neutrons can be added with $^{15}$N and $^{13}$C respectively.

To test if soil DNA could be labeled sufficiently to use H$_2^{18}$O in SIP of soil microbial communities, 0.2 ml of 95 atom% H$_2^{18}$O was added to one gram of Ponderosa Pine soil, with a moisture content of 8%. DNA was extracted from soil and isopycnic centrifugation produced 2 or 3 DNA bands after 7 or 21 days of incubation, respectively (Schwartz, 2007). The third band appears to form after microorganisms feed on $^{18}$O-labeled organic matter while in H$_2^{18}$O. DNA extracted from Ponderosa pine soil incubated with H$_2^{16}$O for 6 or 21 days did not produce multiple bands after isopycnic centrifugation indicating that changes in % GC of bacterial genomes in soil did not cause the formation of multiple DNA bands.
There are important advantages in using H$_2^{18}$O over other types of labels, such as NH$_4^+$, in order to measure growth in soil. These include:

1. More neutrons can be added to DNA when H$_2^{18}$O is used than when $^{15}$N is added as a label in SIP experiments. As a result labeled DNA separates from non-labeled DNA along a CsCl gradient whereas in experiments with $^{15}$N a continuous smear of DNA forms. Migration of DNA within this smear is controlled by both DNA label incorporation and GC content of the DNA whereas in $^{18}$O-SIP changes in % GC are not sufficient to cause DNA to migrate to the lower labeled band. In $^{18}$O-SIP, but not $^{15}$N-SIP, only two DNA fractions are retrieved (labeled and unlabeled DNA) making subsequent analysis much more simple and cost effective.
2. In contrast to an organic or nitrogenous molecule, water by itself does not induce growth because it is not used as an energy, carbon or nitrogen source. As a result fertilization artifacts by adding large quantities of labeled substrate to soil are avoided.

3. Water can easily be distributed homogenously throughout soil because it is a small molecule that does not interact with the cation exchange capacity of soil. As a result microorganisms in soil are uniformly exposed to the label. In SIP with \(^{15}\text{NH}_4^+\), microorganisms will incorporate unlabeled ammonium, formed through N mineralization, as well as labeled ammonium into their DNA. Because some organisms may grow in N-mineralization hot spots not all microorganisms are exposed to the same amount of \(^{15}\text{NH}_4^+\).

4. During precipitation events water is normally added to soil while semiarid soils in northern Arizona never receive pure ammonium solutions.

5. \(\text{H}_2^{18}\text{O}\) SIP allows study of environmental manipulations that do not involve substrate assimilation including the impact of temperature, moisture, soil bulk density and pH on ammonia oxidizing microorganisms. These environmental parameters could be very important in determining which genotype grows fastest in soils.

\textbf{In SIP experiments a majority of atoms in “labeled” DNA must be the heavy isotope}

As previously mentioned the majority of atoms of an element in nucleic acids must be the heavy isotope in order to have sufficient difference in buoyant density to separate labeled and non-labeled nucleic acids along a CsCl or CsTFA density gradient. Consequently SIP experiments should only be designed for labeled substrates that may
be obtained with 50 atom% heavy isotope or higher. Furthermore the researcher needs to consider to what extent the added labeled substrate will be diluted by non-labeled substrates already present in the environmental sample. It may, for instance be difficult to do SIP with $^{13}$C-methane in an environmental sample that produces large quantities of $^{12}$C-methane. Similarly when using $^{15}$N$_2$, it may be prudent to replace the atmosphere of the sample thereby removing the $^{14}$N$_2$ from the incubation.

**Adding high concentrations of a labeled substrate may introduce a fertilizer artifact into the experiment**

Because high concentrations of substrate are required in small environmental samples, SIP experiments are susceptible to fertilizer artifacts. When studying glucose assimilators in soil, for instance, it is important to recognize that addition of glucose will alter the active microbial community in soil. Therefore results may not reflect the community that would assimilate low concentrations of substrate. In soils microorganisms are often limited by labile carbon and adding a large amount of labile carbon to a soil may not reflect average conditions in the environment. Often in bioremediation experiments microorganisms are exposed to high concentrations of carbon substrates but SIP studies of nutrient cycling or soil quality will need to be designed in such a way that the fertilization artifact is avoided.

**The labeled substrates, once added to soil, will be incorporated into a variety of compounds, making interpretation of experimental results more difficult with extended incubations.**

Once microorganisms assimilate the added labeled substrate and produce new biomolecules, a wide range of labeled organic compounds are formed in soil including proteins, lipids, nucleic acids, carbohydrates, cell wall components and smaller
metabolites. These compounds also serve as excellent substrates for microbial growth so that the longer the incubation proceeds the more types of microorganisms will become labeled. While it is reasonable to presume that the first labeled nucleic acids are derived from organisms that assimilated the original added labeled substrate, once the incubation proceeds further it will become increasingly difficult to ascribe labeled nucleic acids to specific substrate assimilators. By conducting a SIP time series analysis it is feasible to track an element such as carbon through different microbial populations as the incubation proceeds. This approach may be highly suitable for studies of nutrient cycling and soil quality.

III Stable Isotope Probing Experimental Protocol

**Incubation of soil with labeled substrate**

1. Combine 1 g of soil with 200 μL of H$_2^{18}$O (95 atom%) or other labeled substrate and place in a 15-mL Falcon tube. Stir the soil with a small spatula so that it becomes homogenously moist. The moisture content of the soil matters because unlabeled H$_2$O present in the soil, prior to addition of labeled H$_2$O, will dilute the label present during the incubation. If the soil is too wet, so that addition of 200 μL of H$_2^{18}$O results in a soil completely saturated with H$_2$O, it may be necessary to air dry the soil before adding the H$_2^{18}$O. If $^{13}$C or $^{15}$N labeled substrates are used it is important to add a sufficient quantity of substrate to cause growth of the microbial population. It is likely that at least 50μg substrate/g soil is required. The more substrate is added the more likely there will be a fertilization artifact.

2. Incubate the soil with the labeled H$_2$O or labeled substrate for ~1 week in a Falcon tube at room temperature (approximately 20 °C). Keep the tube closed to avoid
evaporation of H$_2$O and drying of the soil sample. A preliminary experiment to determine the optimum incubation time to allow formation of labeled DNA which can be detected along the CsCl gradient is highly recommended. More than one week may be required to produce sufficient labeled DNA. If the sample is incubated too long the label will turnover and DNA of organisms that did not originally assimilate the substrate will become labeled. For many $^{13}$C labeled substrates incubation times are shorter than one week; 24 to 72 hours may be sufficient to label a large fraction of the microbial population.

3. After incubation, the soil may be frozen at -20°C or -80°C until time is available for DNA extraction. If the soil is frozen at -20°C, DNA should be extracted within a month, whereas soil may be stored at -80°C for at least a year.

4. A non-labeled substrate control must be included in the experiment to ascertain that nucleic acids in the labeled treatment did become labeled during the incubation. There must be significantly greater quantities of heavy nucleic acids (for DNA usually greater than 1.72 g/mL) in the labeled treatment than in the non-labeled substrate control.

**DNA extraction**

Extract the DNA from soil using a commercially available soil DNA extraction kit according to the manufacturer’s instructions. The frozen soil should not be thawed prior to extraction. Extract only half of the incubated sample at one time, leaving the other half frozen, in case the first attempt at SIP analysis is unsuccessful. During the extraction procedure it is important to maximize yield, not purity, of the DNA because subsequent centrifugation of the DNA on a CsCl gradient will further purify the DNA. The yield can be improved by eluting the DNA, in the final step of purification, with larger amounts of elution buffer, because the DNA does not need to be concentrated for
centrifugation. Some researchers include a phenol/chloroform step in their protocol to improve DNA yield. One microgram of DNA is sufficient to perform SIP.

**DNA quantification**

Nucleic acids should be quantified before they are loaded onto the cesium chloride gradient. Quantification can be done via fluorescent methods such as the use of picogreen a fluorescent DNA binding dye and the QUBIT system. Alternatively a spectrophotometer can be used to quantify the DNA. If using a spectrophotometer it is important to measure the absorbance at 230, 260 and 280nm. The absorbance at 260 nm will be used to calculate the concentration of DNA and the ratio of absorbance at 260 nm over 230 nm or 260 nm over 280 nm are indicative of the cleanliness of the DNA. If the A260/A280 or the A260/A230 are not over 1.5 the DNA is not very clean and the A260 is not a reliable measurement of DNA concentration.

Change in position of DNA bands as the samples are spun in an ultracentrifuge

DNA.
Centrifugation

Rotor Choice

Both fixed angle and nearly vertical rotors may be used in SIP experiments. Vertical rotors appear to set up the density gradient faster and therefore require shorter spin times. However, it appears that greater separation is feasible with a fixed angle rotor. Furthermore contaminants in DNA such as humic acids may be pelleted in a fixed angle rotor.

Vertical Rotor

An example of a vertical rotor used in SIP analysis is the TLN-100 from Beckman. It positions the tubes at a 9 degree angle, so it is referred to as a nearly vertical rotor.

In a nearly vertical rotor, such as the Beckman TLN-100 the tubes are nearly upright in the rotor
If the samples are spun too fast CsCl will precipitate in the centrifuge tubes. This graph shows how fast the TLN-100 rotor can be spun without having CsCl precipitate
In isopycnic centrifugation the gradient media needs to be of a density similar to the molecules the researcher is attempting to hang in the middle of the tube. DNA has a density of approximately 1.7 g/mL. The number is not exact since it varies due to differences in GC content. As a result Cesium Chloride is a good salt to use for DNA-SIP. RNA has a higher buoyant density and will pellet when centrifuged in a cesium chloride media. For RNA SIP Cesium Tri Fluoro Acetate is routinely used as a media to set up a density gradient. Cesium chloride will precipitate from the media if the samples are spun too fast. The graph above shows the relationships, at different temperatures between the density of the cesium chloride solution and how fast the samples are spun. Cesium chloride will not precipitate at any point below these curves. Precipitation of cesium chloride during isopycnic centrifugation should be avoided as it will impact separation of labeled and non-labeled DNA.

![Relative Centrifugal Fields for the TLN-100 Rotor](Image)

The speed at which a rotor is spun generates varying levels of RCF in different rotor. The graph shows the relationship between RCF and rpm for the TLN-100 rotor.
The speed at which samples are spun, in rotations per minute, is not very informative in SIP studies. The density gradient that forms is dependent on the amount of g-force, often expressed in rotational centrifugal force (RCF). While RCF is dependent on how fast the samples are spun in rpm the relationship between rpm and rcf varies between different rotors. Therefore, SIP publications should always report isopycnic procedures in rcf and not rpm.

One of the challenges in SIP is to separate labeled DNA from non-labeled DNA even though there may be only small differences in buoyant density. This is especially the case when the nucleic acids are not fully labeled with the isotopes because non-labeled substrate was present in the incubation. The speed at which the centrifuge is spun will determine the difference in densities from the top of the centrifuge tube to the bottom. If the samples are spun very fast, there will be a large difference in densities along the gradient, resulting in relatively tight nucleic acid bands that are positioned close together in the tube. If the samples are spun more slowly the bands will be more diffuse but also positioned further apart. It may require more time to set up a fully formed gradient when samples are spun more slowly.
The faster the samples are spun the steeper the density gradient becomes. This graph shows the relationship between centrifugation speed and the steepness of the density gradient for the TLN-100 rotor.
**Fixed Angle Rotor**

In the fixed angle rotor the tubes are positioned at more of an angle than in the near vertical rotor. In the case of the TLA-110 rotor, a rotor commonly used in SIP, the tubes are placed at a 28 degree angle. This allows contaminating compounds to pellet more readily from the samples.

Position of tubes in a fixed angle rotor. The Beckman TLA-110 is shown
If the samples are spun too fast CsCl will precipitate in the centrifuge tubes. This graph shows how fast the TLA-100 rotor can be spun without having CsCl precipitate.
The faster the samples are spun the steeper the density gradient becomes. This graph shows the relationship between centrifugation speed and the steepness of the density gradient for the TLA-110 rotor.
The compromise between speed and length of centrifuge spin

The faster the samples are spun the greater the difference in buoyant density between the top and bottom of the centrifuge tubes. As a result compounds that differ only slightly in buoyant density such as partially labeled nucleic acids and non-labeled nucleic acids will position very close together along the density gradient if the centrifuge tube is spun fast. However, if the tubes are spun very slowly it may take a relatively long time to form the density gradient, so that the researcher must make a compromise between length of spin and degree of separation between labeled and non-labeled nucleic acids.

Guanine/cytosine content affects density of DNA

Incorporation of heavy isotopes is not the only determinant of buoyant density of nucleic acids. Nucleotide composition, specifically the ratio of guanidine cytosine (GC) over adenosine thymidine (AT) nucleotides, will also affect the buoyant density of nucleic acids by as much as 0.05 g ml\(^{-1}\). Nucleic acids with high GC content are more dense than nucleic acids with high AT content. The GC content can vary substantially between different microbial genomes. Consequently non-labeled DNA extracted from soil will spread over a range of densities. The impact of GC content on SIP results requires the inclusion of a non-labeled but identically treated control. The scientist must ascertain that the high concentrations of heavy DNA formed during a SIP incubation is due to assimilation of the isotopically labeled substrate and not because a microbial population grew that contained a genome with a high GC content.

DNA binding dyes may be used to exaggerate the impact of GC content

Several studies have used DNA binding dyes, such as bis-benzimide, to preferentially bind AT rich regions to manipulate buoyant density of DNA. This
approach can be exploited in SIP where GC rich DNA can contaminate labeled DNA. By including a second spin in which the DNA binding dye is included GC rich DNA can be separated from labeled DNA. This experimental approach is especially useful when $^{15}$N is the isotope used in SIP experiments.

**Preparing the ultracentrifuge tubes for isopycnic centrifugation**

5. Weigh 1 mL of CsCl solution to determine that it has the correct density. A saturated CsCl solution will have a density of 1.9 g/mL. Be certain that no crystals remain in the solution. By holding the solution up to the light it is possible to see any remaining transparent CsCl crystals.

6. Add a saturated solution of CsCl solution to each centrifuge tube placed on a scale to confirm that each tube receives an identical amount of CsCl. The amount added to the tube depends on the volume of the tube used. In the case of 4.7 ml TLA-110 rotor tubes we add 3.6 ml of saturated CsCl solution. The final density of the tube contents, after the DNA and gradient buffer are added should be approximately 1.72 g/mL.

7. If the researcher wants to visualize the DNA after isopycnic centrifugation, add 0.5 μL of fresh SYBR Green I DNA stain to the DNA extracted from soil. Older SYBR I stain can increase the time required to get good DNA separation along the cesium chloride gradient. The DNA should still be in the microcentrifuge tube used to elute the DNA from the column in the final step of DNA extraction (Step 4). Alternatively, the researcher may elect to not include a DNA binding dye, in which case the DNA cannot be visualized and no photograph of the tube will be taken after centrifugation.

8. Add the DNA/SYBR-Green mixture to the CsCl solution in the centrifuge tubes. Add 300 μL of gradient buffer (200mM Tris pH 8.0, 200mM KCl, 2mM EDTA) to each tube.
Fill each centrifuge tube to the top with H₂O and invert the tubes several times to thoroughly mix the contents.

9. Weigh each tube to ensure that they all weigh within 0.01 g of each other. If necessary, add sterile H₂O to balance the tubes.

10. Load the tubes in the TLA-110 rotor and centrifuge in an ultracentrifuge at approximately 176,000 X g for approximately 72 h.

11. Decelerate the centrifuge as slowly as possible. When the rotor is removed from the centrifuge and the tubes are extracted from the rotor, extreme care needs to be taken to avoid bumps, so that the gradients established in the tubes are not disturbed.

**Photographing DNA Bands Within the Centrifuge Tubes**

12. Carefully transfer the tubes from the rotor into a rack that has been placed on top of a UV transilluminator in a dark room. The two bands formed during centrifugation should be readily visible.

13. Photograph the tubes at this time using an exposure greater than 1 sec. Because of the long exposure time, immobilize the camera on a test tube rack or camera stand.

**Fractionation of centrifuge tube contents**

Once the isopycnic centrifugation is complete and labeled DNA has separated from non-labeled DNA the fractions will still need to be collected. A needle is used to puncture the bottom of the tube while a second needle is used to pierce the tube on top. The gradient media will drip from the bottom of the tube and 16 to 50 fractions of 100 to 250 microliter each may be collected. The density of each fraction is measured with a digital refractometer before the DNA is precipitated from each fraction.
Purifying DNA from CsCl Solution

16. Add ~500 μL of H₂O and ten μL of glycogen solution (10 μg/μL) to each DNA fraction and shake the tubes by hand.

17. Add 1 mL of isopropanol to each tube and vigorously mix the contents.

18. Centrifuge the tubes in a microcentrifuge at full speed for 30 min.

19. Discard the supernatant from the tube, taking care not to dislodge the pellet.

20. Add 500 μL of 70% ethanol to the tube and shake gently. Centrifuge at full speed for 5 min.

21. Discard the supernatant from the tube into a waste beaker.

22. Remove the last bit of liquid from the bottom of the tube with a pipette. Air dry the pellet for ~30 min.

23. Dissolve the pellet in sterile H₂O or TE buffer. The DNA is now ready for subsequent analysis including production of clone libraries, generation of T-RFLP patterns, or real-time PCR analysis.

24. Quantify the DNA in each fraction using a QUBIT from Invitrogen inc.
The SIP experiment is successful if significantly more heavy DNA is present in the labeled substrate treatment than the non-labeled substrate treatment.

Most of the DNA isolated from the centrifuge tubes will have a lower buoyant density (1.68 to 1.72 g/mL) but in the labeled treatment there should also be DNA with a higher buoyant density. The fractions with a density greater than 1.72 g/mL and that contain significantly more DNA in the labeled treatments than in the control non-labeled treatments contain the genomes of microorganisms that assimilated the added substrate.

This graph shows the DNA concentrations in different fractions taken from the tube. In the heavy isotope treatments (samples 2, 4 and 6, represented by filled symbols) there is DNA in heavy fractions that is absent from the non-labeled control treatments (sample 4W represented by open circles)
**Analysis of DNA fractions**

The labeled fractions may be combined into one labeled DNA pool or, alternatively each fraction can be analyzed separately. Any type of DNA analysis is feasible but quantitative PCR and next generation sequencing are the two most common types of analyses.

**Quantitative PCR**

In quantitative PCR the fluorescence generated by dyes binding to PCR product is measured after each cycle. The resulting sigmoidal curve can be related to a set of standard curves to determine the original abundance of gene copies in a DNA fraction. This approach quantifies the growth or the extent to which a pre-determined microbial population has assimilated the added substrate. The benefit from this approach is that it is highly quantitative. The downside is that it can only be used for microbial populations or functional genes for which PCR primers have been developed.

**Sequencing of fractions**

The DNA in the fractions may also be used in next generation sequencing, including pyrosequencing and illumina based sequencing. Usually an amplicon, such as a fragment of the bacterial 16S rRNA gene, is analyzed. However, increasingly scientist are shot gun sequencing all the DNA in SIP samples in order to identify booth functional genes and genes indicative of taxonomic structure. However, the quantity of DNA obtained through SIP analysis is not sufficient to analyze directly in shotgun sequencing. Therefore the DNA has to first be used in whole genome amplification. Commonly multiple displacement amplification, a non-PCR based amplification technique, is used to amplify small amounts of DNA to obtain sufficient quantities for genomic analyses. PCR based amplification approaches are likely to introduce biases into the analysis.
Use of SIP in elucidating the role of microorganisms in nutrient cycling and soil quality

The majority of SIP studies have focused on degradation of pollutants in environmental samples or assimilation of simple carbon compounds. Studies of nutrient cycling or soil quality, however, require understanding of how complex structures with multiple compounds, such as plant biomass, are assimilated by microorganisms in soil. There are two possible approaches to identifying microorganisms that assimilate complex organic structures. The first is to label the plant with $^{13}$CO$_2$. The challenge is that for SIP to work the majority of C atoms in the plant need to be of the $^{13}$C variety, thus the plant should be grown from a seed in an atmosphere consisting predominately of $^{13}$CO$_2$. This is expensive, though not impossible. In the second approach newly growing organisms are labeled with $^{18}$O during $^{18}$O-water incubations. In this approach the growing microorganisms in soil with only $^{18}$O-water are compared to the growing microorganisms in soil with $^{18}$O-water and plant litter. This approach is likely cheaper and allows comparison of many different kinds of naturally grown litter but does not provide a direct connection between litter and labeled organisms. Rather the organisms that are labeled in the litter treatment but absent from labeled DNA in the treatment without litter are identified as the litter assimilators.
Assimilation of complex organic structures such as plant litter can be studied with $^{18}$O-water SIP. In this approach, the labeled DNA of an environmental sample without plant litter is compared to the labeled DNA of a sample with plant litter. The microorganisms present in the labeled DNA from the litter treatment but absent in the control represent the organisms that grew due to the presence of plant litter.

Bottom DNA bands are compared to determine the impact of treatment on growth of microorganisms in soil.
There have been several SIP studies that relate to soil quality and nutrient cycling and these are described in the sections below:

**Carbon Cycle**

The few SIP studies that focused on the carbon cycle have employed either simple sugars such as glucose or more complex polymers such as cellulose in order to identify the organisms that assimilate and therefore decompose these carbon compounds in soil. To date there have been no shotgun sequencing studies of SIP fractions in order to identify genes involved in carbon catabolism.

**Nitrogen Cycle**

**Nitrogen Fixation**

SIP with $^{15}$N is more difficult than SIP with $^{13}$C or $^{18}$O because of the small changes in buoyant density caused by incorporation of $^{15}$N into nucleic acids. The list of publications that employed $^{15}$N are shown earlier in the report and several of these studies investigated nitrogen fixers in environmental samples via SIP with $^{15}$N. However, considering the large interest in nitrogen fixation among the soil microbial ecology community, it is surprising not more reports have followed these initial publications. It is likely that nitrogen fixation studies with $^{15}$N-SIP are extremely challenging.

**Ammonia oxidation**

Ammonia oxidizers catalyze the first rate limiting step in nitrification, converting ammonia to nitrite. These organisms may be bacterial or archaeal and are autotrophic
so that their nucleic acids can be labeled with $^{13}\text{CO}_2$. Alternatively, it is feasible to study the impact of environmental parameters such as ammonia availability on ammonia oxidizers with $^{18}\text{O}$-water SIP. Here the abundance of ammonia oxidizing genes such as bacterial amoA or archaeal amoA is compared in the labeled DNA between environmental treatments of elevated ammonia and ambient ammonia.

**Food web analysis**

If environmental samples are exposed to labeled substrates for extended periods of time the isotope will turn over, first being incorporated into biomolecules of the organism that assimilated the substrate but subsequently becoming part of organisms that feed on the original assimilating microorganisms. This can be an artifact if the objective is to identify the organism that assimilated the labeled substrate. But it can also be exploited in studies of the soil food web. Here samples are taken over time and the isotope is followed into nucleic acids of different groups of organisms. There are very few SIP studies of soil food webs but it is likely that SIP will provide new insights into the soil food web.

**Characterization of microorganisms in the plants rhizosphere**

Plants interact with a large number of microorganisms in the rhizosphere, releasing root exudates which may consist of simple sugars and amino acids and forming symbiotic relationships with mycorrhizal fungi to obtain nutrient and/or water from soil. Several studies have tried to follow organic carbon from the plant into nucleic acids of microorganisms in the rhizosphere. Plants are exposed to high concentrations of $^{13}\text{CO}_2$ before soil attached to roots is used for nucleic acid extraction. One challenge
with this experimental approach is that often the plant contains large amounts of $^{12}\text{C}$-organic compounds including sugars that will be released as exudate. Consequently it is difficult to label the nucleic acids of microorganisms in the rhizosphere sufficiently for SIP. There have been successful RNA-SIP studies of the rhizosphere but DNA-SIP studies have required extensive periods of exposing the plant to labeled $^{13}\text{CO}_2$, often in excess of a month. As a result it is not possible to determine if the organisms represented in the labeled DNA are rhizosphere organisms or of they feed on rhizosphere organisms. To date there have been no metagenomic studies of SIP –DNA obtained from rhizosphere organisms.
List of SIP studies that employ $^{15}$N labeled molecules


A list of SIP studies that employed $^{18}$O isotopes


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A list of SIP publications related to the soil carbon cycle:


A list of SIP studies that investigate ammonia oxidizers in environmental samples:


A list of SIP studies related to food webs:


A list of SIP studies focused on the rhizosphere


Alphabetical List of Published SIP Studies


an impact on microbes in the rhizosphere and plant roots. *The ISME journal*, 3(11), 1243–57. doi:10.1038/ismej.2009.68


Lu, H., & Chandran, K. (2010). Diagnosis and quantification of glycerol assimilating denitrifying bacteria in an integrated fixed-film activated sludge reactor via 13C


microbial diversity with microbial community function. *Rapid communications in mass spectrometry: RCM*, 16(23), 2179–83. doi:10.1002/rcm.782


