



CHARLES UNIVERSITY



Bioinformatics and Microbiome Analysis

MB140P94

Methods for studying microbial communities – Stable Isotope Probing (SIP)

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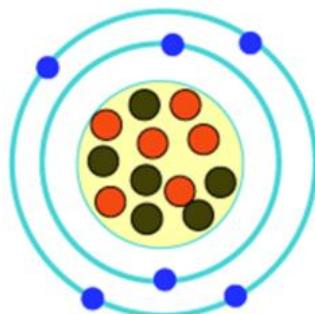
What are isotopes?

Isotopes - atoms of the same element that differs in mass number

→ Same number of protons but different number of neutrons

Some isotopes are **stable**, while other isotopes are unstable (**radioactive**)

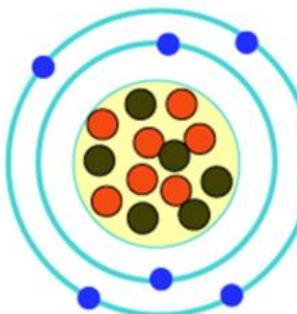
Carbon-12



$$(6P + 6N)$$

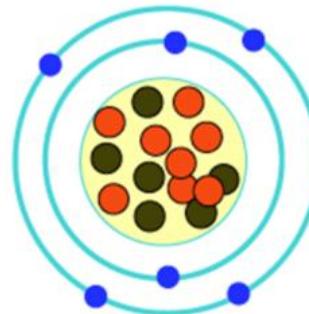
Stable isotopes

Carbon-13



$$(6P + 7N)$$

Carbon-14



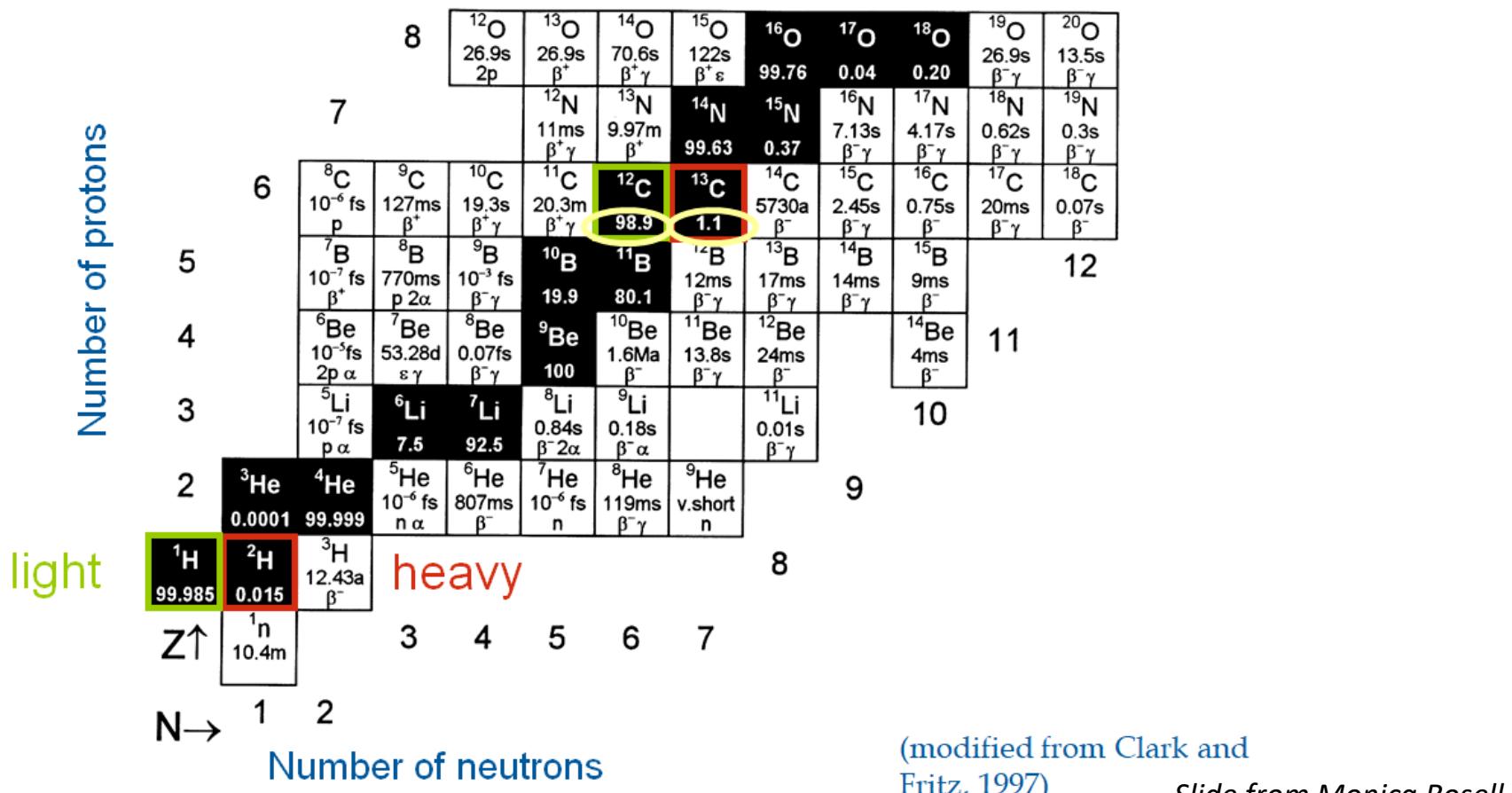
$$(6P + 8N)$$

Radioactive isotope

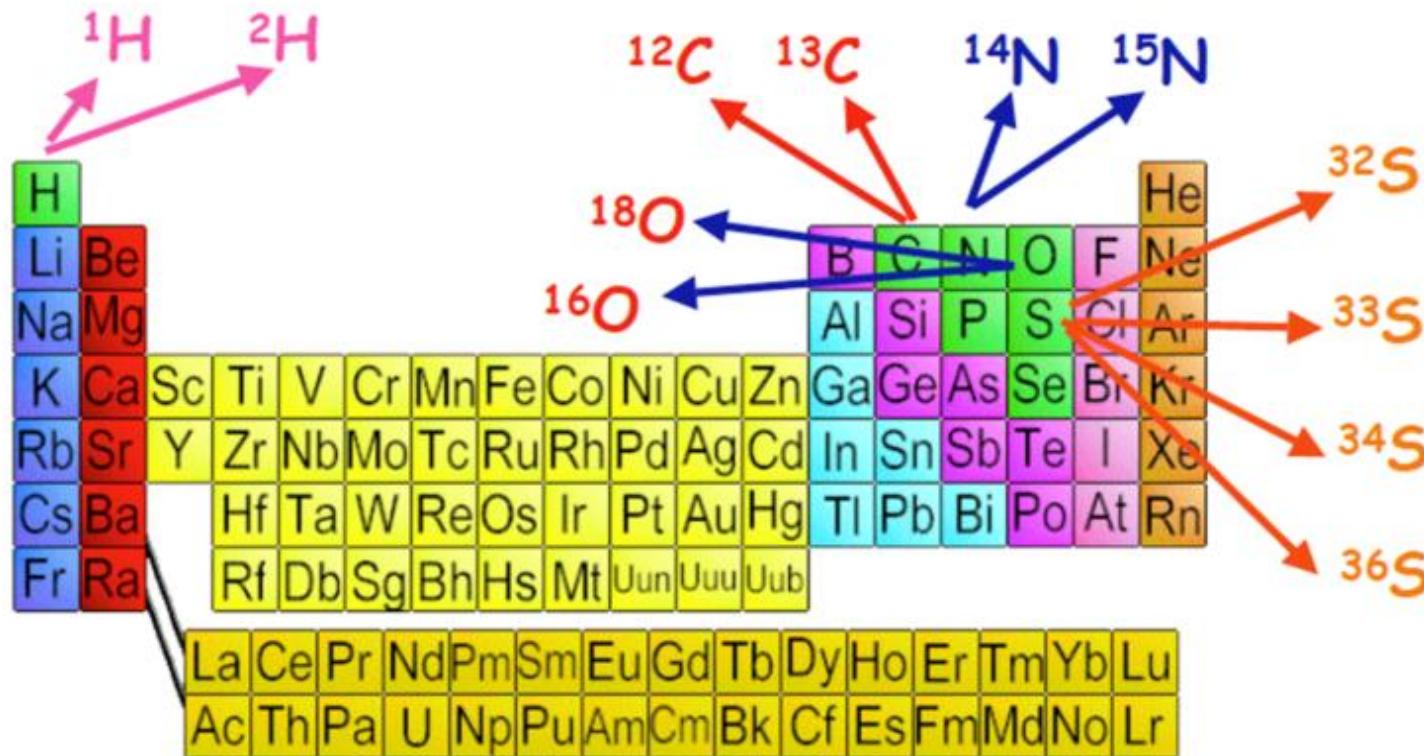
What are stable isotopes?

A_Z^X $A = [Z \text{ (protons)} + N \text{ (neutrons)}]$ atomic mass → isotope

Different isotopes of one chemical element that are **not** subject to radioactive decay



How many stable isotopes?



Many elements in the periodic table have **more than one** stable isotope.

Some elements are important for life and biogeochemical processes

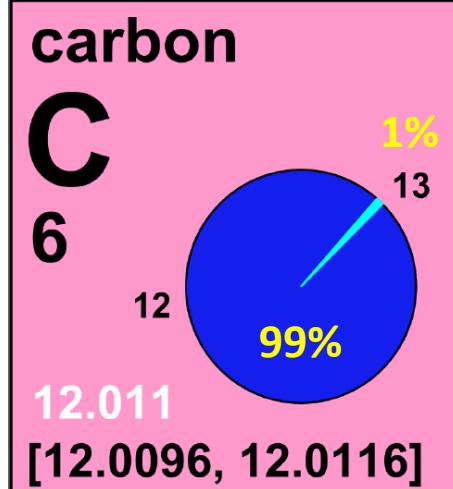
Abundance of stable isotopes in nature

One isotope (the lightest) is always more common than the others

Element	Isotopes	Abundance
Hydrogen	^1H , ^2H	$^1\text{H} = 99.985\%$ $^2\text{H} = 0.015\%$
Carbon	^{12}C , ^{13}C	$^{12}\text{C} = 98.89\%$ $^{13}\text{C} = 1.11\%$
Nitrogen	^{14}N , ^{15}N	$^{14}\text{N} = 99.633\%$ $^{15}\text{N} = 0.366\%$
Oxygen	^{16}O , ^{17}O , ^{18}O	$^{16}\text{O} = 99.759\%$ $^{17}\text{O} = 0.037\%$ $^{18}\text{O} = 0.204\%$
Sulfur	^{32}S , ^{33}S , ^{34}S , ^{36}S	$^{32}\text{S} = 95.00\%$ $^{33}\text{S} = 0.76\%$ $^{34}\text{S} = 4.22\%$ $^{36}\text{S} = 0.014\%$

- Lighter isotopes are more common
- Lighter isotopes move faster, and react more quickly
- Biological processes 'prefer' lighter isotopes
- Lighter isotopes form weaker bonds

Isotope ratios



Natural abundance (atom %)
Carbon ^{12}C 98.89 ^{13}C 1.11

Isotope ratio

$$\frac{^{13}\text{C}}{^{12}\text{C}} = \frac{1.11}{98.89} = 0.011237 = R$$

$$\delta^{13}\text{C} (\text{\textperthousand}) = \left(\frac{\frac{^{13}\text{C}/^{12}\text{C}_{\text{sample}} - ^{13}\text{C}/^{12}\text{C}_{\text{standard}}}{^{13}\text{C}/^{12}\text{C}_{\text{standard}}}}{^{13}\text{C}/^{12}\text{C}_{\text{standard}}} \right) \times 1000$$

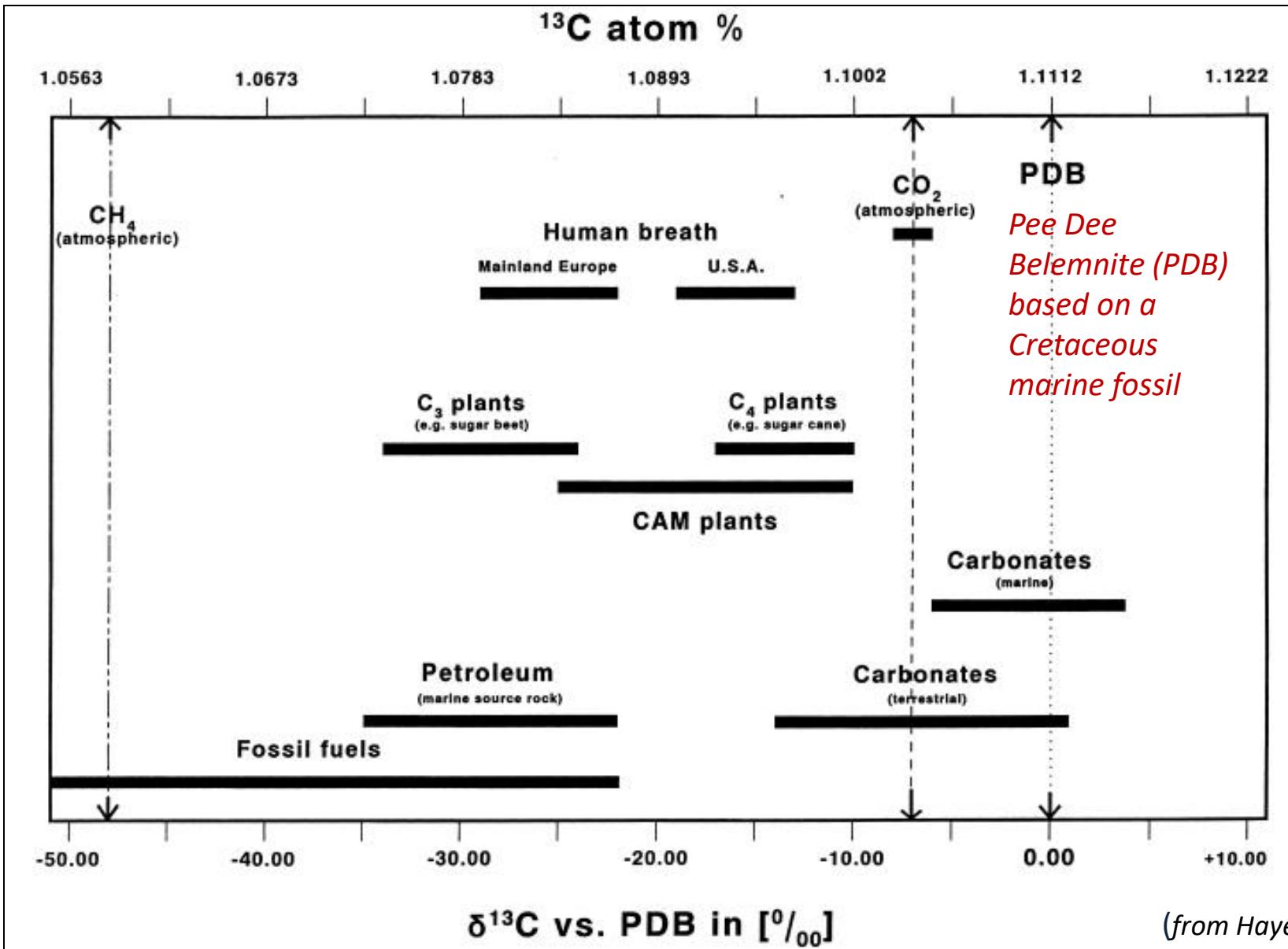
International standard: V-PDB (Pee Dee Belemnite (PDB)) for C

IPTEI, IUPAC

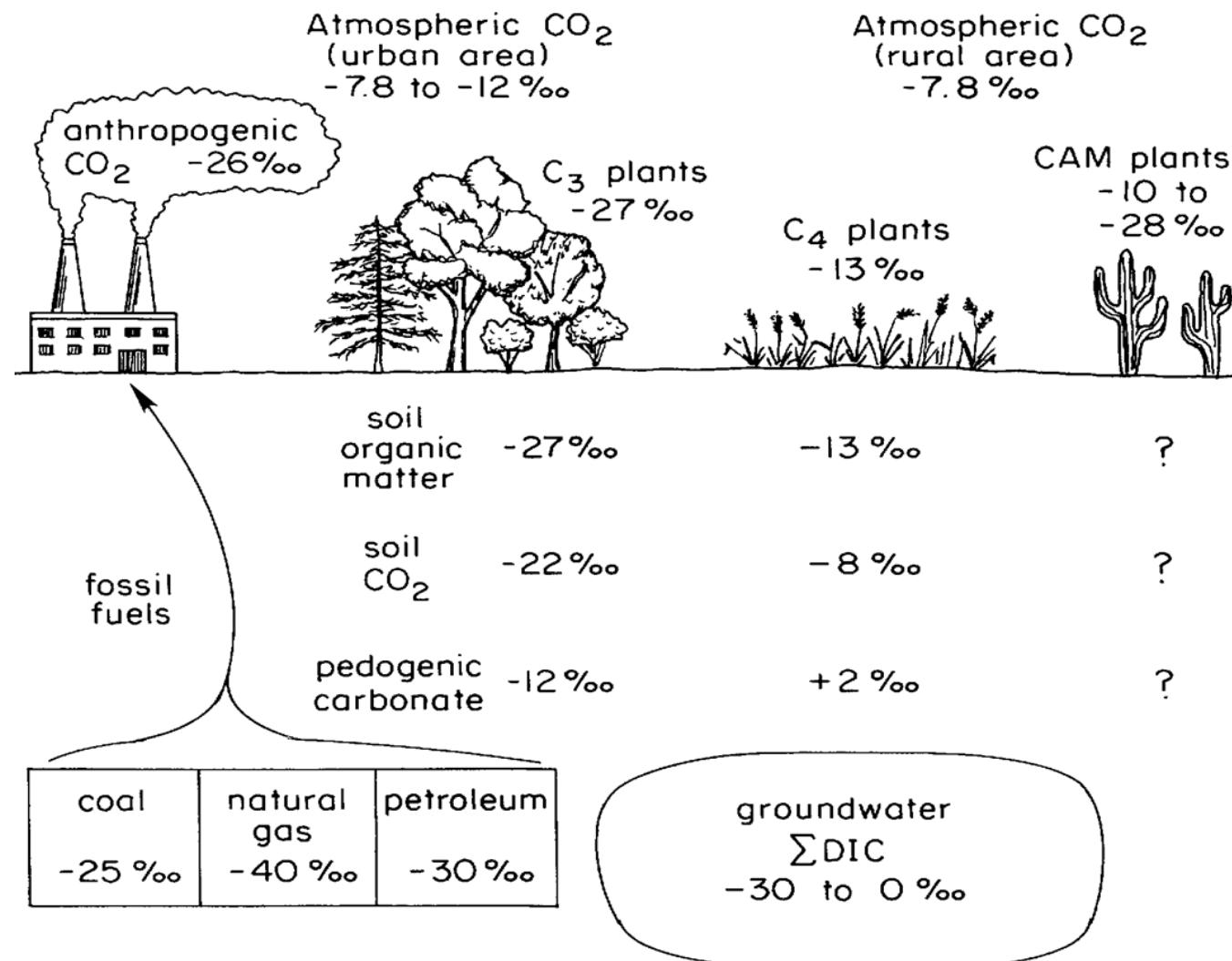
Isotope ratios are reported in **δ notation** relative to an international standard per mil (‰)

Slide modified from Monica Rosell

Isotopic signatures of different compounds in nature



Isotopic signatures of different compounds



Applications of stable isotopes

Different disciplines use isotopes in their studies: Hydrology, Forensics, Chemistry, Environmental studies, Bioremediation, Ecology

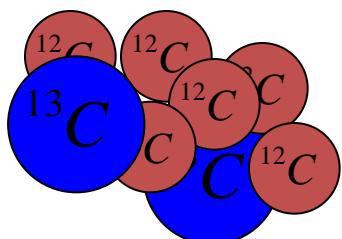
- **Fractionation of isotopes:**
 - Water cycle and biogeochemical cycles
 - Physiology and biochemistry
 - Isotope forensic / authentification / source of chemicals
 - Characterization and quantification of processes
- **Added heavy isotopes as tracers:**
 - Ex. $^{13}\text{CO}_2$, $^{13}\text{CH}_4$
 - Stable Isotope Probing (SIP)
 - DNA/RNA-SIP
 - Protein-SIP
 - PLFA-SIP

STABLE ISOTOPE TOOLS

Compound Stable Isotope Fractionation (CSIA)

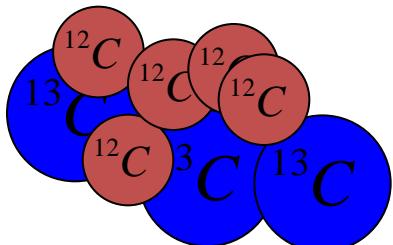
Stable Isotope Tracer (SIP)

“natural conditions”
substrate
 $^{12}\text{C}:\text{^{13}\text{C}} = 99:1$

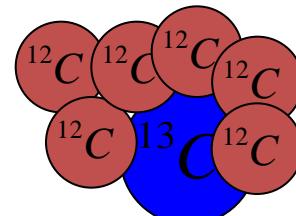


Biotic or abiotic
reaction
(Kinetic isotope
effect)

residual substrate +
 $\text{^{13}\text{C}} > 1\%$



product
 $^{12}\text{C} > 99\%$



$\text{^{13}\text{C}}$ -enriched substrate

Degraders



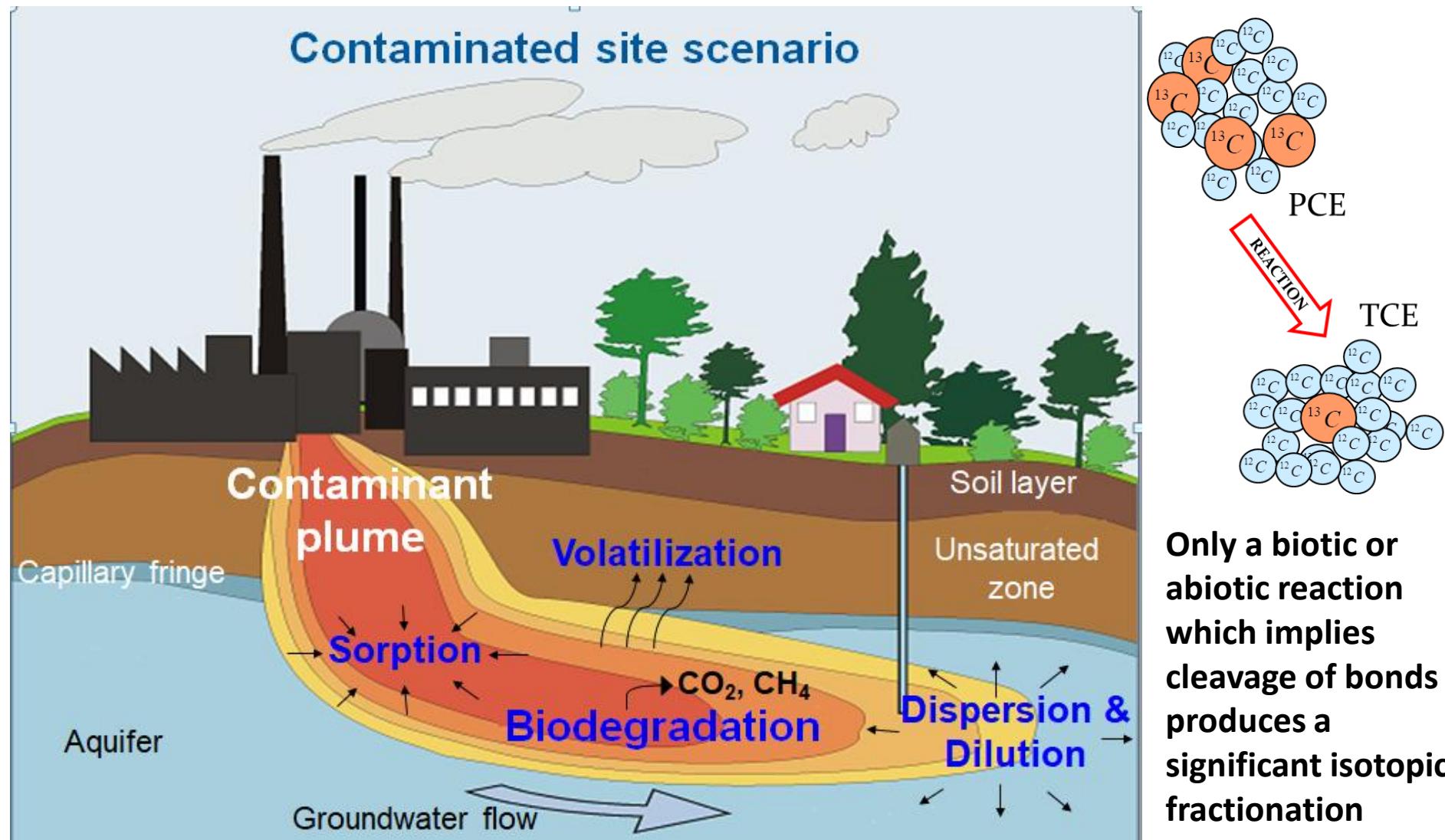
$\text{^{13}\text{C}}$ -CO₂ or $\text{^{13}\text{C}}$ -methane

$\text{^{13}\text{C}}$ -metabolites

$\text{^{13}\text{C}}$ -biomass

- fatty acids
- DNA/RNA
- proteins
- amino acids

Example of an application of Isotope fractionation (CSIA): bioremediation of contaminants in nature



Gas Chromatography Isotope Ratio Mass Spectrometer



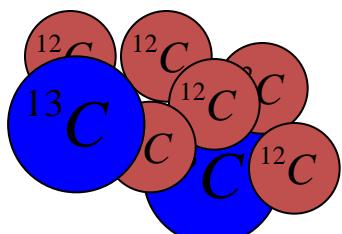
Photo from Hans H. Richnow, UFZ-Leipzig

STABLE ISOTOPE TOOLS

Compound Stable Isotope Fractionation (CSIA)



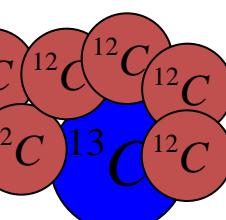
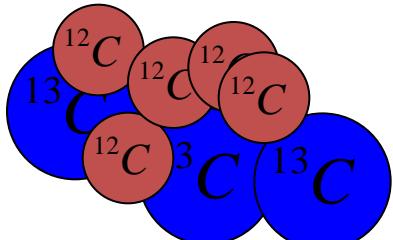
“natural conditions”
substrate
 $^{12}\text{C}:\text{^{13}\text{C}} = 99:1$



*Biotic or abiotic
reaction
(Kinetic isotope
effect)*

residual substrate + product

$\text{^{13}\text{C}} > 1\%$



Slide modified from Monica Rosell

Stable Isotope Tracer (SIP)



$\text{^{13}\text{C}}$ -enriched substrate

Degraders



$\text{^{13}\text{C}}\text{-CO}_2$ or $\text{^{13}\text{C}}$ -methane

$\text{^{13}\text{C}}$ -metabolites

$\text{^{13}\text{C}}$ -biomass

-fatty acids

-DNA/RNA

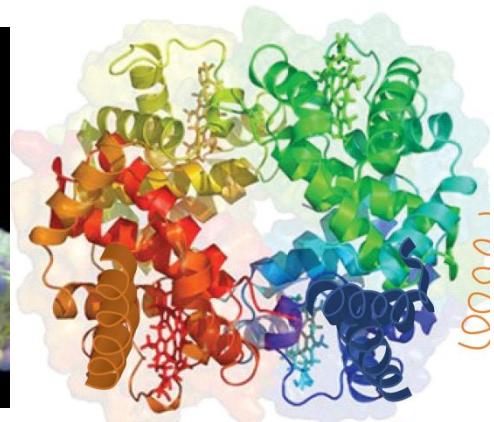
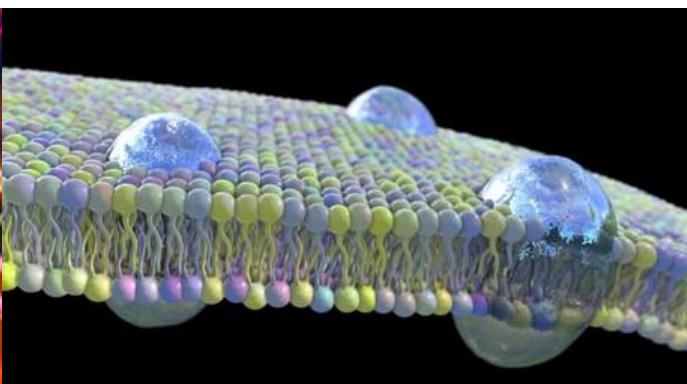
-proteins

-amino acids

Use of heavy isotopes as tracers by SIP

Stable Isotope Probing (SIP) is a technique used to track the incorporation of isotopes into biological molecules, allowing researchers to identify and trace specific microorganisms or metabolic pathways.

SIP links metabolic activity and taxonomic identity of environmental microbes



Stable Isotope Probing (SIP)

The term **SIP** was first attributed to the method for identifying labeled microbes through the incorporation of a stable isotope into their DNA.

DNA is the gold-standard for taxonomic classification of organisms and for hypothesizing about potential functions. **DNA-SIP is the most widely used.**

Additionally, DNA amplification and sequencing technologies are affordable and widespread in most molecular and microbiological labs

Types of SIP based on the target molecule

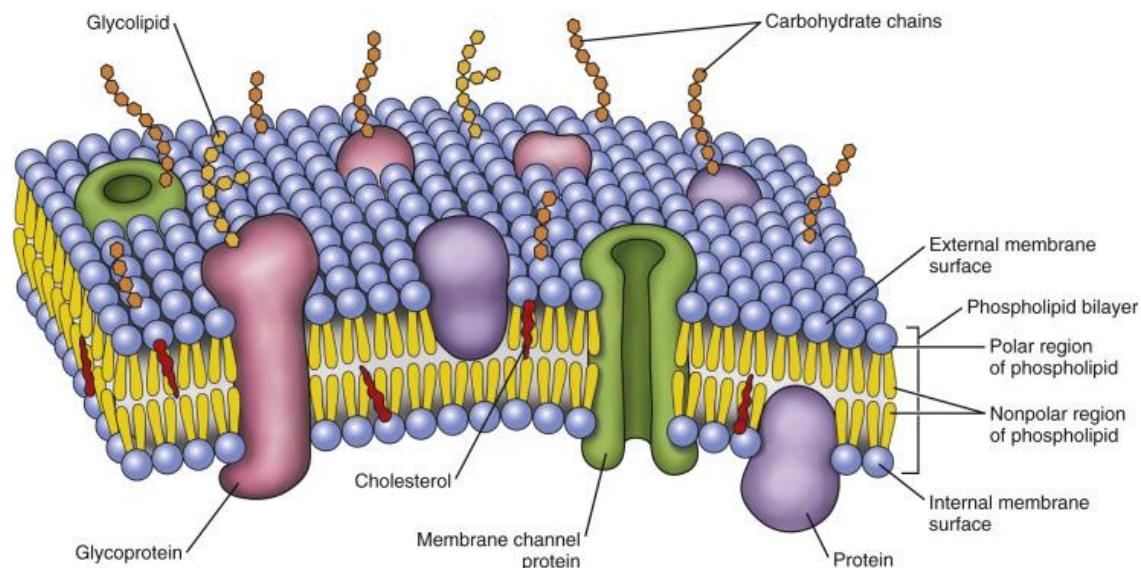
While **DNA-SIP** is the most commonly used type of SIP, other types are also popular, since in essence nearly every stable bio-molecule in the cell can be used as a target for SIP.

Other commonly targetted molecules:

- **Lipids, e.g., PLFAs**
- **Protein-SIP**
- **RNA-SIP**

PLFA-SIP

Phospholipid Fatty Acid (PLFAs) Stable Isotope Probing, which is a technique used to study microbial community structure and activity based on the incorporation of stable isotope tracers (such as ^{13}C or ^{15}N) into PLFAs, which are integral components of microbial cell membranes.



Measurement of isotopes per GC-IRMS

Gas Chromatography Isotope Ratio Mass Spectrometer

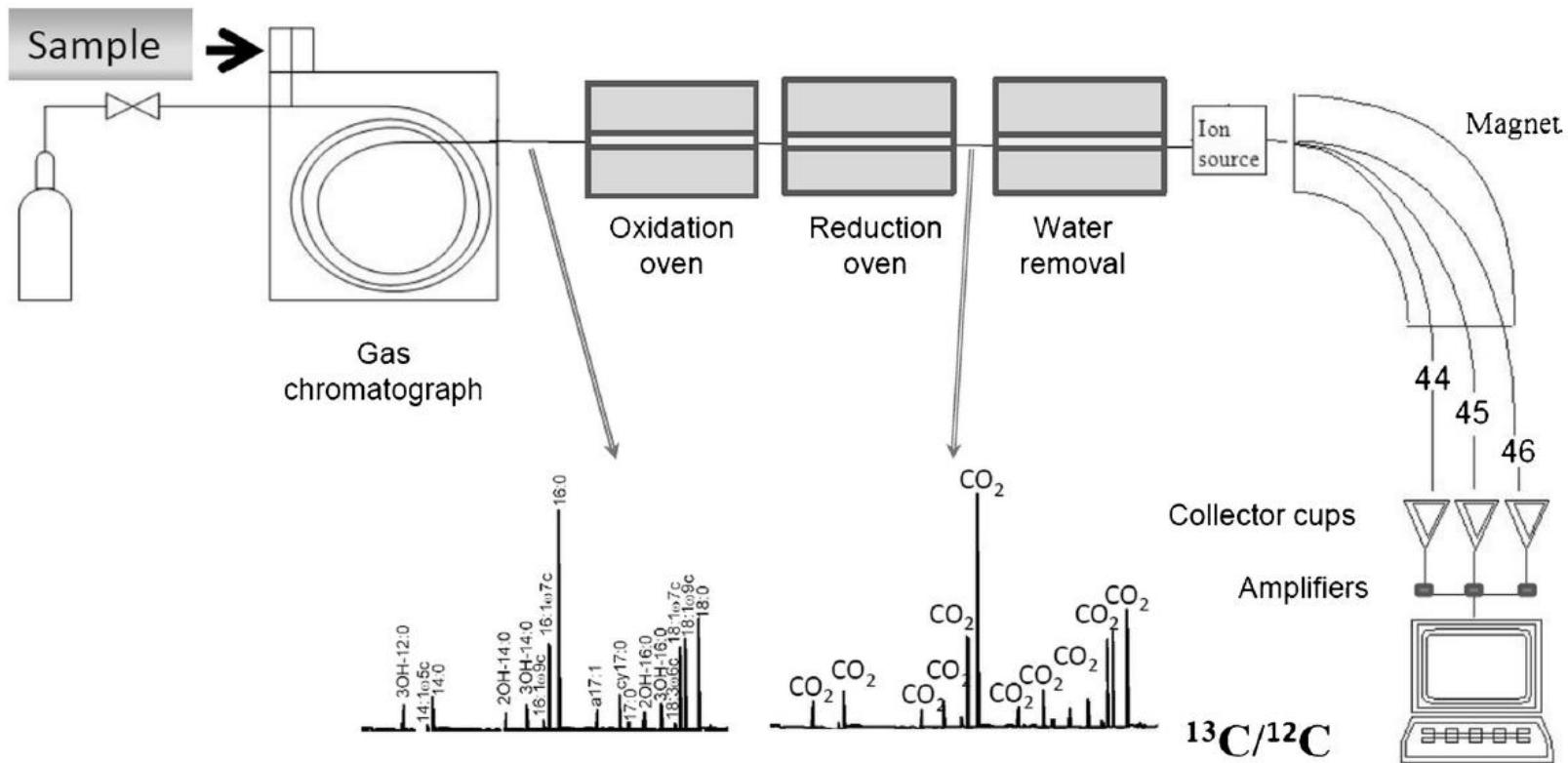


Fig 1 Scheme of carbon isotope ratio determination after gas chromatographic separation of a sample. The sample (here: fatty acid methyl esters) is injected into the gas chromatograph, the compounds are separated, followed by oxidation to CO_2 , NO_x , and water; NO_x is then reduced to N_2 and, finally, the water is separated using a Nafion membrane. During

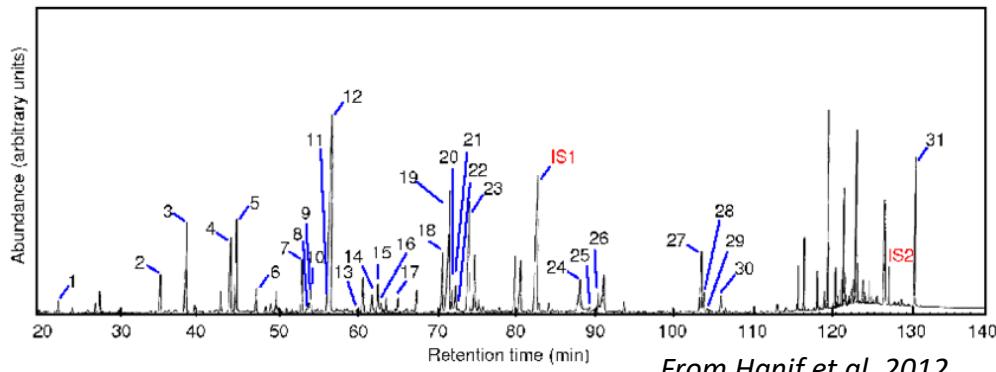
these steps, the separation of the compounds is maintained allowing isotope determination for every separated compound. In the isotope ratio mass spectrometer ions of the masses m/z 44 ($^{12}\text{C}^{16}\text{O}^{16}\text{O}$), 45 ($^{13}\text{C}^{16}\text{O}^{16}\text{O}$) and $^{12}\text{C}^{17}\text{O}^{16}\text{O}$) and 46 ($^{13}\text{C}^{17}\text{O}^{16}\text{O}$) are collected. From these data $^{13}\text{C}/^{12}\text{C}$ is calculated

PLFA-SIP

PLFAs are commonly used as biomarkers to profile microbial communities.

PLFA	Organism
18:2w6,9	Fungi (Eucaryota)
10Me 18:0	Actinobacteria
10Me 16:0	Actinobacteria
10Me 17:0	Actinobacteria
i14:0	GramPositive
i15:0	GramPositive
a15:0	GramPositive
i16:0	GramPositive
i17:0	GramPositive
a17:0	GramPositive
16:1w7	GramNegative
18:1w7	GramNegative
cy 17:0	GramNegative
cy19:0	GramNegative
16:1w5	GramNegative
17:0	other_bacteria
16:1w9	other_bacteria
15:0	other_bacteria

Fungi



Measured with a gas chromatography-mass spectrometry (GC-MS)

Total Bacteria
Sum-up

The presence of isotopically labeled fatty acids in the PLFAs indicates which microorganisms are actively metabolizing the labeled substrate, providing insights into their functional roles within the ecosystem

DNA-SIP

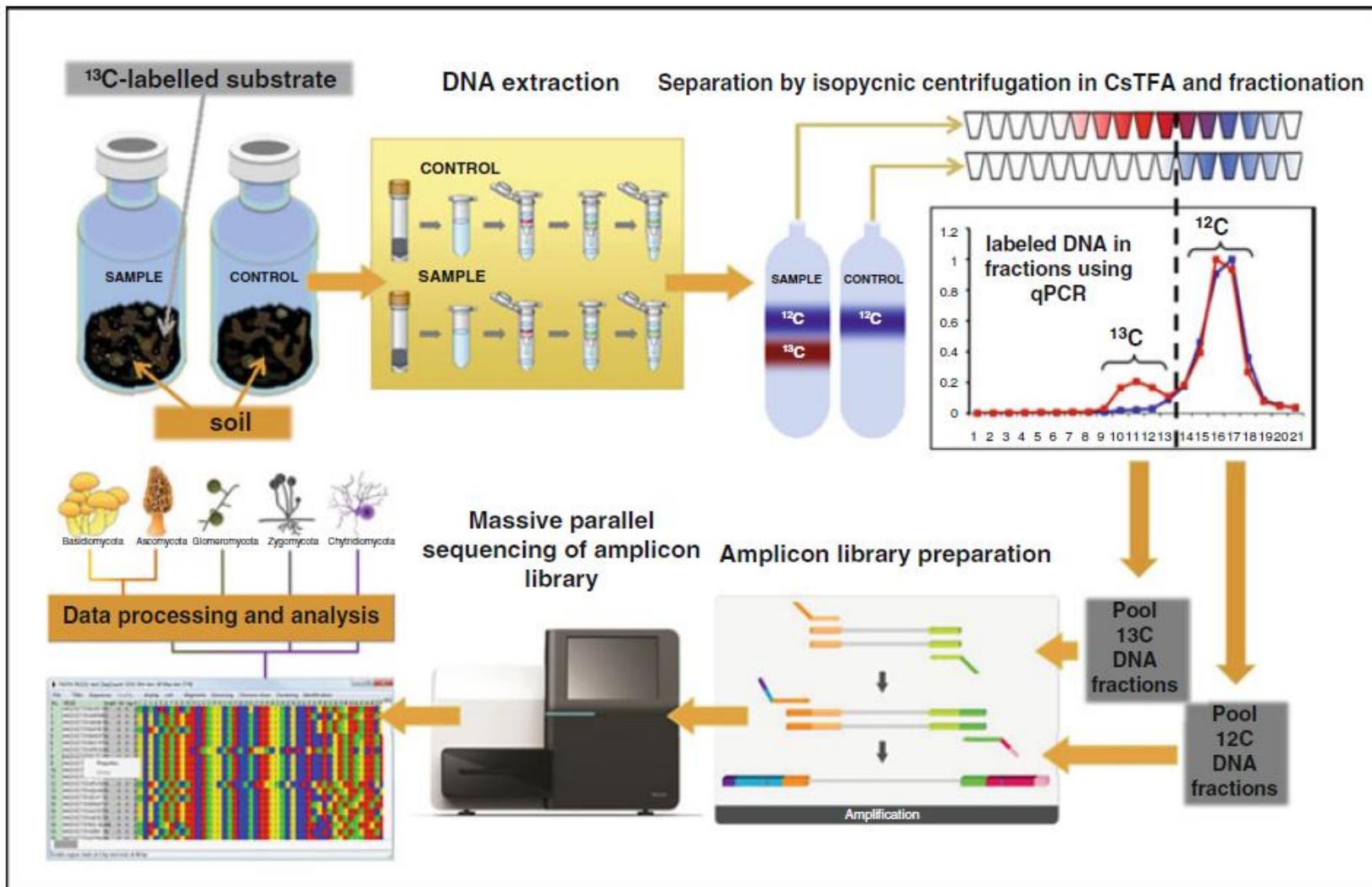


Fig. 1 Analysis of the members of fungal community involved in the decomposition of biopolymers using stable isotope probing

From Vetrovsky, Stursova, Baldrian, 2016

Methods: Stable Isotope Probing (SIP)

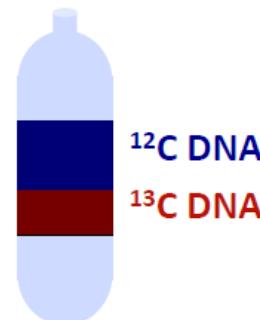
¹³C-labelled
cellulose



↓
¹³CO₂ sample;
total respiration

→ DNA
extraction

DNA separation by
isopycnic centrifugation
in CsTFA



↓
Precipitation of DNA in fractions



↓
DNA presence in fractions
detected by qPCR

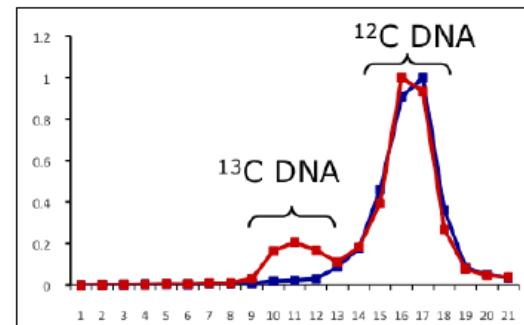
Incubation time 0, 8, 15, and
22 days
microcosms contained either
5 grams of litter (L) or
humic (H) horizon material
3 parallels per incubation
time for each horizon

Identification of organisms

Titanium pyrosequencing:
16S rRNA & ITS 1+2 regions

Community fingerprint

T-RFLPs



Separation and pooling of ¹³C and
¹²C DNA samples

From Martina Stursova

Isopycnic centrifugation

DNA-SIP methodology



Refractometer

Measurement of temperature and refractive index in each fraction to calculate density



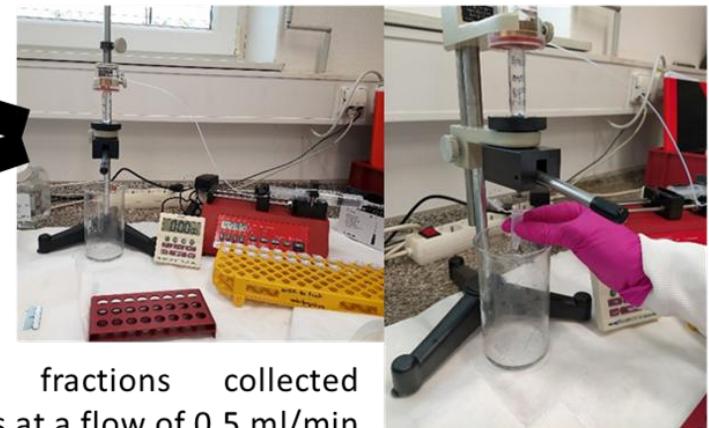
Quantitative PCR

Estimation of the DNA distribution in DNA-SIP fractions



DNA separation by density:
light ^{12}C -DNA and heavy ^{13}C -DNA

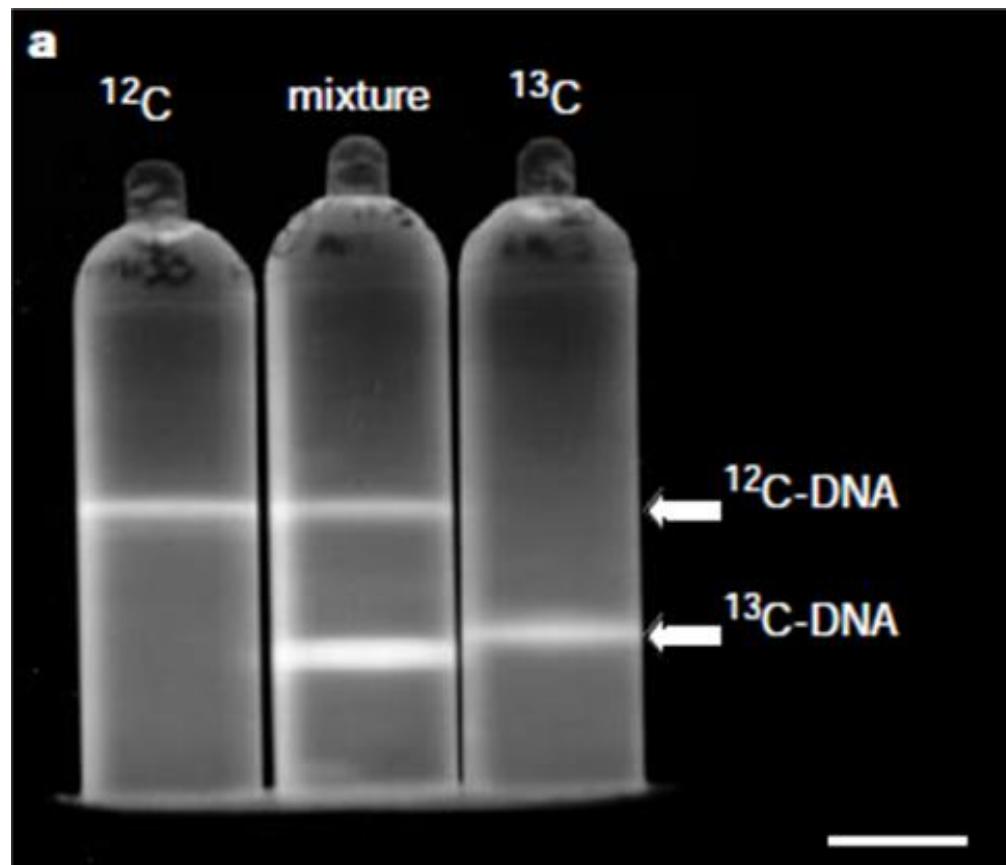
Fractionation



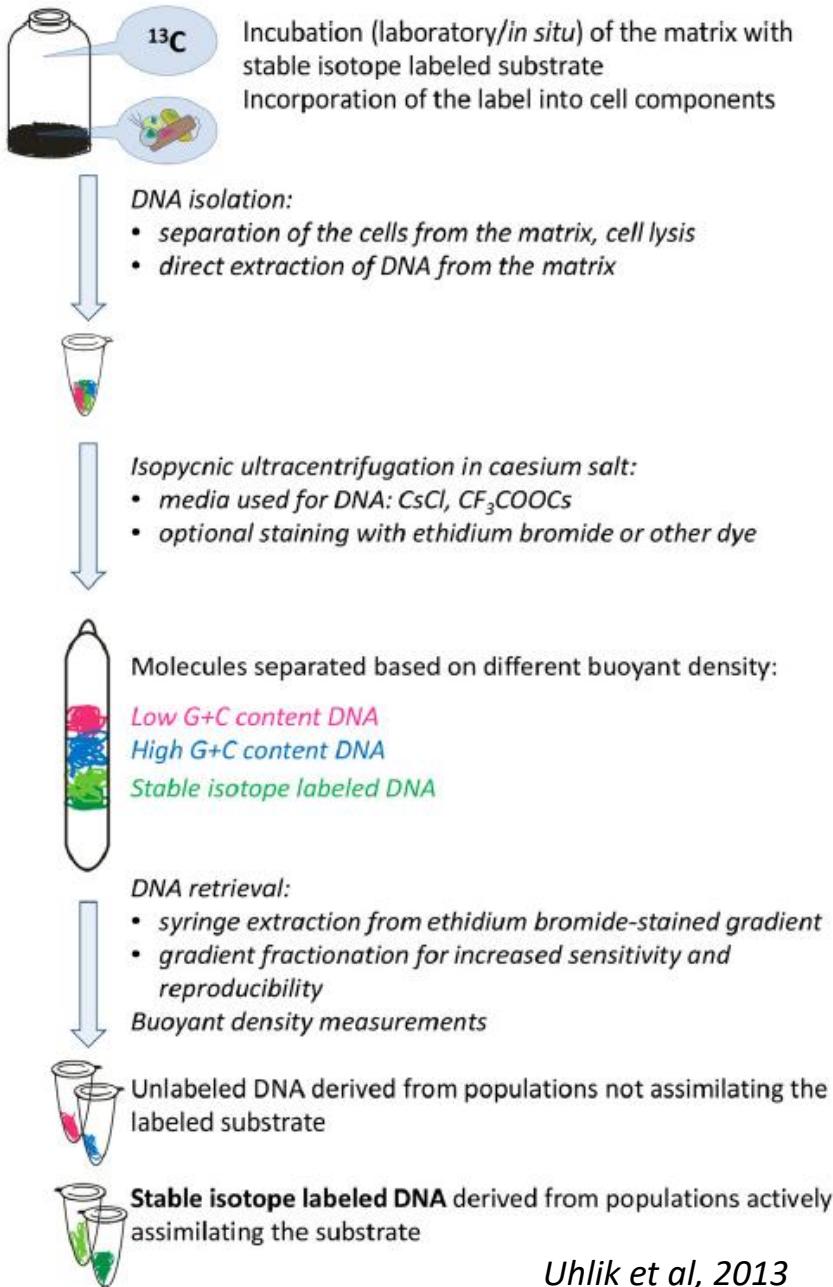
DNA-SIP fractions collected every 30s at a flow of 0.5 ml/min with a fractionator

DNA-SIP

Goal: separate heavy ^{13}C -DNA from light ^{12}C -DNA



From Neufeld, 2007



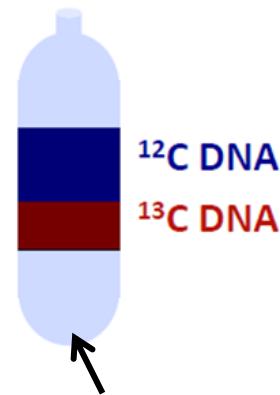
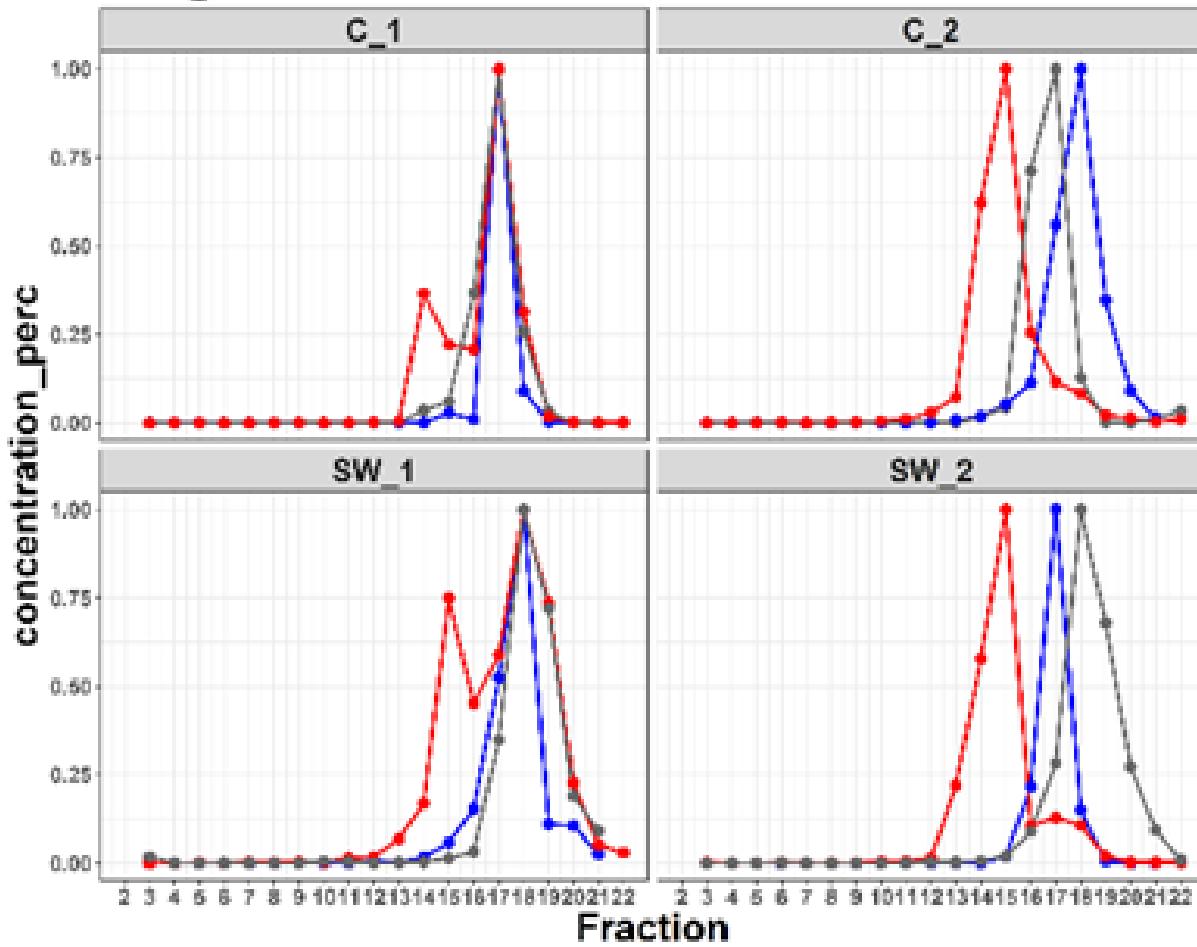
Uhlik et al., 2013

Fig. 1. Scheme of a DNA-based stable isotope probing (SIP) experiment with ^{13}C -labeled substrate.

Sometimes, there is not a clear separation of the band of ^{12}C and ^{13}C in the ultracentrifuge tubes due to the **G+C content** of bacteria. There are bacteria with high G+C content whose DNA is naturally heavier.

For that reason, always a comparison of incubations amended with a substrate **^{12}C vs ^{13}C** is necessary to compare microbial populations shifting.

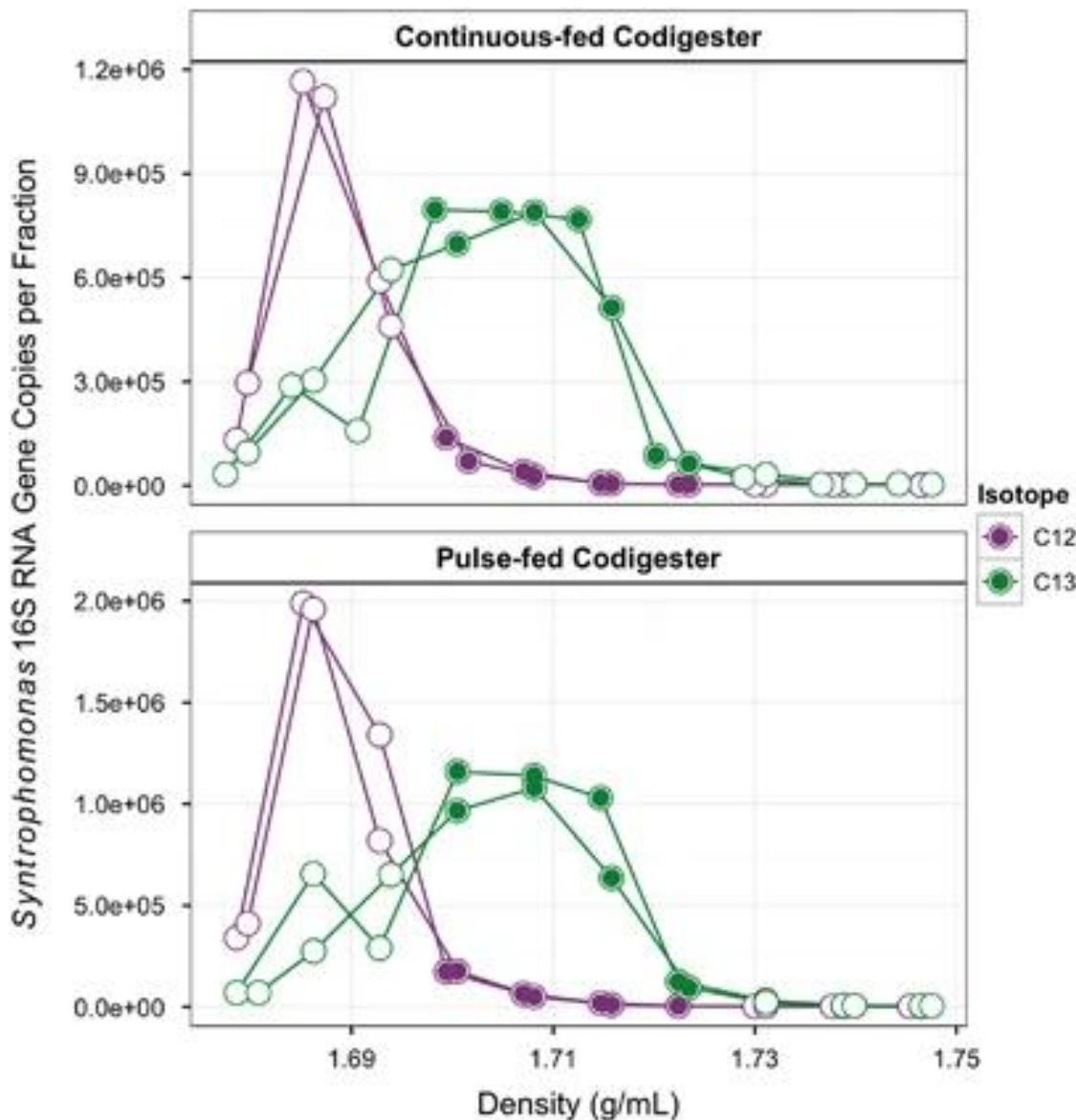
Quantitative-PCR of fractions



label

- 12A → 12C-labeled
- 13A → 13C-labeled
- N

Quantitative PCR per density



From Ziels et al, 2018, ISME J

Identifying the taxa that incorporated the label

Sequencing a pool of fractions per sample:

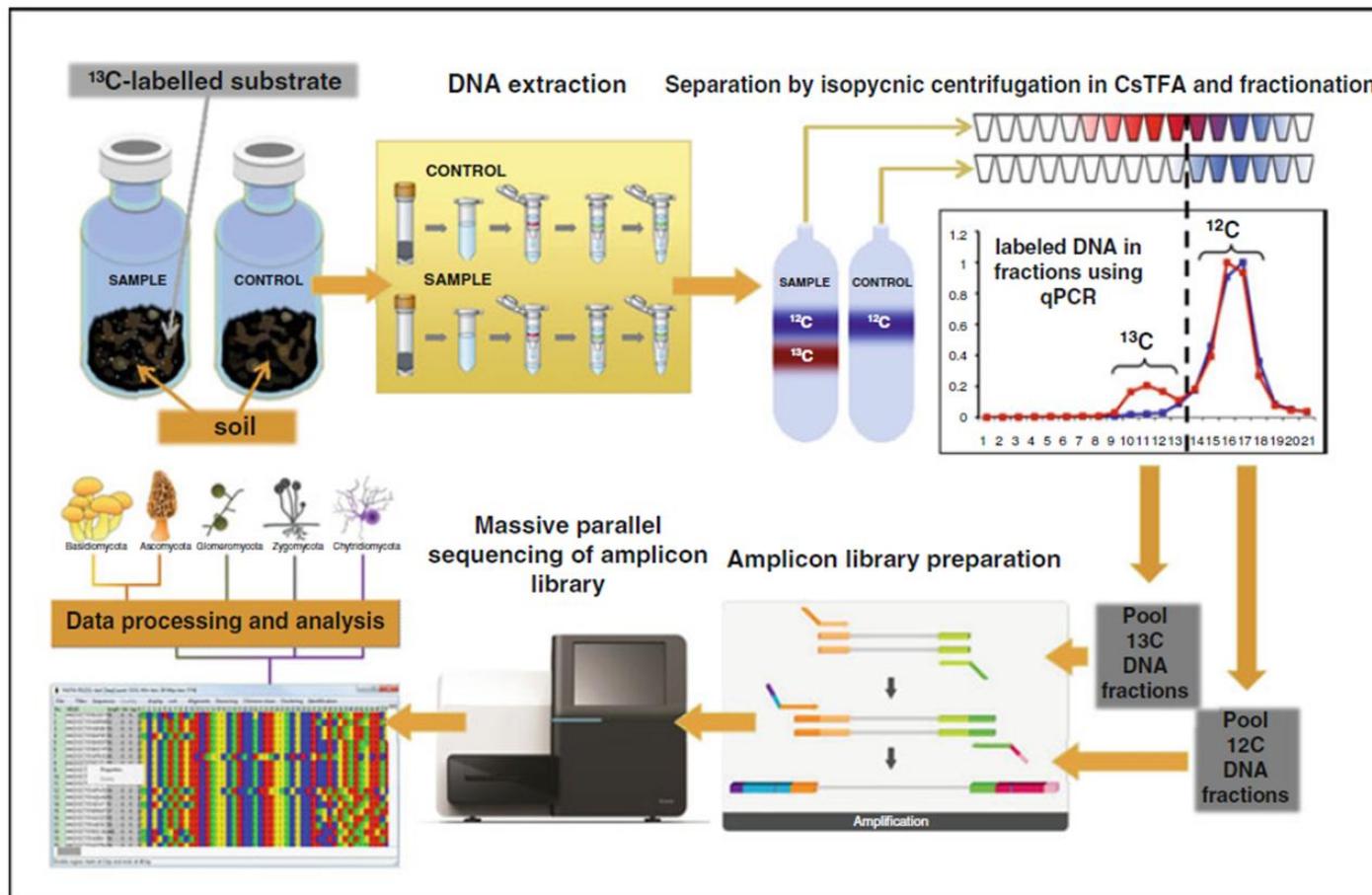


Fig. 1 Analysis of the members of fungal community involved in the decomposition of biopolymers using stable isotope probing

From Vetrovsky, Stursova, Baldrian, 2016

Identifying the taxa that incorporated the label

IF each fraction is sequenced separately, one could do →
Quantitative SIP for quantifying

- modification to SIP that enables the isotopic composition of DNA from individual bacterial taxa to be determined after exposure to isotope tracers
- **Taxon-specific density curves** are produced for labeled and nonlabeled treatments, from which the shift in density for each individual taxon in response to isotope labeling is calculated
- It estimate the extent of isotope assimilation as **atom fraction excess (AFE)**, which is the increase in the isotopic composition of DNA above background levels

qSIP

Quantitative Microbial Ecology through Stable Isotope Probing

Bruce A. Hungate^{a,b}, Rebecca L. Mau^a, Egbert Schwartz^{a,b}, J. Gregory Caporaso^{a,b,c}, Paul Dijkstra^{a,b}, Natasja van Gestel^a, Benjamin J. Koch^a, Cindy M. Liu^{d,e}, Theresa A. McHugh^{a,*}, Jane C. Marks^{a,b}, Ember M. Morrissey^a, Lance B. Price^{d,f}

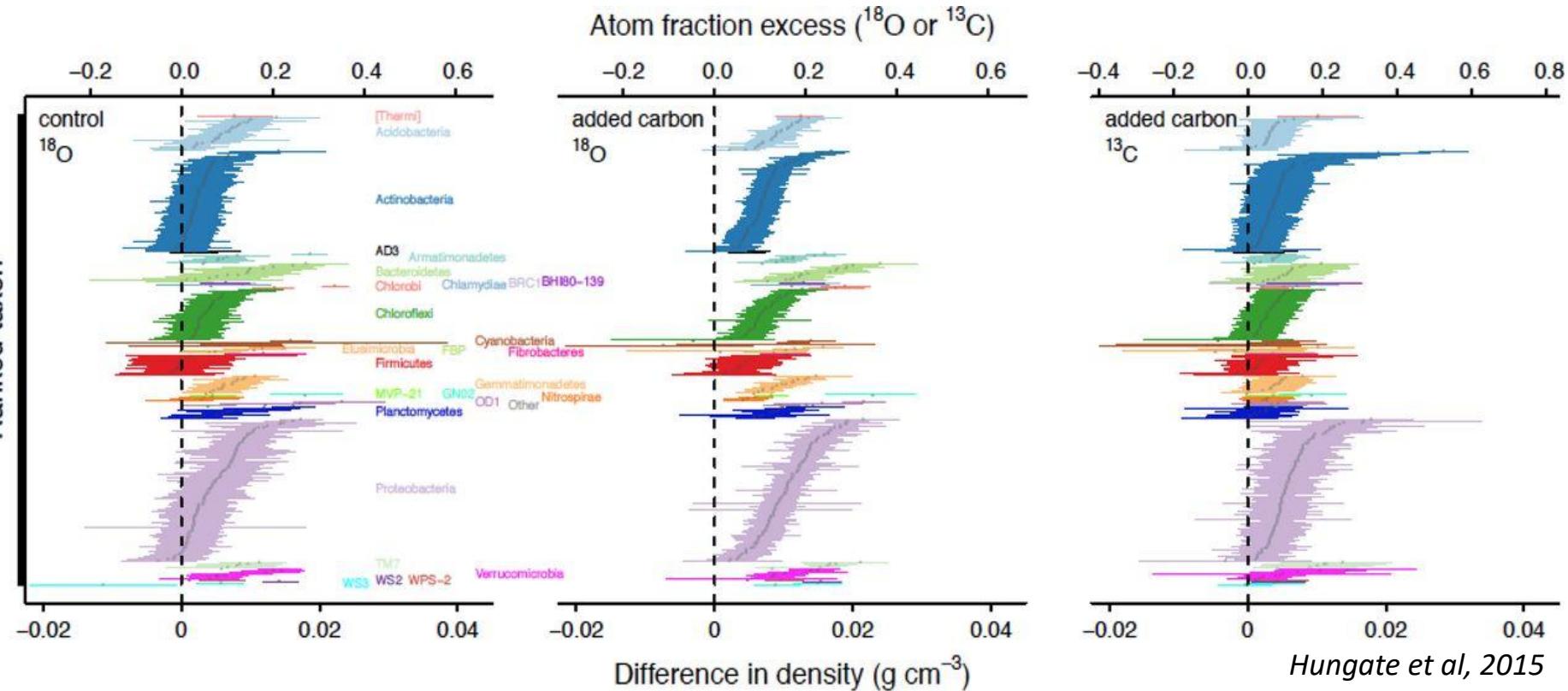
^aCenter for Ecosystem Science and Society, Northern Arizona University, Flagstaff, Arizona, USA

Expressing each **taxon's density shift** relative to that **taxon's density measured without isotope enrichment** accounts for the influence of nucleic acid composition on density and isolates the influence of isotope tracer assimilation.

The shift in density translates quantitatively to isotopic enrichment

qSIP

Taxon-specific density curves



The incorporation of the isotope tracer is expressed as **atom fraction excess**, which is the increase above the natural abundance isotopic composition and ranges from a minimum of 0 to a maximum of 1 minus the natural abundance background for a given isotope-element combination

qSIP analysis



RESEARCH ARTICLE

HTSSIP: An R package for analysis of high throughput sequencing data from nucleic acid stable isotope probing (SIP) experiments

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OPEN ACCESS

Citation: Youngblut ND, Barnett SE, Buckley DH (2018) HTSSIP: An R package for analysis of high throughput sequencing data from nucleic acid stable isotope probing (SIP) experiments. PLoS ONE 13(1): e0189616. <https://doi.org/10.1371/journal.pone.0189616>

Abstract

Combining high throughput sequencing with stable isotope probing (HTS-SIP) is a powerful method for mapping *in situ* metabolic processes to thousands of microbial taxa. However, accurately mapping metabolic processes to taxa is complex and challenging. Multiple HTS-SIP data analysis methods have been developed, including high-resolution stable isotope probing (HR-SIP), multi-window high-resolution stable isotope probing (MW-HR-SIP), quantitative stable isotope probing (qSIP), and Δ BD. Currently, there is no publicly available software designed specifically for analyzing HTS-SIP data. To address this shortfall, we have developed the *HTSSIP* R package, an open-source, cross-platform toolset for conducting HTS-SIP analyses in a straightforward and easily reproducible manner. The *HTSSIP* pack-

SIP-metagenomics

Adapting SIP analysis tools for the genomic level, rather than the 16S rRNA gene level, enables **genome-centric metagenomic SIP experiments** that establish stronger links between genomic information and in situ activity

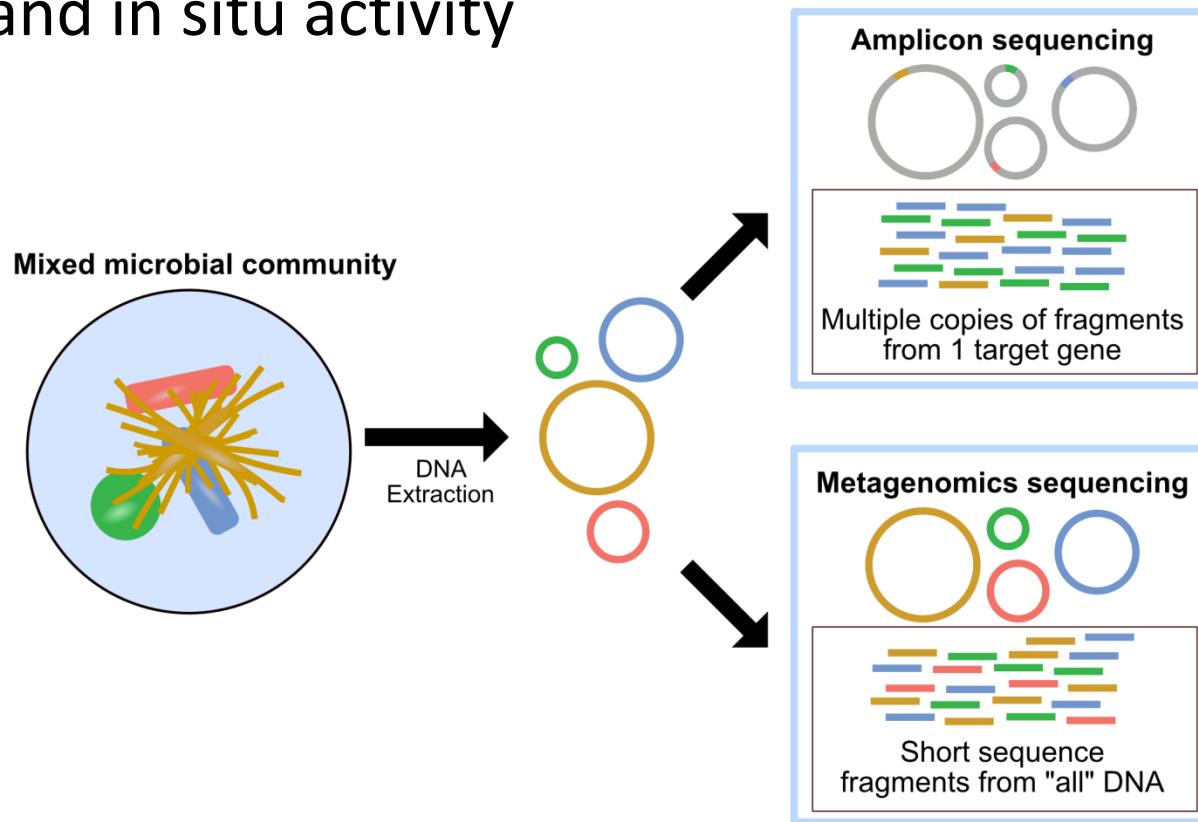


Figure from:
[https://astrobiomike.github.io/
misc/amplicon_and_metagen](https://astrobiomike.github.io/misc/amplicon_and_metagen)

SIP-metagenomics

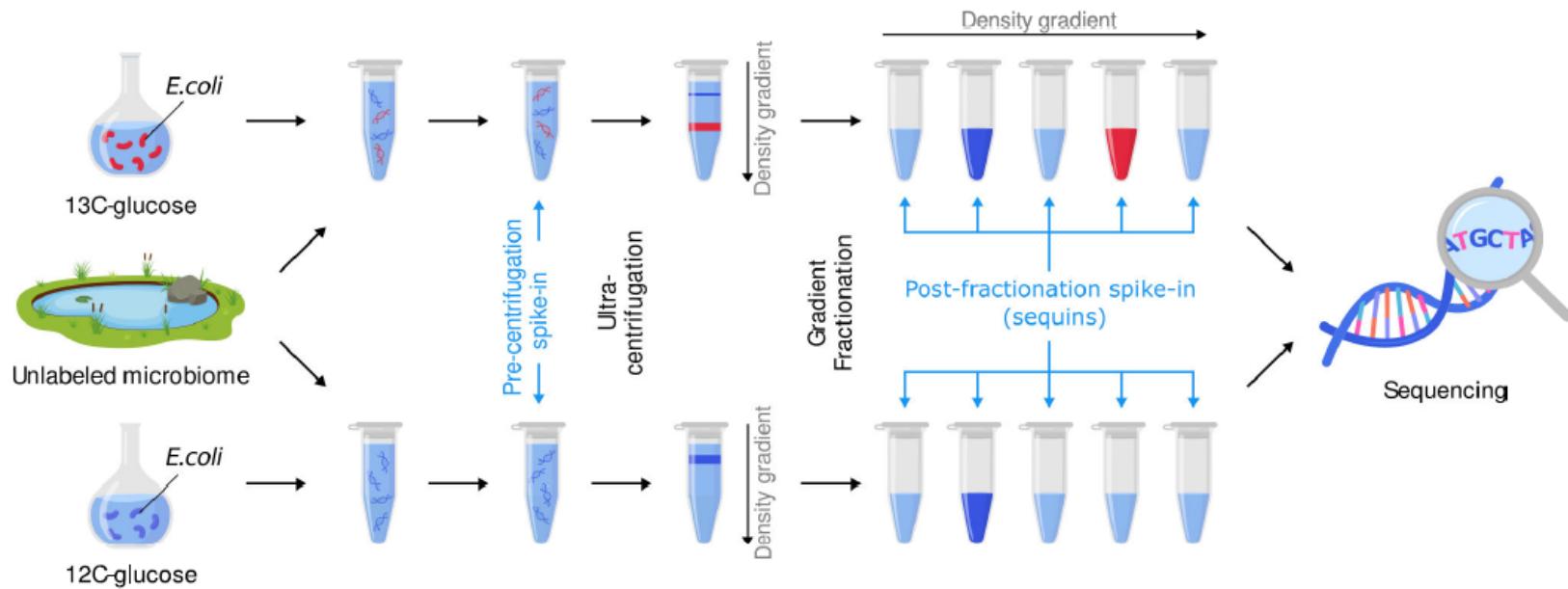
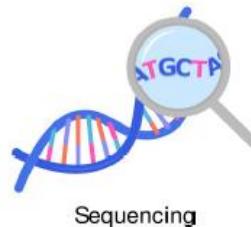


FIG 1 Experimental design and overview of laboratory steps in the SIP metagenomics workflow. To create a defined SIP experimental sample, DNA extracted from an unlabeled freshwater microbial community was amended with either labeled (¹³C) or unlabeled (¹²C) *E. coli* DNA. Pre-centrifugation spike-ins were added to each sample prior to ultracentrifugation in a CsCl gradient, and post-fractionation spike-ins (sequins) were added to each fraction after density gradient fractionation and collection. These two sets of synthetic DNA oligos served as internal standards to monitor the quality of density separations and normalize genome coverage levels.

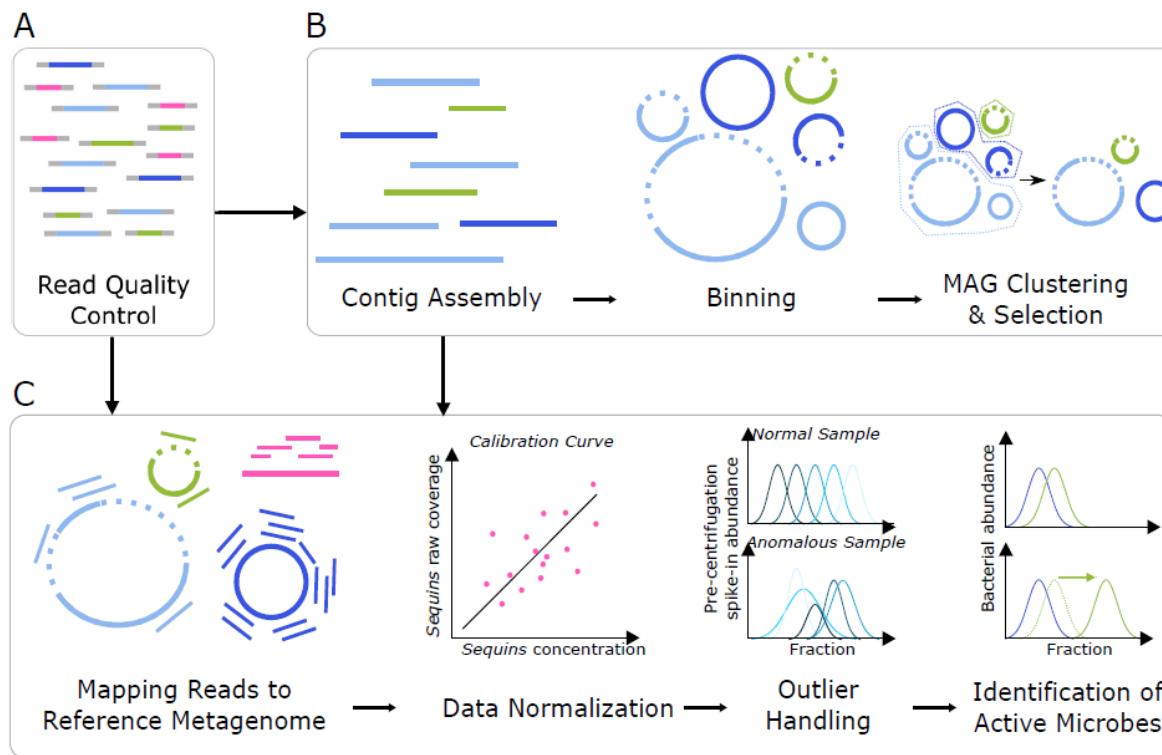
FIG 1

Vyshenska et al, 2021

SIP-metagenomics



SIPmg, an R package to facilitate the estimation of absolute abundances and perform statistical analyses for identifying **labeled genomes within SIP metagenomic data**



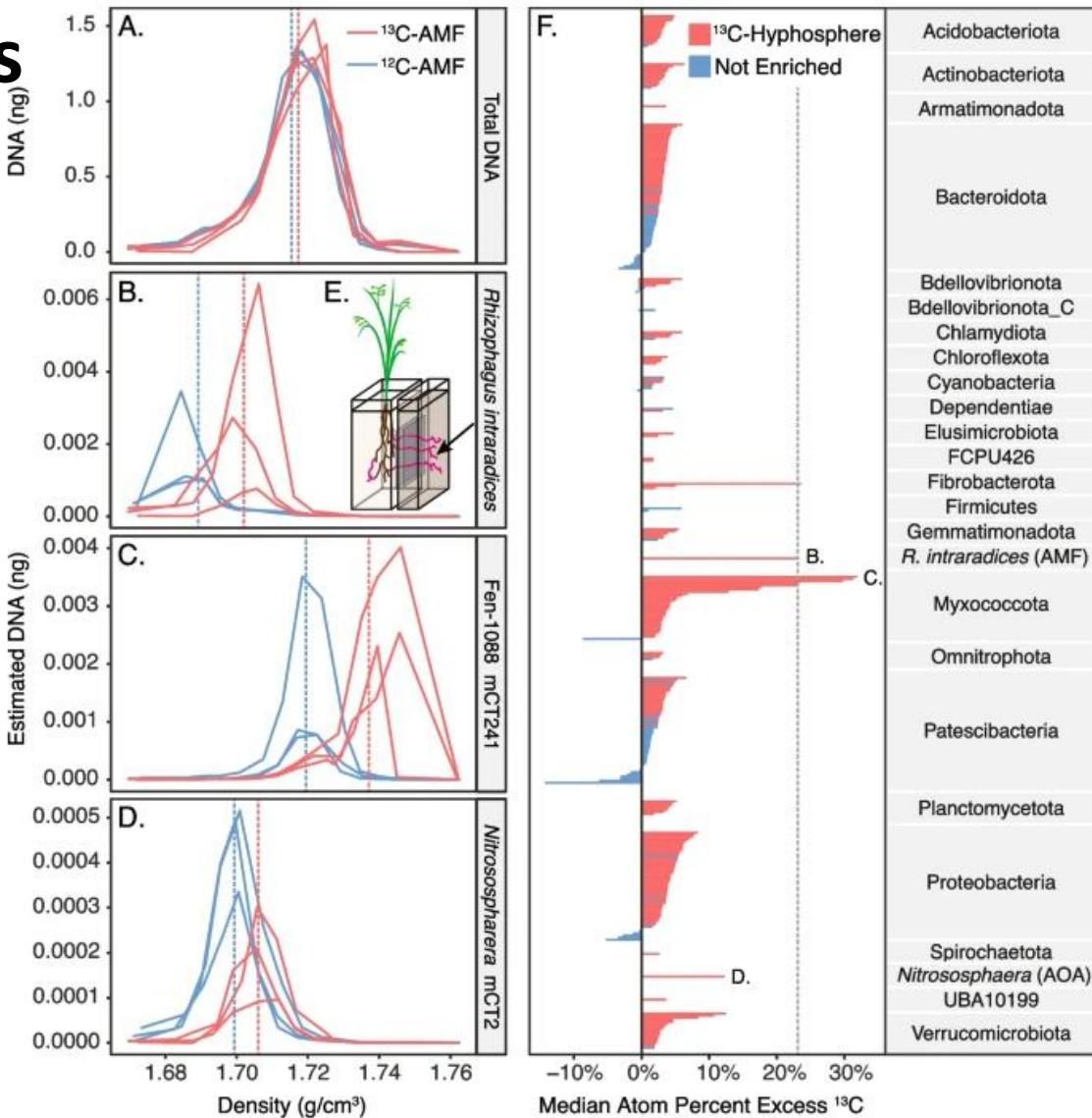
Vyshenska et al, 2021

FIG 2 The workflow scheme for SIP metagenomic data analysis includes (A) quality filtering of the raw reads and (B) generation of a unique set of medium- and high-quality MAGs used for (C) quantification of absolute taxa abundances and identification of isotope incorporators. The addition of sequins provides the means for calculating absolute bacterial abundances (C, Data Normalization), and pre-centrifugation spike-ins aid in the detection of anomalous samples (C, Outlier Handling).

qSIP-metagenomics

Estimated median atom percent excess (APE) for all assembled MAGs, where red bars indicate the 212 ^{13}C -hyphosphere MAGs with detectable isotopic enrichment and blue bars indicate 87 bulk soil MAGs that were unenriched.

MAGs are grouped by phylum, and letters indicate the APE of the taxa shown in panels B–D. The dashed gray line indicates the APE of the AMF, which supplied ^{13}C to the no-plant compartment.



Stable Isotope Probing: elements

Tracing the major elements in the food web to investigate the cycle of elements and its recycling.

Most used stable isotopes:

- ^{13}C
- ^{18}O
- ^{15}N
- ^{34}S

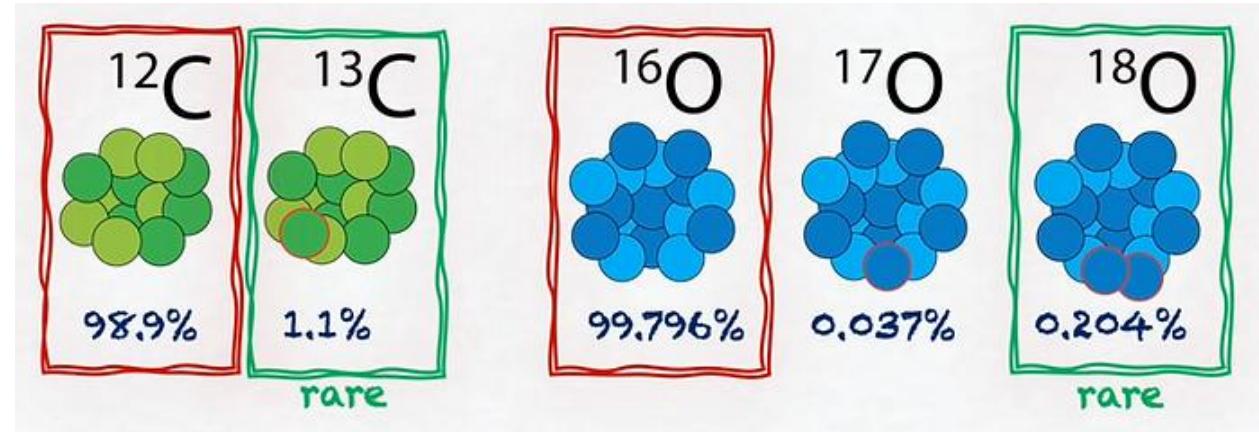


Figure modified from fabianarichter.com

Use of ^{13}C for investigating the C-cycle

Isotope: Carbon-13 (^{13}C), a stable isotope of carbon.

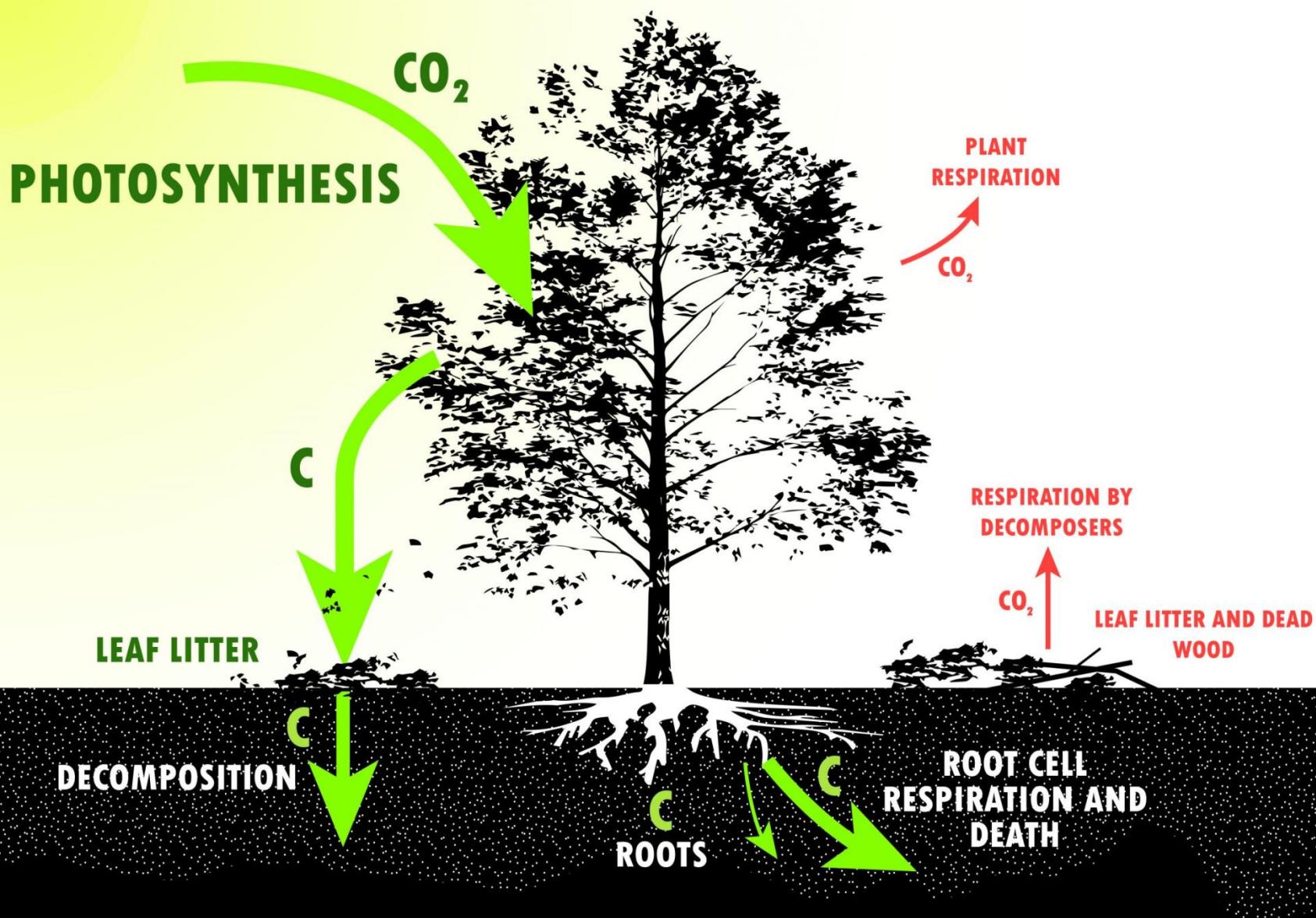
Application: It is the most commonly used SIP, mostly for feeding microorganisms a substrate labeled with ^{13}C (e.g., glucose- ^{13}C , acetate- ^{13}C , cellulose- ^{13}C) and tracing the incorporation of ^{13}C into microbial DNA, RNA, proteins, or lipids.

Purpose: To study microbial communities and their metabolic processes, such as carbon cycling and substrate utilization in environments like soils, water, or the gut.

Who is eating what?

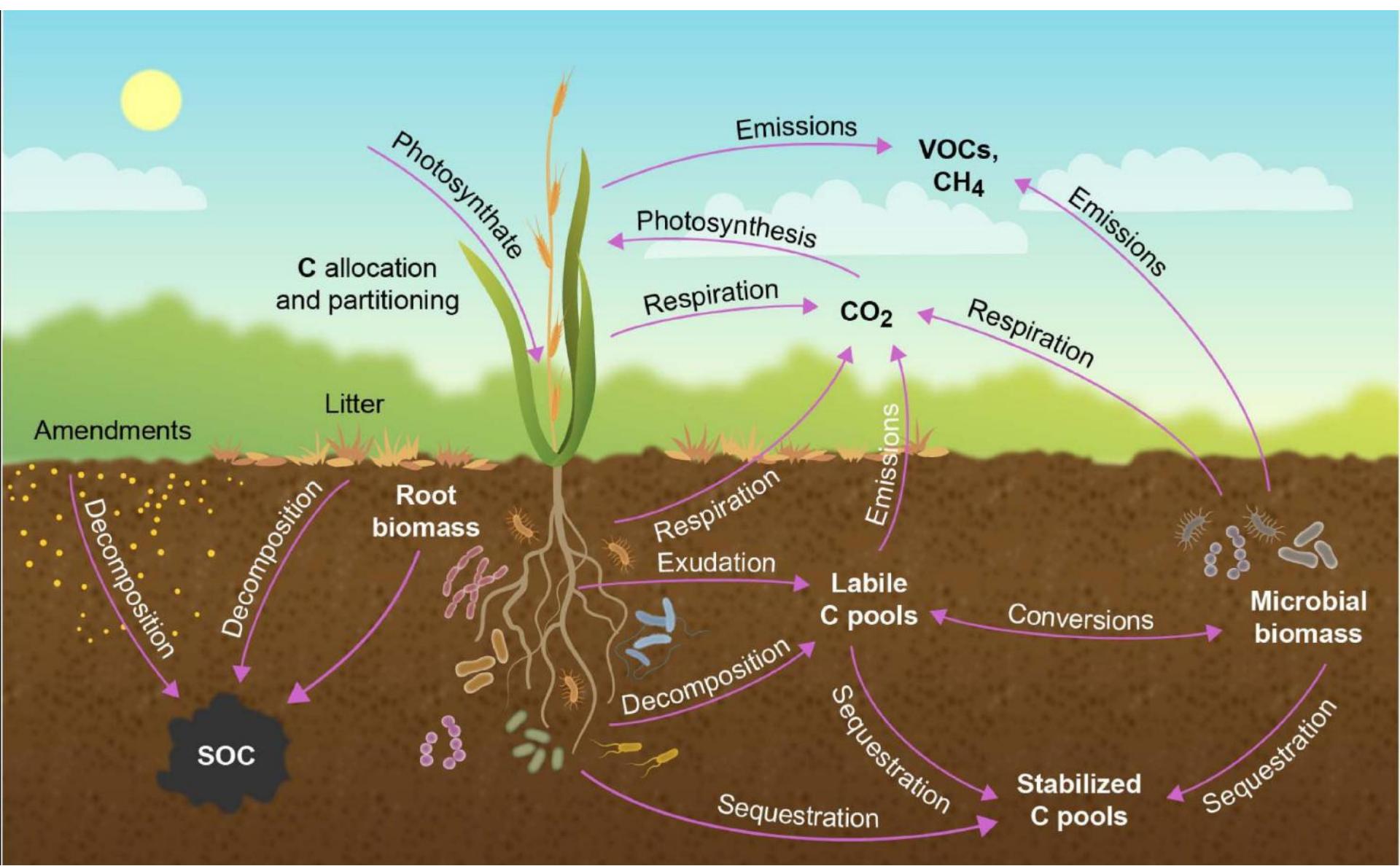
The Carbon cycle in forest soils

[Figure from dlnr.hawaii.gov](http://dlnr.hawaii.gov)



The Carbon cycle in soils

Figure from Jansson et al, 2021 *Front. Plant Sci.*
<https://doi.org/10.3389/fpls.2021.636709>



Experiment

Stable Isotope-Labeled Substrates

¹³C Plant biomass



¹³C Cellulose



¹³C Fungal biomass
(*Phanerochaete velutina* PV29)



¹³C Hemicellulose



¹³C Bacterial biomass
(*Streptomyces* sp. PR6)



¹³C Glucose



- Parallel microcosms with **unlabeled substrates**
- Control with **no substrate** addition
- Incubation 10°C (dark)
- Sampling points at: **0, 7, 14 and 21 days**



Respiration
(CO₂-SIP)

Biomass
(PLFAs-SIP)

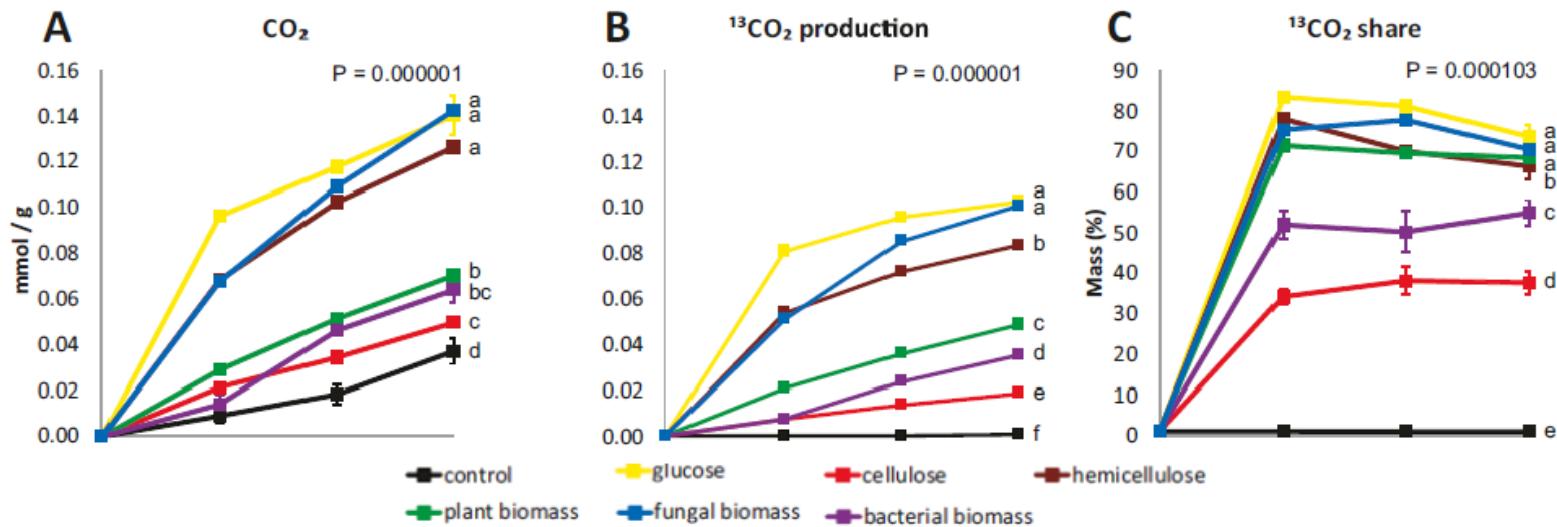
Community analysis
(DNA-SIP)

Amplicon sequencing
(16S & ITS)

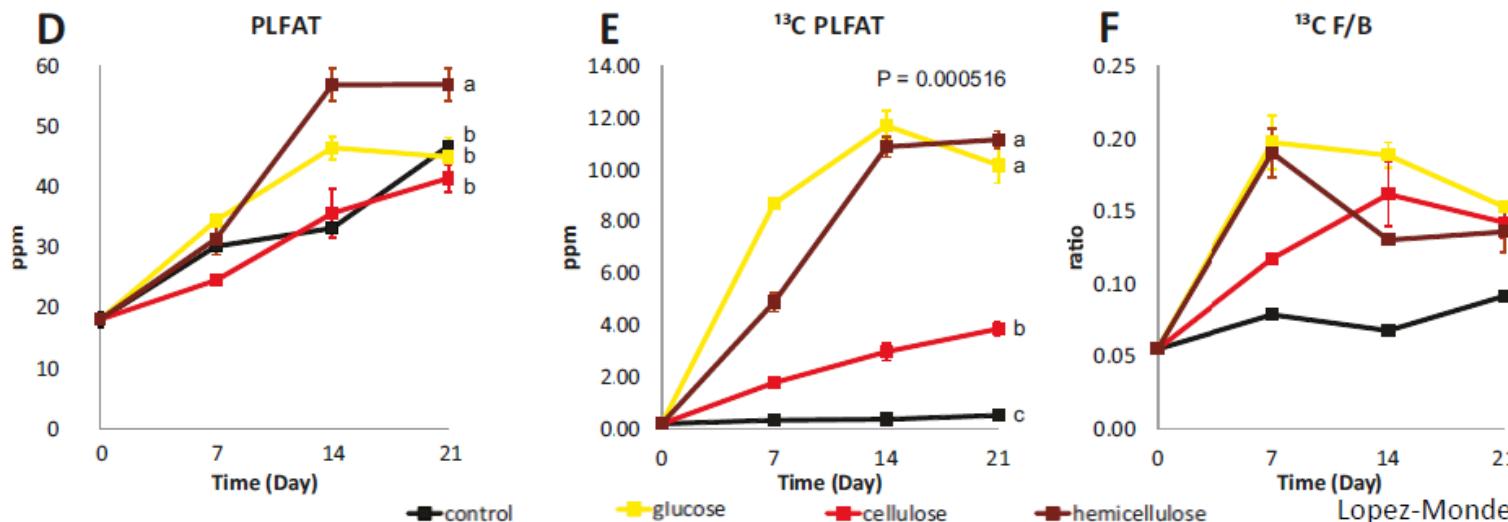
Shotgun sequencing
(CAZymes & MAGs)

Results

1) Respiration of different substrates (CO_2 -SIP)



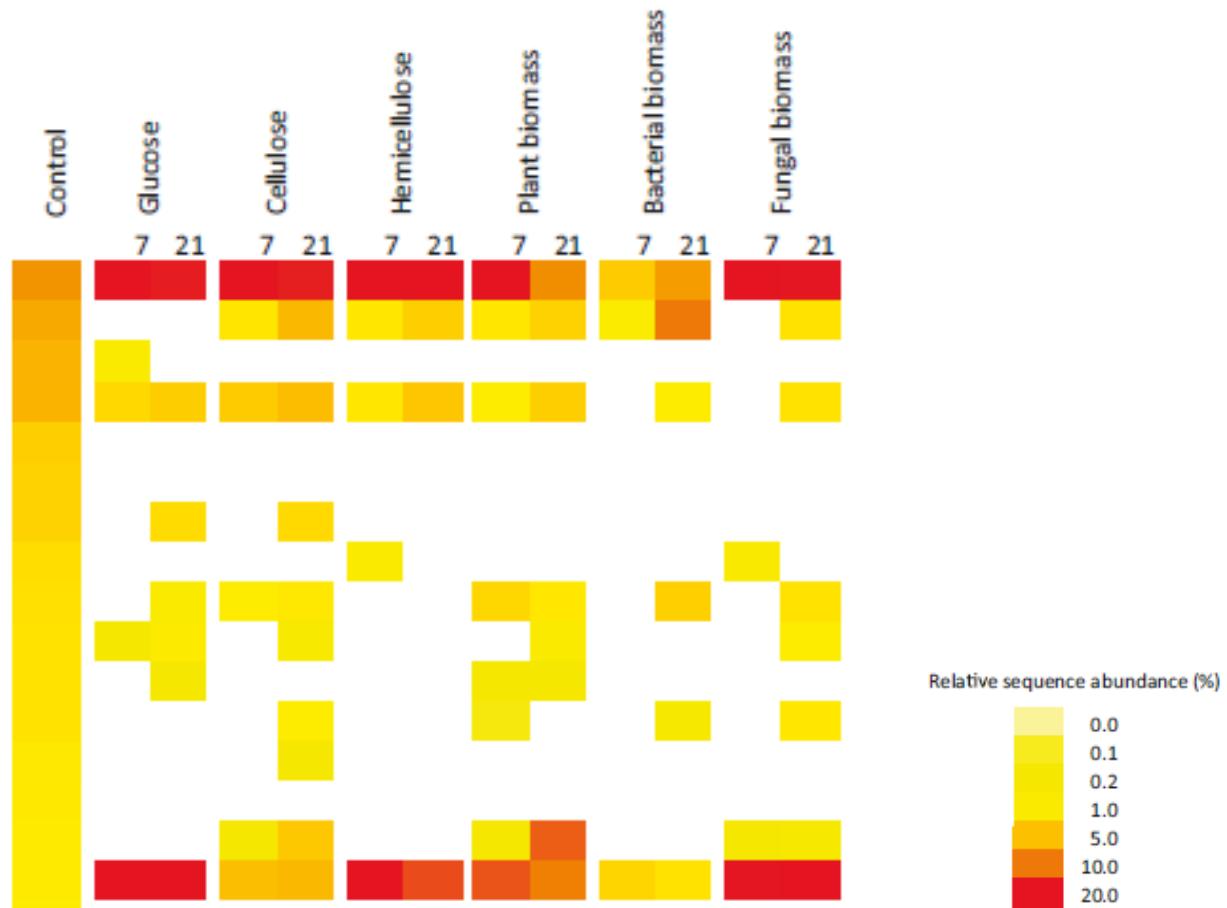
2) Microbial biomass in different substrates (PLFAs-SIP)



Microbial incorporators: Bacteria

Bacteria

Mucilaginibacter (Bacteroidetes)
Granulicella (Acidobacteria)
Actinoallomorus (Actinobacteria)
Rhizomicrombium (Alphaproteobacteria)
Bradyrhizobium (Alphaproteobacteria)
Actinomadura (Actinobacteria)
Conexibacter (Actinobacteria)
Pseudomonas (Gammaproteobacteria)
Acidobacterium (Acidobacteria)
Chthoniobacter (Verrucomicrobia)
Adipipila (Acidobacteria)
Prosthecobacter (Verrucomicrobia)
Candidatus Solibacter (Acidobacteria)
Mycobacterium (Actinobacteria)
Cytophaga (Bacteroidetes)
Burkholderia (Betaproteobacteria)



Microbial incorporators: Fungi



From Lopez-Mondejar, ISME J, 2018

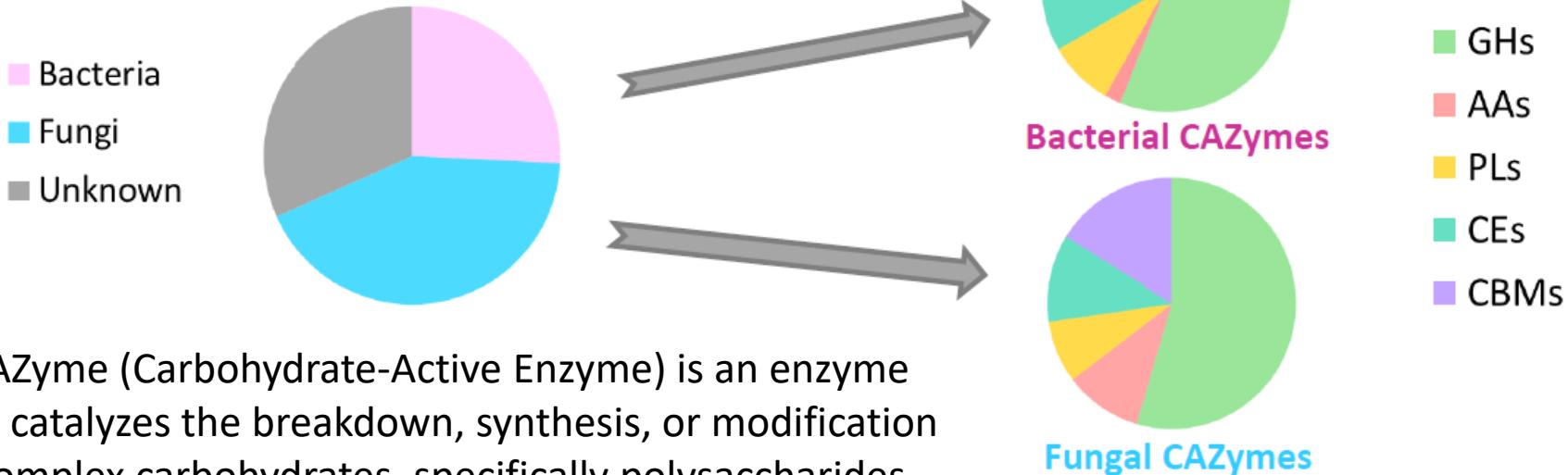
DNA-SIP-metagenomics

Results II

Shotgun sequencing
(CAZymes & MAGs)

Relative abundance of CAZymes in fungal and bacterial communities

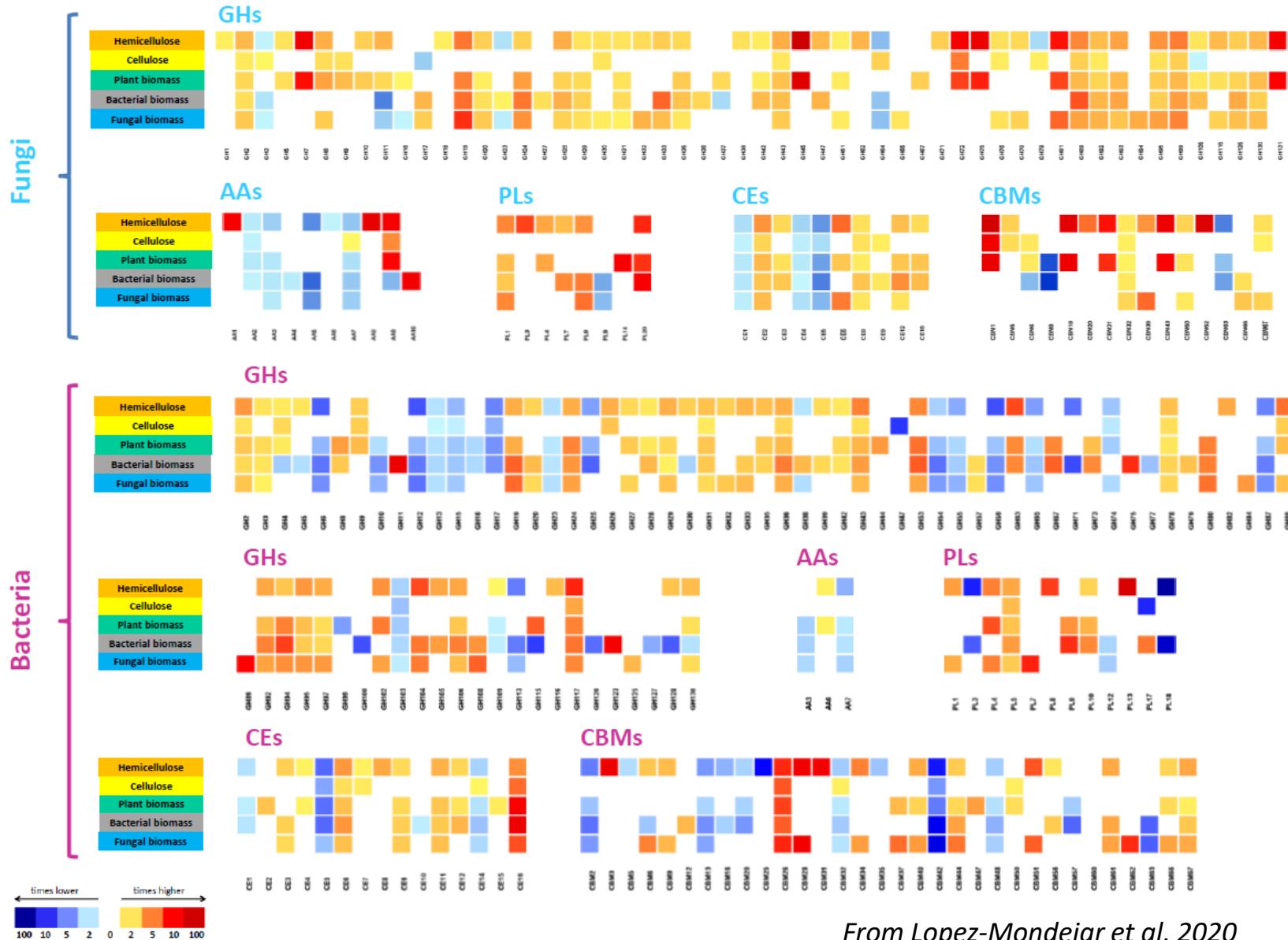
- We found 7.2 millions of predicted proteins in the whole metagenome
- > 132 000 CAZymes in the metagenome



From Lopez-Mondejar

DNA-SIP-metagenomics

Abundance of CAZy families observed in each substrate respect to the control



From Lopez-Mondejar et al, 2020

Nitrogen Stable Isotope Probing (^{15}N -SIP)

Isotope: Nitrogen-15 (^{15}N), a stable isotope of nitrogen.

Application: Similar to ^{13}C -SIP, but the labeled substrate is nitrogen-based (e.g., ammonium- ^{15}N , nitrate- ^{15}N). The incorporation of ^{15}N into microbial DNA or other cellular components is then traced.

Purpose: Used to study nitrogen cycling, such as nitrogen fixation, nitrification, and denitrification processes in microbial communities.

An example using ^{15}N and RNA-SIP

environmental
microbiology

Environmental Microbiology (2018) 20(1), 44–61



doi:10.1111/1462-2920.13954

Application of stable-isotope labelling techniques for the detection of active diazotrophs

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Summary

Investigating active participants in the fixation of dinitrogen gas is vital as N is often a limiting factor

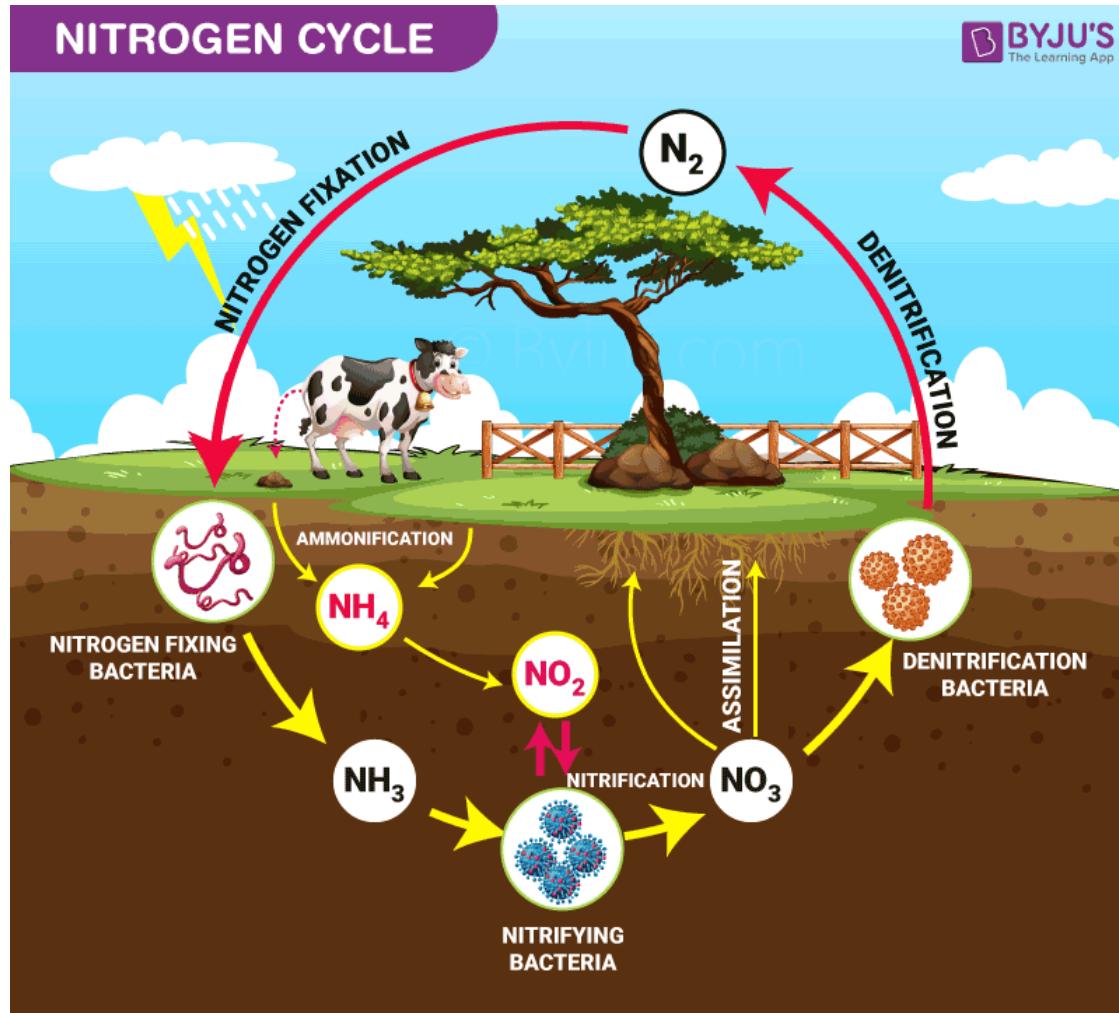
using SIP-Raman microspectroscopy for detecting ^{15}N -labelled cells. Taken together, these tools allow identifying and investigating active free-living diazotrophs in a highly sensitive manner in diverse environments, from bulk to the single-cell level.

Introduction

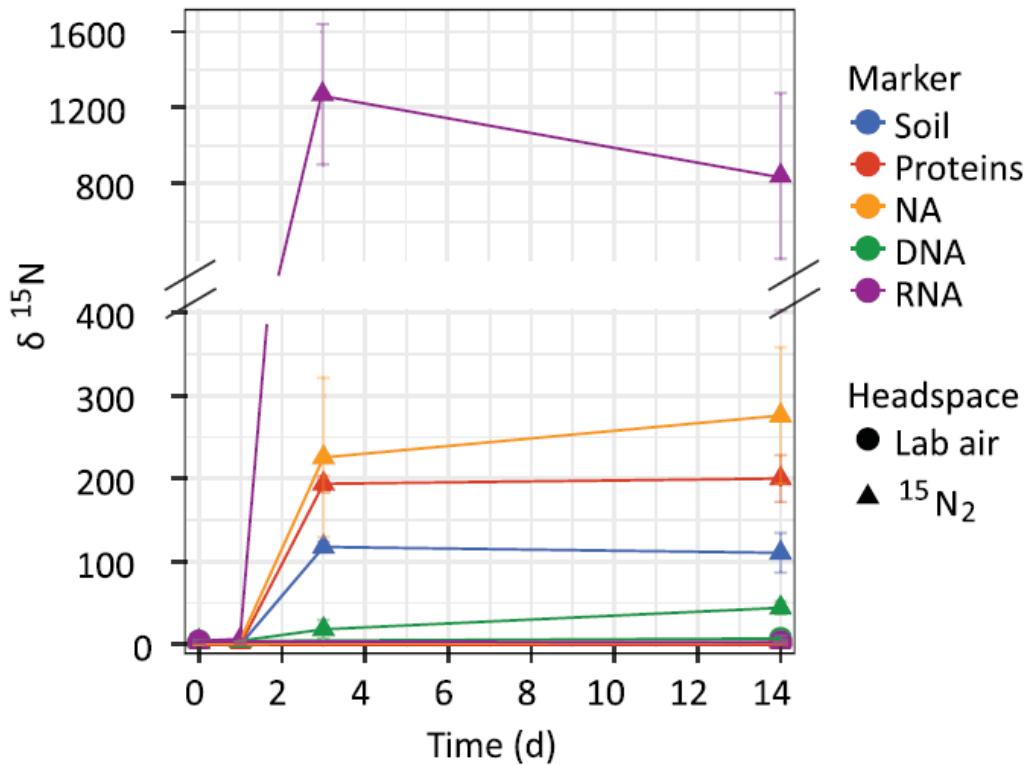
The terrestrial nitrogen (N) cycle is essential for the Earth’s biosphere and is intimately linked to microbial activity. Biological N_2 fixation, that is, the reduction of atmospheric dinitrogen gas (N_2) to ammonia, is of paramount importance as N is often a limiting factor for primary production. Excluding anthropogenic contributions, most of the fixed N in nature is provided through biological N_2 fixation, which is performed by one guild of microorganisms (bacteria and archaea) – the N_2 fixers or diazotrophs. Symbiotic (such as

Purpose of study / background

Diazotrophs are bacteria or archaea that fix N_2



An example using ^{15}N and RNA-SIP



RNA-SIP has multiple advantages over DNA-SIP, such as

- rapid tracer incorporation,
- labelling which is independent of cellular replication
- no suffering from the G+C effect

Fig. 2. Average \pm standard error of ^{15}N enrichment in bulk soil and different microbial biomolecules measured by EA-IRMS. Forest soil samples were incubated for up to 14 days in an atmosphere containing $^{15}\text{N}_2$, or in lab air as a control.

The ^{15}N was determined by elemental analysis-isotope ratio mass spectrometry (EA-IRMS)

Identifying specific OTUs involved in $^{15}\text{N}_2$ fixation using RNA- and DNA-SIP

50 R. Angel et al.

Table 1. Taxonomic classification of the enriched OTUs in the RNA- and DNA-SIP gradients.

RNA-SIP	Domain	Phylum	Class	Order	Family	Genus
OTU 2 ^a	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium_sensu_stricto</i>
OTU 4	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium_sensu_stricto</i>
OTU 398	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium_sensu_stricto</i>
OTU 20	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>
OTU 83	Bacteria	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	<i>Streptomyces</i>
OTU 27	Bacteria	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	<i>Streptomyces</i>
OTU 54	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	<i>Paenibacillus</i>
OTU 272	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Rhodopseudomonas</i>
OTU 86	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	<i>Arthrobacter</i>
OTU 292	Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Unclassified
OTU 344	Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Unclassified
DNA-SIP	Domain	Phylum	Class	Order	Family	Genus
OTU 2	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium_sensu_stricto</i>
OTU 398	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium_sensu_stricto</i>
OTU 15	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium_sensu_stricto</i>
OTU 43	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Idiomarinaceae	<i>Aliidiomarina</i>
OTU 157	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Clostridium_sensu_stricto</i>
OTU 204	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	<i>Brevundimonas</i>

a. Rows in bold represent highly enriched OTUs.

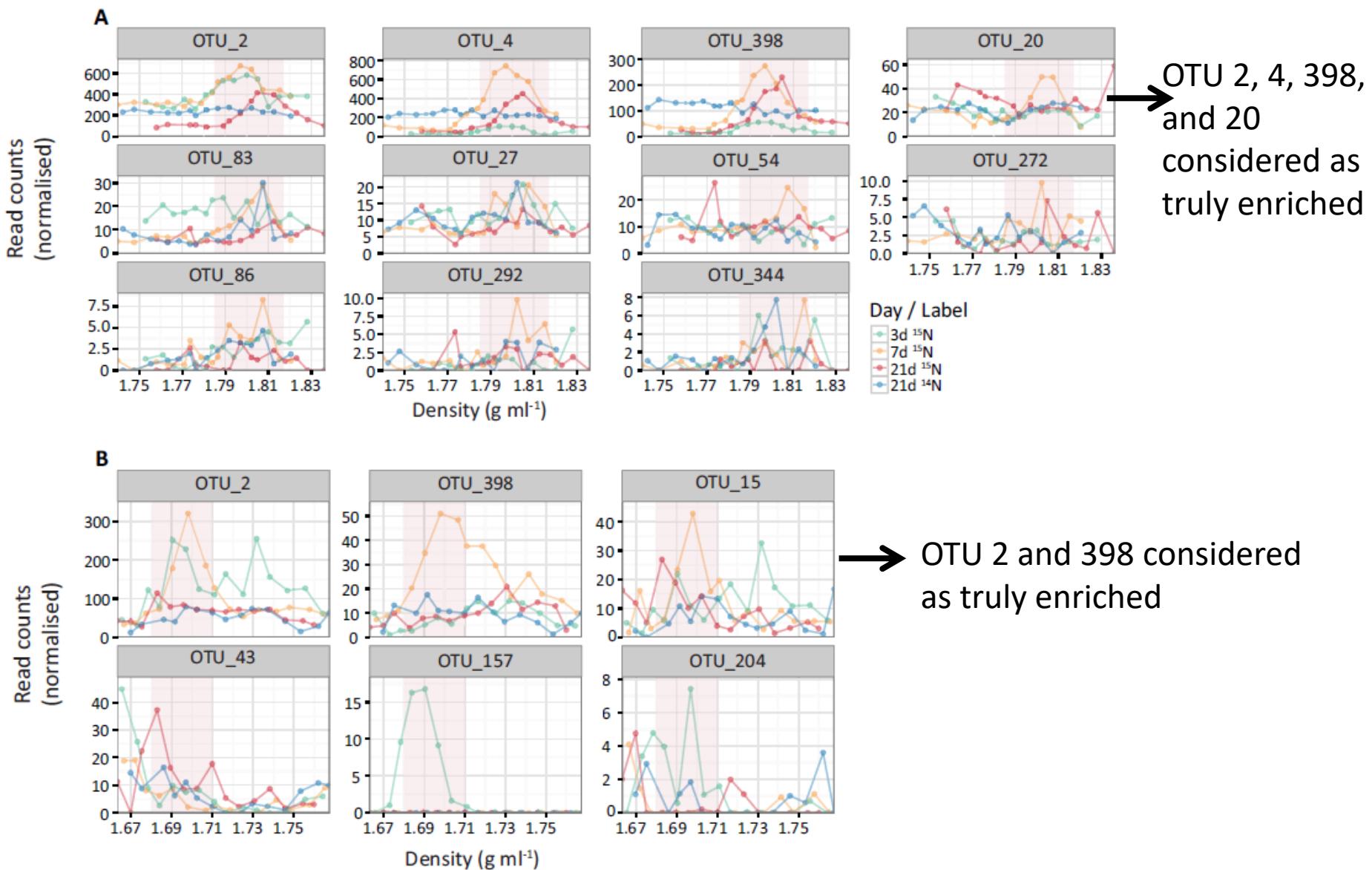


Fig. 6. Normalised OTU abundances of enriched OTUs along the density gradients from RNA-SIP (panel A) and DNA-SIP (panel B). Shaded areas indicate where labelled template is expected to concentrate.

Use of heavy water for investigating microbial activity



RESEARCH ARTICLE

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<https://doi.org/10.1128/msystems.00417-22>

Quantitative Stable-Isotope Probing (qSIP) with Metagenomics Links Microbial Physiology and Activity to Soil Moisture in Mediterranean-Climate Grassland Ecosystems

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Use of heavy water for investigating microbial activity, an example

heavy water (H_2^{18}O) DNA-SIP → ***Who is active?***

Purpose: identify **actively growing soil microorganisms and their genomic capacities** in three Mediterranean grassland soils across a rainfall gradient to evaluate the hypothesis that historic precipitation levels are an important factor controlling traits

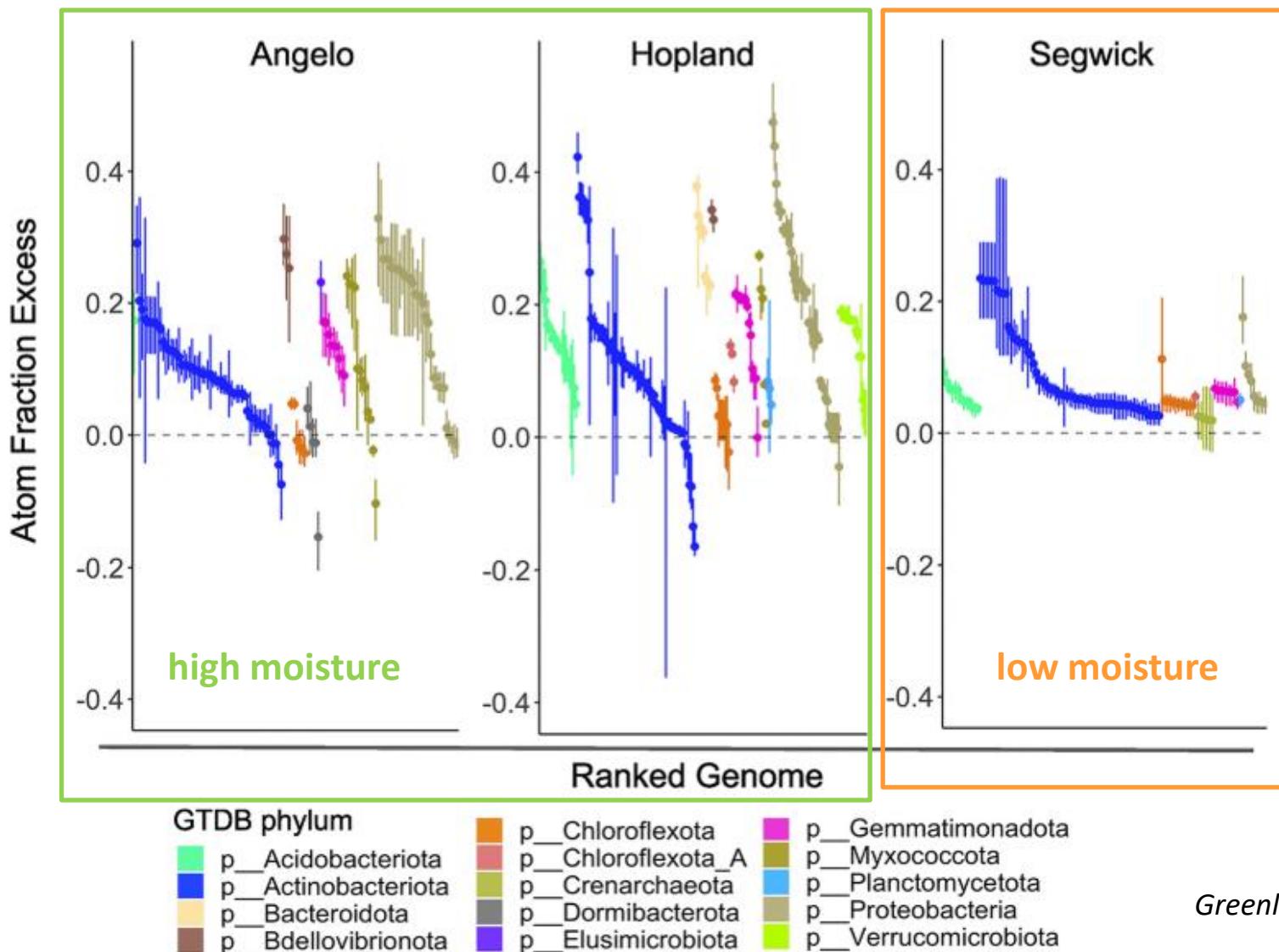
Technique: SIP with **^{18}O -labeled water** to identify growing microorganisms, relying on the universal assimilation of water-derived oxygen during DNA synthesis.

The assimilation of ^{18}O increases the buoyant density of DNA, making it separable from nonlabeled DNA by centrifugation in cesium chloride

Analysis: genome-resolved metagenomic implementation of qSIP

- qSIP enabled measurement of **taxon-specific growth** because isotopic incorporation into microbial DNA requires production of new genome copies (active subset of soil community members)
- qSIP **identify their characteristic ecophysiological traits.**

Use of heavy water, an example

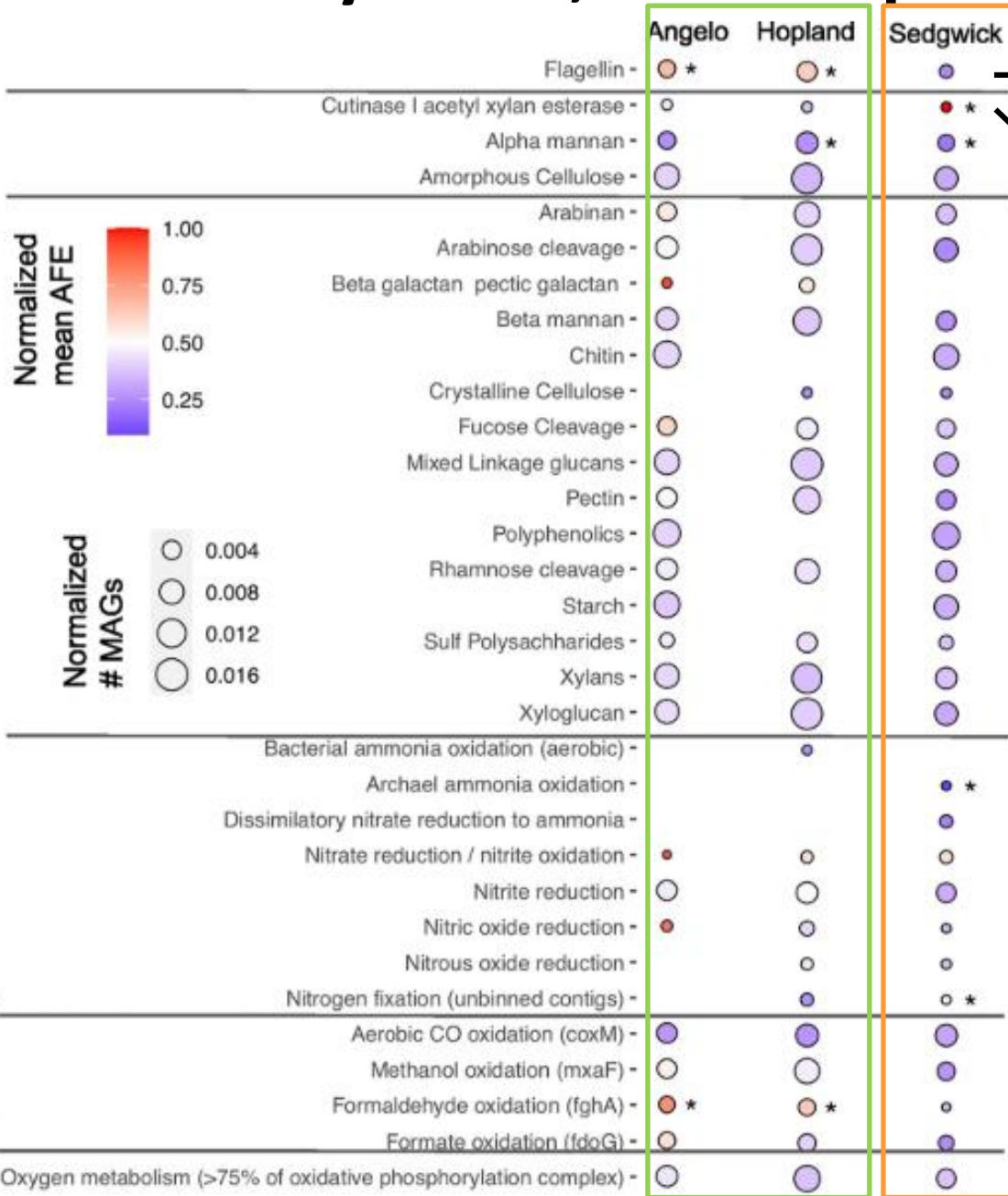


Greenlon et al, 2022

Mean and 95% confidence intervals of ^{18}O atom fraction excess (AFE) for all genome bins measured in the three California grassland soils, colored and ranked by phylum

Use of heavy water, an example

A.
B.



Flagellin, significantly active at **high moisture** sites

Cutinase, significantly active at **low moisture** site, Sedgwick

polysaccharide metabolism

C.

nitrogen metabolism

D.

E.

F.

SIP- Conclusions

- 1. Links Function to Identity** - It connects microbial activity (function) to the specific microbes (identity) responsible for that activity
- 2. Cultivation-independent** - SIP works *in situ* without needing to culture
- 3. Specificity for active populations** SIP only labels organisms that are actively using the labeled substrate, helping distinguish the functionally relevant members of a community from the passive or inactive ones
- 4. Versatile for many isotopes and compounds** - various stable isotopes (^{13}C , ^{15}N , ^2H , ^{18}O , ^{34}S) and substrates (glucose, acetate, CO_2 , methane, etc.) depending on your target metabolic process. Flexibility enables study of diverse processes like methanogenesis, sulfate reduction, nitrogen fixation, etc.
- 5. Works in Complex Environments** - soil, water, sediments, gut microbiomes
- 6. Quantitative Insights (with qSIP)** - measuring the extent of isotope incorporation, which reflects microbial growth or substrate assimilation rates
- 7. Compatibility with Multi-Omics** Metagenomics (see functional genes), Metatranscriptomics (active gene expression), Proteomics (track protein synthesis) Lipidomics/DNA/RNA SIP → to target specific biomolecules.
- 8. Tracks Metabolic Pathways and Nutrient Flows** - SIP provides insight into carbon, nitrogen, sulfur, and other elemental cycles, helping understand who does what in nutrient cycling and energy flow.

