Stable Isotope Probing

EMD Team Fact Sheet—November 2011

This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.

Why is stable isotope probing relevant?

Stable isotope probing (SIP) techniques are used to determine whether biodegradation of a specific contaminant can or does occur at a contaminated site. If a specific biodegradation process is detected, SIP approaches can also be used to identify the microorganisms responsible for this activity. A unique feature of SIP approaches that distinguishes them from virtually all other EMDs is that they do not require any prior knowledge of the microorganisms, genes, or enzymes involved in a specific biodegradation processes and can therefore be applied to novel and otherwise uncharacterized contaminants.

What does SIP do?

Collectively, SIP approaches all use isotopically labeled contaminants to detect and quantify biodegradation processes and to characterize the microorganisms responsible for these activities. These approaches are "culture-independent" methods that minimize biases inherent in other techniques that identify and quantify microorganisms after they have been extracted from environmental samples and cultivated in laboratories as either individual pure strains or mixed microbial communities. SIP is also substantially different from compound specific isotope analysis (CSIA, see the CSIA Fact Sheet), in two key respects. First, SIP approaches require contaminants that have been artificially enriched with high levels of stable isotopes such as ^{13}C and ^{15}N. In contrast, CSIA examines only changes in the naturally occurring low levels of stable isotopes found in contaminants. Second, SIP focuses on analyzing changes in the isotopic composition of biomolecules (e.g., lipids, nucleic acids, proteins) derived from microorganisms. In contrast, CSIA focuses on analyzing the isotopic composition of contaminants themselves. Most SIP approaches have been developed over the last decade, and their inherent simplicity and versatility has led to the introduction of several commercial applications of these powerful techniques in the environmental field. SIP approaches have now been used to characterize the biodegradation of many contaminants including polycyclic aromatic hydrocarbons (PAHs), fuel oxygenates, pesticides, and gasoline constituents, including benzene, toluene, ethylbenzene, and xylenes (see Table 1).

How are the data used?

Data generated from SIP approaches can be used to establish whether biodegradation of a specific contaminant can or does occur at a site. Depending on which approach is used, SIP techniques can also provide additional information that ranges from identification of broad groups of microorganisms through to identification of specific organisms, genes, and enzymes involved in a particular biodegradation process. These data can be used to simply demonstrate the occurrence of a particular biodegradation process. Data from SIP analyses can also be used to confirm the effectiveness of existing remediation processes or aid in the design of remediation approaches.

Example Environmental Remediation Questions SIP Can Help Answer

- **Site Characterization**
  - Are microorganisms present that are capable of degrading the contaminant?
- **Remediation**
  - Can biodegradation of a contaminant occur within a particular set of environmental conditions?
- **Monitoring**
  - Is biodegradation of a contaminant occurring?

Table 1. Examples of recent applications of SIP to important contaminants

Contaminant	Biomolecule	Redox conditions	Isotope	Lab or field	Reference	PMID[a]
Benzene	DNA	Aerobic	^{13}C	lab	Xie et al. 2010	20549308
Benzene	DNA	Sulfate reducing	^{13}C	lab	Herrmann et al. 2010	19840104
Benzene	DNA	Methanogenic	^{13}C	lab	Sakai et al. 2009	19914583
Benzene	DNA	Aerobic and anaerobic	^{13}C	both	Liou et al. 2008	18430012
Benzene	DNA	Iron reducing	^{13}C	lab	Kanapuli et al. 2007	18043671
Benzene	RNA	Aerobic and anaerobic	^{13}C	lab	Kasai et al. 2006	16672506
Benzene and toluene	PLFA	Anaerobic	^{13}C	field	Geyer et al. 2005	16053100
Benzoate	DNA	Aerobic	^{13}C	field	Pumphrey and Madsen 2008	18469130
Biphenyl	DNA	Aerobic	^{13}C	lab	Uhlik et al. 2009	19700551
Methane	PLFA	Aerobic	^{13}C	lab	Bodelier et al. 2009	19194481
Methane	PLFA and RNA	Aerobic	^{13}C	field	Qiu et al. 2008	18385771
Methyl chloride	DNA	Aerobic	^{13}C	lab	Borodina et al. 2005	16104855
Pentachlorophenol	RNA	Aerobic	^{13}C	lab	Mahmood et al. 2005	16104858
Perchloroethylene	DNA	Dehalorespiring	^{13}C	lab	Kittelmann and Friedrich 2008	18211265
Phenol	RNA	Nitrate reducing	^{13}C	lab	Sueoka et al. 2009	19146573
Phenol	DNA	Aerobic	^{13}C	field	DeRito et al. 2005	16332760
Polyaromatic hydrocarbons	DNA	Aerobic	^{13}C	lab	Singleton et al. 2005	15746319
Polychlorinated biphenyls	PLFA and DNA	Aerobic	^{13}C	lab	Tillmann et al. 2005	16329907
Pyrene	DNA	Aerobic	^{13}C	lab	Jones et al. 2008	18165874
Toluene	DNA	Sulfate reducing	^{13}C	lab	Winderl et al. 2010	20428224
Toluene	DNA	Aerobic	^{13}C	lab	Sun et al. 2010	20008173
Toluene	DNA	Sulfate reducing	^{13}C	lab	Bombach et al. 2010	19951369
2,4,6-Trinitrotoluene	DNA	Sulfate reducing	^{15}N and ^{13}C	lab	Gallagher et al. 2010	20081008
Uranium	PFLA and DNA	Anaerobic	^{13}C	field	Chang et al. 2005	16382923

[a] PMID = PubMed reference number. Abstracts of the referenced studies can be obtained at the PubMed web page (www.ncbi.nlm.nih.gov/pubmed) by entering the PMID number in the search box.

How does it work?

All living organisms are made of four key carbon-containing biomolecules (e.g., lipids, sugars, proteins, and nucleic acids). Microorganisms synthesize these biomolecules from simple metabolites they generate during the degradation of chemicals present in their environment. When a microorganism grows and divides, the isotopic composition of the biomolecules in the new cells reflects the isotopic composition of the chemicals on which the microorganism was grown. For instance, if a microorganism is grown on sugars consisting entirely of carbon-12 (^{12}C, the most abundant stable form of carbon), every carbon atom in every biomolecule in the newly formed cells is ^{12}C. However, if the microorganism is grown on sugars enriched in ^{13}C (the other but more rare stable isotope of carbon), the new biomolecules are significantly enriched in ^{13}C. This effect is exploited in SIP, and consequently all SIP approaches involve exposing samples to isotopically enriched contaminants. After a predetermined period of time, all forms of the specific biomolecule under investigation are extracted from the sample and analyzed. If biodegradation of the isotopically enriched contaminant has occurred, some of the extracted biomolecules will contain elevated levels of the stable isotope originally present in the contaminant. As there are no significant alternative routes that allow the stable isotope to migrate from the contaminant to biomolecules, detection of elevated levels of stable isotopes in biomolecules provides compelling evidence for biodegradation.

Most SIP studies use ^{13}C-labeled compounds, although compounds enriched with nitrogen (^{15}N) and oxygen (^{18}O) can also be used. The most frequently used and commercially available form of SIP involves analysis of fatty acids from microbial phospholipids. This technique, known as phospholipid fatty acid (PLFA)-SIP, uses contaminants that contain a relatively low level of isotopic enrichment (e.g., 10% ^{13}C). After exposure of samples to ^{13}C-labeled contaminants, all forms of phospholipid (labeled and unlabeled) are extracted using solvents. A subsequent analysis of the solvent extracted phospholipids by mass spectrometry (gas chromatography [GC]/mass spectrometry [MS] or GC/isotope ratio mass spectrometry [IRMS]) can quantify total ^{13}C incorporation into PLFAs. This measurement can establish that biodegradation has occurred and can estimate the amount of contaminant that has been degraded. A further identification of specific PLFAs that have elevated levels of ^{13}C can help identify broad groups of microorganisms responsible for the biodegradation process. The analysis of ^{13}C incorporation into PLFAs also can be supplemented by data showing the detection of elevated ^{13}C levels in terminal metabolites such as CO_2 and CH_4.

Other forms of SIP such as DNA-SIP or protein-SIP are currently mainly research and discovery tools and require contaminants with very high levels of isotopic enrichment (ideally 100% ^{13}C). Although not routinely available as a commercial service, the analysis of ^{13}C-labeled DNA using molecular approaches (e.g., quantitative polymerase chain reaction [qPCR], microarrays, DNA sequencing) or ^{13}C-labeled proteins using MS can provide precise information about which species of microorganisms are responsible for a biodegradation reaction and even the specific enzymes and pathways involved in the biodegradation process. These analyses can be particularly useful when little is known about either the microorganisms or the pathways involved in a particular biodegradation process.

How are the data reported?

Positive PLFA-SIP data typically report the level of ^{13}C enrichment in solvent-extracted bulk or individual fatty acids in the form of change in ^{13}C abundance. These data are obtained from either GC/MS or GC/IRMS measurements. Positive DNA/RNA-SIP data simply state that ^{13}C-labeled DNA or RNA was detected based on separation from unlabeled ^{12}C-DNA or -RNA by ultracentrifugation. Additional supporting lines of evidence in all ^{13}C SIP analyses can be expected to include measurements of $^{13}CO_2$ or $^{13}CH_4$ production.

Advantages

- Can be used without any prior knowledge of the organisms responsible for biodegradation of the contaminant.
- Applicable to different environmental media (water, soil, sediment). Although often a laboratory technique, PLFA-SIP approaches have been used with in situ passive sampling devices such as Bio-Traps™.
- Can be conducted on any contaminant as long as isotopically enriched forms are available. SIP approaches are most appropriate for compounds that are used as carbon and energy sources (e.g., hydrocarbons, gasoline oxygenates, PAHs) for microorganisms, as carbon from these compounds is incorporated into biomolecules during biodegradation.
- Different SIP approaches can provide different information, ranging from whether or not biodegradation has actually occurred to which microbial species are degrading the molecule and which biodegradation pathways and enzymes they are using.

Limitations

- Not appropriate for contaminants used as terminal electron acceptors, such as chlorinated ethenes or perchlorate. Atoms from these compounds are typically not incorporated into biomolecules by organisms that reduce these compounds as electron acceptors.
- Some isotopically labeled compounds can be expensive (especially if they have to be custom-made).
- Different SIP methods can require different lengths of time to implement. In the field, using passive microbial sampling devices such as Bio-Traps, the sampling devices may be deployed for 30–90 days. In the laboratory the time to completion depends on the rate of disappearance of the contaminant in the specific SIP study.
- Care has to be taken in extrapolating the results of SIP studies to field conditions, even when using passive (field) sampling devices. The conditions required to obtain a detectable SIP signal (e.g., high localized concentrations of isotopically labeled contaminant) may be substantially different from the prevailing conditions in the subsurface.
- Permits may be required for the use of passive microbial sampling devices for use in in situ SIP studies although the amounts of isotopically labeled contaminants used in these analyses are typically very small.
- Cross feeding can occur when isotopically labeled metabolites are excreted by one organism and then used by other microorganisms. In DNA-SIP, this effect can potentially lead to identification of organisms that are not directly responsible for the biodegradation process of interest. The extent and significance of cross feeding can be estimated by conducting several SIP analyses over time. Cross feeding would be expected to be the least significant in the earliest time points.

Sampling Protocols

Passive microbial sampling devices are generally used in groundwater wells to conduct in situ SIP studies. The isotopically enriched contaminant (e.g., ^{13}C-methyl *tert*-butyl ether) is applied to media in the passive microbial sampling device, and the sampling device is incubated in a well for a given period of time. The biomolecules of interest (e.g., DNA, PLFA) are then extracted from the media and analyzed. Additional details on passive microbial sampling devices are provided in the EMD Sampling Methods Fact Sheet. An alternative to these studies is laboratory incubations in which the isotopically enriched contaminant is added to field samples (e.g., microcosms with groundwater and aquifer sediment from the site of interest) and these samples are incubated under relevant conditions (e.g., similar temperature, oxidation-reduction potential to the field site). Subsamples can then be collected from the laboratory incubations over time for analysis of stable isotope incorporation into biomolecules. In all cases biomolecules such as DNA and lipids are extracted from samples using standard laboratory techniques used in other analyses described in other EMD fact sheets (e.g., qPCR, Microbial Fingerprinting). Users should work with the analytical laboratory to ensure sampling protocols for collecting, handling, and transporting samples are in place and understood.

Quality Assurance/Quality Control

To date, most EMDs do not have standardized protocols approved by the U.S. Environmental Protection Agency or other government agencies. However, most laboratories work under standard operating procedures (SOPs) and good laboratory practices, which can be provided to the user (e.g., consultant, state regulator) on request.

Currently, users can best ensure data quality by detailing the laboratory requirements in a site-specific quality assurance project plan (QAPP). This plan includes identification of the EMDs being used; the field sampling procedures, including preservation requirements; the SOPs of the laboratory performing the analysis; and any internal quality assurance (QA)/quality control information available (such as results for positive and negative controls). SIP approaches all detect incorporation of isotopes of specific elements into new biomolecules. While the absolute degree of isotopic enrichment of a pure chemical is not a major QA issue (i.e., the same organisms/biomolecules are labeled in samples that are incubated with a pure compound that has 10% ^{13}C enrichment as with the same pure compound with 100% ^{13}C enrichment), the purity of the parent chemical is of critical importance. Ambiguous results can be obtained in a SIP study using a ^{13}C-enriched chemical that is impure, even if the chemical of interest is 100% enriched with ^{13}C. For example, a SIP study evaluating benzene degradation that uses 99% pure benzene that is enriched 100% with ^{13}C may yield inaccurate results if the 1% impurity is also ^{13}C enriched (e.g., ^{13}C-toluene). In this case, the organisms detected may actually be degrading toluene rather than benzene, even though the toluene is present as a minor constituent. Consequently, the use of highly purified isotopically labeled compounds is of paramount importance in SIP studies.

Whenever possible, SIP analyses should involve use of control incubations in which isotopically labeled compounds are added to sterilized or inhibited samples as well as active samples. Although no standards currently exist, isotopically labeled biomolecules should also be extracted from samples either using protocols described by commercial kit manufacturers or individual laboratory SOPs.

Additional Information

See Table 1 for current references for SIP applications. The table includes PubMed reference numbers (PMID). The abstract of a published study can be obtained at the PubMed web page (www.ncbi.nlm.nih.gov/pubmed) by entering the PMID number in the search box.

References

EPA (U.S. Environmental Protection Agency). 2004. *Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples.* EPA/815/B-04/001. Office of Water. www.epa.gov/nerlcwww/qa_qc_pcr10_04.pdf.

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Glossary

biomolecules—Classes of compounds produced by or inherent to living cells including phospholipids, nucleic acids (e.g., DNA, RNA), and proteins.

DNA (deoxyribonucleic acid)—A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (EPA 2004).

enzyme—Any of numerous proteins or conjugated proteins produced by living organisms and facilitating biochemical reactions (based on EPA 2004).

gene—A segment of DNA containing the code for a protein, transfer RNA, or ribosomal RNA molecule (based on Madigan et al. 2010).

isotopically labeled contaminant—A contaminant that has been specially synthesized to deliberately contain specific isotopes at elevated levels above those found in either natural or commercial bulk forms of the same chemical.

lipids—A diverse range of organic compounds that are defined as being insoluble in water but soluble in nonaqueous solvents. Lipids include oils, waxes, and sterols.

microcosm—A sample that is regarded as a small but representative portion of something larger. In environmental studies microcosm are typically small samples of soil, sediment, or water incubated in enclosed containers under laboratory conditions.

phospholipid—A type of biomolecule that is a primary structural component of the membranes of almost all cells.

PLFA—Phospholipid fatty acids derived from the two hydrocarbon tails of phospholipids.

stable isotope—A form of an element that does not undergo radioactive decay at a measureable rate.

terminal electron acceptors—Compounds used by microorganisms to support respiration. In aerobic organisms the terminal electron acceptor is oxygen (O_2). Anaerobic organisms use compounds other than O_2. These include common naturally occurring compounds such as nitrate (NO_3^-) or sulfate (SO_4^{2-}) or anthropogenic contaminants such as chlorinated ethenes (e.g., perchloroethene). Atoms from electron acceptors are typically not incorporated into biomolecules made by organisms that reduce these compounds during respiration.

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